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ICG-Sulfo-OSu

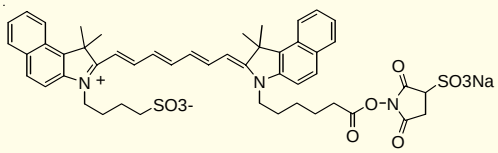
----------Page 35

Application: Indocyanine green labeling of proteins

Product Description

ICG is one of the dyes which is used for determining cardiac output, hepatic function, and liver blood flow, as well as for ophthalmic angiography. It has a long excitation wavelength and emission wavelength of about 780 nm and 800 nm, respectively. Due to such a long wavelength near the infrared region and low cytotoxicity, ICG is used to label antibodies for in vivo assay.

Chemical Structure

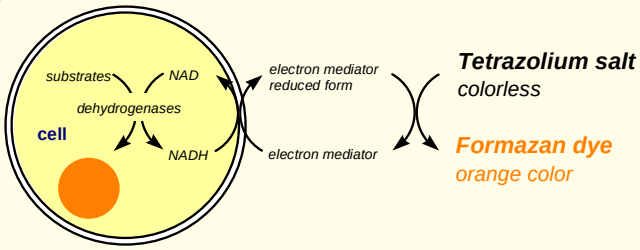


Cell Counting Kit-SK

----------Page 59

Application: Cell viability and cytotoxicity detection

Features: Colorimetric microplate assay
One solution type
No washing required
No radioisotopes or organic solvents required
High sensitive



Principle of cell viability detection with Cell Counting Kit-SK

Product Description

Cell Counting Kit-SK (CCK-SK) is an alternative kit to CCK-8(Cell Counting Kit-8, code#:CK04, page 55) for cells or cell lines with low metabolism. The O.D. of the CCK-8 for leukocyte cells is sometimes very low, such as 0.2-0.4 after 2-4 hours incubation because of lower metabolism compared with adherent type cells. CCK-SK enables a shorter incubation time to obtain a higher O.D. increase within 30 min to 1 hour. CCK-SK is suitable for the purpose of determining the cell viability.

Microbial Viability Assay Kit-WST

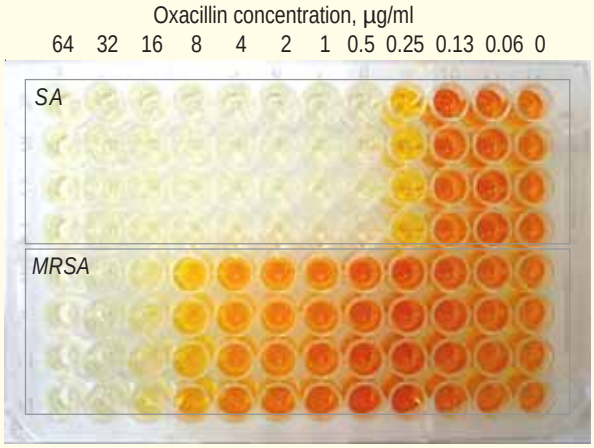
----------Page 62

Application: Bacterial cell viability detection

Features: Colorimetric microplate assay
Wide variety of microorganism detection
No harvesting or washing required

Product Description

Viable bacterial cell detections are very important for analyzing bacteria contamination in food or evaluating the cleanliness of facilities in order to protect us from food poisonings and infections. Bacterial cell detections are also used for the screening of sanitizing agents and drug resistance detections. Generally, counting the number of colonies on an agar plate is the standard method for determining the number of viable bacterial cells in samples. However, colony formations require one to several days. Dojindo's Microbial Viability Assay Kit-WST can be used to determine the number of viable bacterial cells in a sample by a colorimetric method and can be applied to 96-well microplate assays.



Susceptibility test of SA and MRSA against Oxacillin.
The data indicated that MRSA has lower Susceptibility than SA. The MICs of *MRSA* (32 µg/ml) and *SA* (0.5 µg/ml) are close to the MICs determined by the CLSI (Clinical and Laboratory Standards Institute) method.

-*Bacstain*-CTC Rapid Staining Kit

for Flow cytometry (BS01-10), for Microscopy (BS02-10)

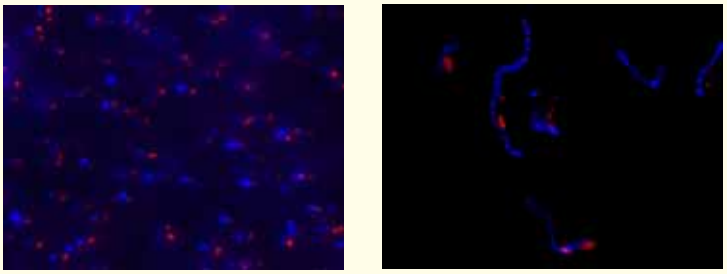
----------Page 66

Application: Aerobic bacterial cell staining
Hard to culture bacteria detection

Features: Highly sensitive fluorescence detection
No washing required

Product Description

In order to count bacterial cells, colony formation using an agar plate is a very common and reliable method. However, it takes quite a long time to incubate. Therefore, alternative detection methods have been developed. Bacteria-specific gene amplification methods such as PCR, LAMP, and nucleus staining are quite rapid, but these methods count dead bacteria as well. Therefore, detection of live cell functions is essential to determining the actual number of living bacteria in a sample. Tetrazolium salts can be used to detect respiratory activity of bacterial cells or mitochondria. CTC is one of the tetrazolium salts and is reduced by this respiratory activity to form fluorescent CTC formazan on the cell surface. Therefore, CTC is used for specific staining of an aerobic live bacteria and can be applied to hard to culture bacteria (VBNC: viable but non-culturable). CTC-Rapid Staining Kit contains an enhancing reagent which improves the CTC staining efficiency. Compared with CTC only staining, this staining kit enables rapid and sensitive staining of microorganism.



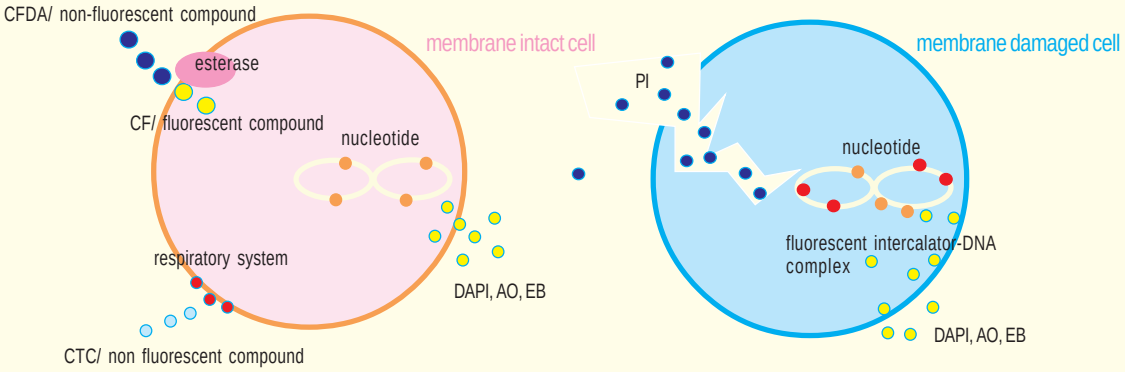
E. coli staining (left) and L. casei staining (right) with CTC and DAPI.
Bacterial cells were stained with CTC first, and then 1 µl of DAPI solution was added. The cells were incubated at room temperature for 5 min.
Formaldehyde fixation with 1-4% formaldehyde can be performed before DAPI staining.

-*Bacstain*-Series

----------Page 69

Products Description

The *Bacstain* series can be used for microorganism staining. CFDA is used for staining of viable microorganisms. CFDA is bacterial cell wall and cell membrane permeable, and hydrolized by esterase of the cell to stay inside of the cell. Other *Bacstain* reagents are used for nucleotide staining and are cell wall permeable except for PI. Therefore, using one of these nucleotide staining reagents and CFDA or *Bacstain*-CTC Rapind Staining Kit, it is possible to stain both membrane intact cells and membrane damaged cells simultaneously. Since PI can stain only membrane damaged cells, membrane intact cells are not stained by this compound. PI is also used for double staining coupled with CFDA and CTC.



ACE Kit-WST

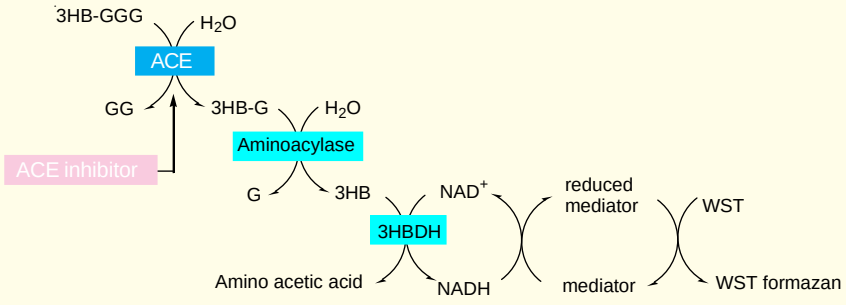
----------Page 90

Application: ACE activity detection. Screening of ACE inhibitors

Features:
- Colorimetric microplate assay
- Simple protocol
- No organic solvent required
- High reproducibility

Product Description

This kit is used for the determination of ACE (angiotensin-converting enzyme) inhibition activity. ACE works in the Renin-Angiotensin system, which is one of the mechanisms of blood pressure control, to convert Angiotensin I to the vasopressor Angiotensin II. This enzyme also contributes to elevated blood pressure due to the activity of breaking down the antihypertensive peptide Bradykinin. In recent years, food and supplements containing ingredients that have ACE blocking activity have received attention for their use in preventing high blood pressure.The conventional method of measuring ACE inhibition employs the synthetic substrate Hippuryl-His-Leu. Hippuric acid from the synthetic substrate is extracted with ethyl acetate, condensed, redissolved, and then read at an absorbance of 228 nm. This method is cumbersome and measurement is subjected to error due to residual ethyl acetate. ACE inhibition Assay Kit enzymatically detects 3-Hydroxybutyric acid (3HB) which is made from 3-Hydryoxybutyryl-Gly-Gly-Gly (3HB-GGG). Using a 96-well format, it is possible to test multiple samples at one time. In addition, there is no need to use harmful organic solvents, resulting in a safe, simple, and highly reproducible assay.



Principle of the assay system to determine ACE activity or inhibition activity.

Spy-LHP

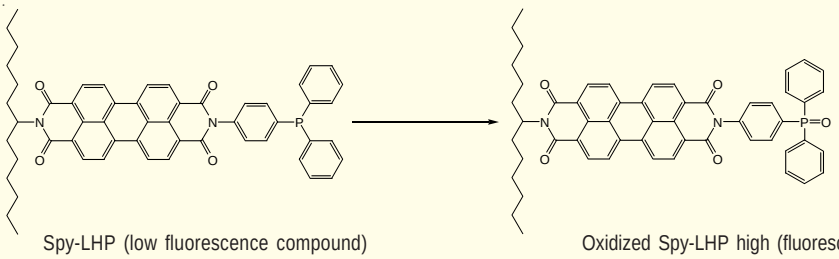
----------Page 105

Application: Phospholipid peroxidase detection
Selective to lipid hydroperoxidase

Features: Fluometric detection

Product Description

Spy-LHP is a newly developed fluorescent probe for live cell imaging of phospholipid peroxide. There are several detection methods available for lipid peroxides, such as iodide titration method, colorimetric method, or chemiluminometric method to determine malondialdehyde or 4-hydroxynonenal. Malondialdehyde or 4-hydroxynonenal are derivatives from lipid hydroperoxide prepared by oxidation with reactive oxygen species. Thiobarbituriic acid and 1-Methyl-2-phenylindole are used for the derivertization of malondialdehyde for the colorimetric or fluorometric analysis. Spy-LHP is a low-fluorescent compound, but is oxidized with lipid hydroperoxide to become a high fluorescent compound as indicated below. A similar product, DPPP, is oxidized by a lipid hydroperoxide and becomes a fluorescence compound which can be excited at 352 nm to emit fluorescence at 380 nm. However, the UV excitation for DPPP gives significant damage to a live cell. Since the oxidized Spy-LHP emits strong fluorescence (quantum yield: ~1) with maximum wavelength at 535 nm when excited at 524 nm, damage to a live cells is very small. Spy-LHP has two alkyl chains to improve the affinity to the lipid bilayer. Spy-LHP is highly selective to lipid hydroperoxide and does not react with hydrogen peroxide, hydroxy radicals, superoxide anion, nitric oxides, peroxynitrite, and alkylperoxy radicals.



Spy-LHP (low fluorescence compound)

Oxidized Spy-LHP high (fluorescence compound)

CarryMax

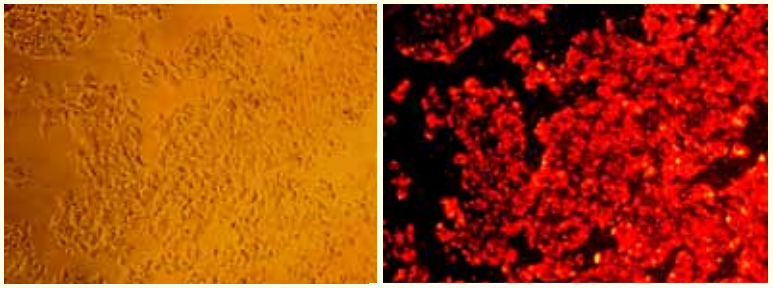
----------Page 139

Application: Protein delivery to viable cells

Features: Simple procedure to optimize delivery conditions
Low toxicity to cells

Product Description

CarryMax is a protein delivery kit for animal cells. This kit is designed to acheive the best performance of protein delivery to various animal cells. Since the kit contains two different types of delivery reagents, the protein delivery conditions can be optimized for higher delivery with a lower cytotoxicity rate. The delivery mechanism of the protein by CarryMax is similar to the gene delivery by cationic liposomes. The protein-cationic liposome complex binds on the cell membrane and then the complex is taken inside cells by endocytosis where upon the protein is released to the cytoplasm. The location of the protein delivered by this method will be concluded by various experiments. Currently, the delivered protein in the cell such as apoptosis-related proteins works in the same manner as a protein synthesized in the cell by gene expression. This delivery method will be very useful in understanding the function of the protein in the cell. The total time to deliver proteins is about 2-4 hours if no further cell culture is required. Therefore, much quicker experiments will be possible to check the function of the protein than when using a DNA transfection method. The delivered protein is stable over several hours to several days.



R-Phycoerythrin-delivered CHO cells
left) Phase-contrast microscope, x100
right) Fluorescent microscope, x100 G-filter
Delivery conditions:
Complex prepared by adding 1 µg of R-Phycoerythrin and 5 µl of PT-H to CHO cells in a 24 well plate, and incubated for 4 hours.

HilyMax

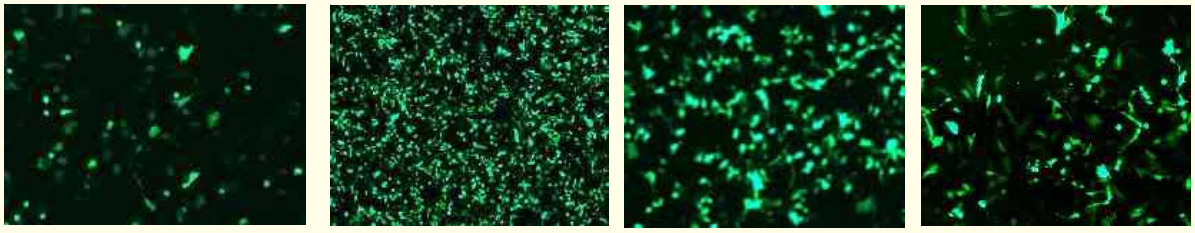
----------Page 142

Application: DNA and RNA transfection to mammalian cells

Features: High transfection efficiency to a wide variety of cells
Simple protocol for maximum transfection conditions
Better response in signal transduction research
Efficient suppression in siRNA transfection

Product Description

HilyMax is a newly developed gene transfection reagent that forms a liposome to be used for highly efficient gene transfection to a wide variety of cells. HilyMax can be applied to siRNA as well. Since serum in the growth medium does not interfere with the transfection using HilyMax, no exchange of the media during the transfection is required. HilyMax does not contain biological components that might interfere with the transfection.



A549 CHO HEK293 HeLa

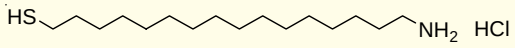
GFP expressed cells transfected by HilyMax/ G filter, x100

16-Amino-1-hexadecanethiol, hydrochloride

----------Page 155

Application: SAM preparation, amine group coating

Chemical Structure

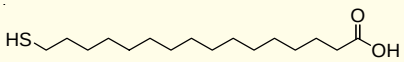


15-Carboxy-1-pentadecanethiol

----------Page 159

Application: SAM preparation, carboxylate coating

Chemical Structure

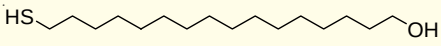


16-Hydroxy-1-hexadecanethiol

----------Page 165

Application: SAM preparation, dilution of functional alkanethiols

Chemical Structure

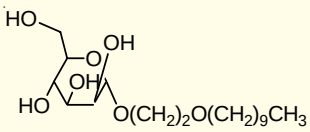


3-Oxatridecyl-α-D-mannoside

----------Page 178

Application: Membrane protein crystalization

Chemical Structure



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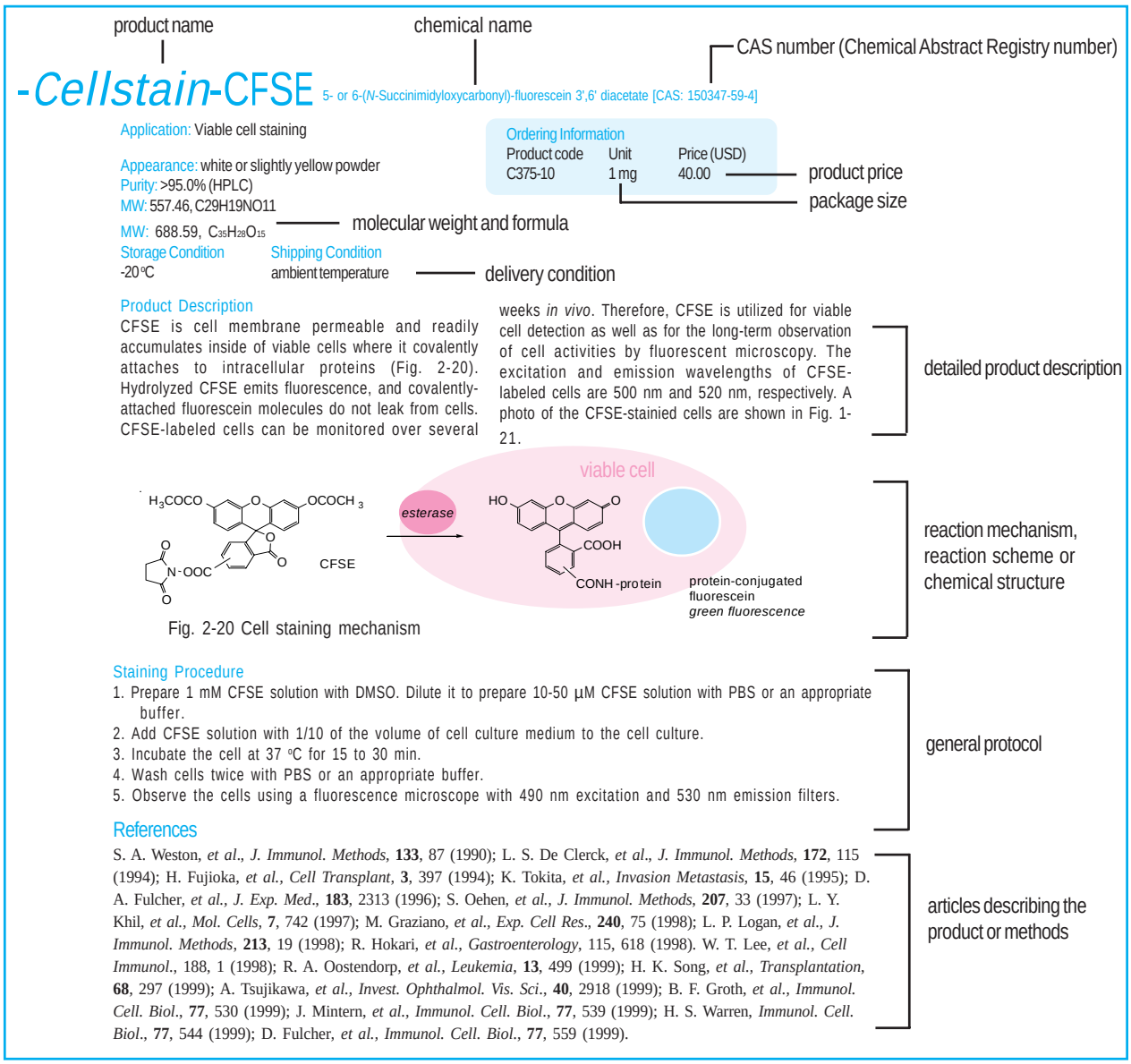


Fig. 2-20 Cell staining mechanism

S. A. Weston, *et al*., *J. Immunol. Methods*, **133**, 87 (1990); L. S. De Clerck, *et al*., *J. Immunol. Methods*, **172**, 115 (1994); H. Fujioka, *et al.*, *Cell Transplant*, **3**, 397 (1994); K. Tokita, *et al.*, *Invasion Metastasis*, **15**, 46 (1995); D. A. Fulcher, *et al.*, *J. Exp. Med*., **183**, 2313 (1996); S. Oehen, *et al.*, *J. Immunol. Methods*, **207**, 33 (1997); L. Y. Khil, *et al.*, *Mol. Cells*, **7**, 742 (1997); M. Graziano, *et al.*, *Exp. Cell Res*., **240**, 75 (1998); L. P. Logan, *et al.*, *J. Immunol. Methods*, **213**, 19 (1998); R. Hokari, *et al.*, *Gastroenterology*, 115, 618 (1998). W. T. Lee, *et al.*, *Cell Immunol*., 188, 1 (1998); R. A. Oostendorp, *et al.*, *Leukemia*, **13**, 499 (1999); H. K. Song, *et al.*, *Transplantation*, **68**, 297 (1999); A. Tsujikawa, *et al.*, *Invest. Ophthalmol. Vis. Sci*., **40**, 2918 (1999); B. F. Groth, *et al.*, *Immunol. Cell. Biol*., **77**, 530 (1999); J. Mintern, *et al.*, *Immunol. Cell. Biol*., **77**, 539 (1999); H. S. Warren, *Immunol. Cell. Biol*., **77**, 544 (1999); D. Fulcher, *et al.*, *Immunol. Cell. Biol*., **77**, 559 (1999).

Abbreviations:

Ac: acetyl
ADP:adenosine 5'-diphosphate
AM: acetoxy methyl ester
AMC: aminomethylcoumarin
ALP: alkaline phosphatase
APC: Allophycocyanin
ATP: adenosine 5'-triphosphate
Br: bromide
B-PE: B-phycoerythrin
Cl: chloride
DMF: *N*,*N*-dimethylformamide
EIA: enzyme immunoassay
ESR: electron spin resonance
GC: gas chromatography
GSH: glutathione reduced form
GSSG: glutathione oxidized form
HCl: hydrogen chloride
H_2O_2: hydrogen peroxide
HPLC: high performance liquid chromatography
HRP: horseradish peroxidase
Kd: (metal-chelate) dissociation constant
NAD: β-nicotinamide adenine dinucleotide
NADH: β-nicotinamide adenine dinucleotide, reduced form
NADP: β-nicotinamide adenine dinucleotide phosphate
NADPH: β-nicotinamide adenine dinucleotide phosphate, reduced form
NHS: N-hydroxysuccinimidyl ester
NO: nitric oxide
NOS: nitric oxide synthase
OSu: N-hydroxysuccinimidyl ester
PVC: polyvinyl chloride
ROS: reactive oxygen species
R-PE: R-phycoerythrin
SH: sulfhydryl
THF: tetrahydrofuran
TLC: thin layer chromatography
ε: molar absorptivity
λem: emission maximum wavelength
λex: excitation maximum wavelength
λmax: maximum wavelength

I. Protein Labeling

Introduction

Protein labeling reagents are used for various assays that are coupled with antigen-antibody reaction. Of these, fluorescent, biotin, and enzyme labeling reagents are used most frequently for antibody labeling. Most protein labeling reagents have similar reactive sites, such as succinimidyl ester (NHS) for amino groups and maleimide or bromoacetamide for sulfhydryl groups. Dojindo offers the following protein labeling reagents and kits for protein research and proteomics.

There are several reactive sites on proteins as indicated in Fig. 1-1. The most common reactive site used is the NH_2 group from lysine residue because of the very simple process of labeling an NH_2-terminal under physiological conditions. Sometimes, protein labeling with NH_2-reactive compounds is not favorable due to the potential blockage of the active site of the protein. However, such activity loss due to the blocking of active sites by labeling is very rare in the case of antibody labeling with NH_2-reactive reagent. Therefore, labeling at the NH_2 group on a protein is fairly reliable and simple for preparing a conjugate. The second most common labeling method is the use of SH-reactive compounds. Most proteins do not have an SH group, just a disulfide group. The disulfide group can be converted to SH groups by reducing agents such as dithiothreitol (DTT) or beta-mercaptoethanol. Then, SH-reactive compounds such as maleimide and bromoacetyl compounds are applied in the labeling of SH groups of the protein. Since the location of the disulfide group on an antibody is more specific than that of NH_2 groups, activity loss by SH group labeling is minimal. The reduction process may cause activity loss of an antibody because of possible cleavage between a heavy chain and a light chain. However, a conjugate prepared by SH labeling gives a better signal than that by NH_2 labeling due to the site specific labeling. Overall, a conjugate prepared by NH_2 labeling is adequate for general assays while a conjugate prepared by SH labeling can be used for high sensitivity assays. The sugar chain of a protein is also a possible labeling site with hydrazide compounds. Sugar chains have to be reduced to convert to an aldehyde group prior to labeling with a hydrazide labeling agent.

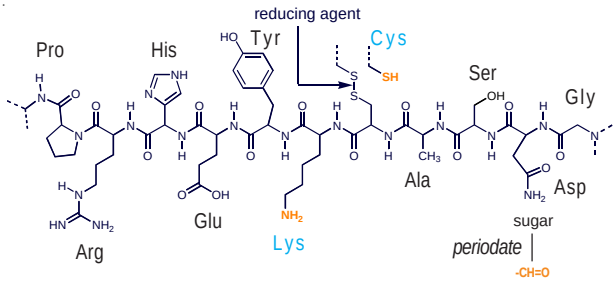


Fig. 1-1 Reaction site for protein labeling.

Labeling Kits

Dojindo offers a wide variety of protein labeling kits. These kits can be used to prepare conjugates with enzyme, fluorophore, and biotin (Table 1-1). There are two types of kits available; NH_2 labeling (Kit-NH_2) and SH labeling (Kit-SH). Dojindo's labeling kits contain centrifugal filtration tubes which are for used protein purification, conjugation reaction, condensation, and buffer exchange. This single filtration tube format is the most unique feature of the kit. (Fig. 1-2). The Labeling process requires 1) purification of protein and buffer exchange, 2) labeling reaction, and 3) purification and recovery of the conjugate. Since these three processes can be performed in a single filtration tube, recovery of proteins is always high; nearly 100% of protein can be recovered from the tube. The filtration tube has a membrane that allows smaller molecules to pass through while larger molecules remain on the membrane. Through this simple centrifuge process, small-sized proteins can be removed, leaving only conjugated proteins to be recovered by pippetting with a buffer solution. Therefore, recovery of the conjugated protein and reproducibility of the labeling are high.

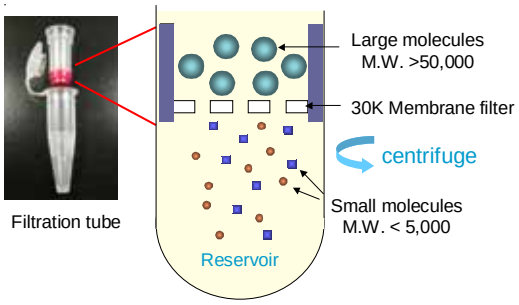


Fig. 1-2 Filtration tube with a 30K membrane filter and its molecule separation mechanism.

Additionally, the unique buffer system in the kit prevents aggregation of the protein and the conjugate during the labeling process and storage of the conjugate. The conjugate recovered with the buffer (Storage buffer) is stable for several months at 4 °C or several years if it's stored at -20 °C. There is a10K membrane filter available for protein smaller than 50K. The enzyme labeling kits can be used for small molecule labelings such as organic chemicals, amino acids, peptides, or oligonucleotides. For more detailed information, please reveiw the product information section. Kit-NH_2 and Kit-SH include buffer solutions for washing, labeling reaction and recovery of proteins, activated compounds, 30K filtration tubes, and an easy-to-follow protocol with photos. Kit-SH additionally contains reducing agents for converting a disulfide group to sulfhydryl groups (SH). The overall protocols for Kit-NH_2 and Kit-SH for macromolecule and small molecule labelings are indicated in Fig. 1-3 and 1-4. Most small molecules, such as buffer components, sodium azide (preservative), amine compounds, thiol compounds, detergents, glycerol, etc. are removed from proteins by the first step. Then, the appropriate labeling reagent is added directly to the Filtration tube and incubated at 37 °C for a certain time period. The conjugate is then recovered with Storage buffer or WS buffer. The average concentration of protein will be 500-1,000 µg per ml as antibody. In most cases, the conjugates can be applied for downstream experiments such as ELISA, western blotting, tissue staining, and flowcytometry without further purification.

Table 1-1 Labeling Kits

Category	Labeling material	Target group
Enzyme labeling	Peroxidase	amine, thiol
	Alkaline phosphatase	amine, thiol
Biotin labeling	Biotin	amine, thiol
Fluorophore	Phycobiliproteins	amine, thiol
labeling	Fluorescent dyes	amine, thiol

I. Protein Labeling

Labeling Process

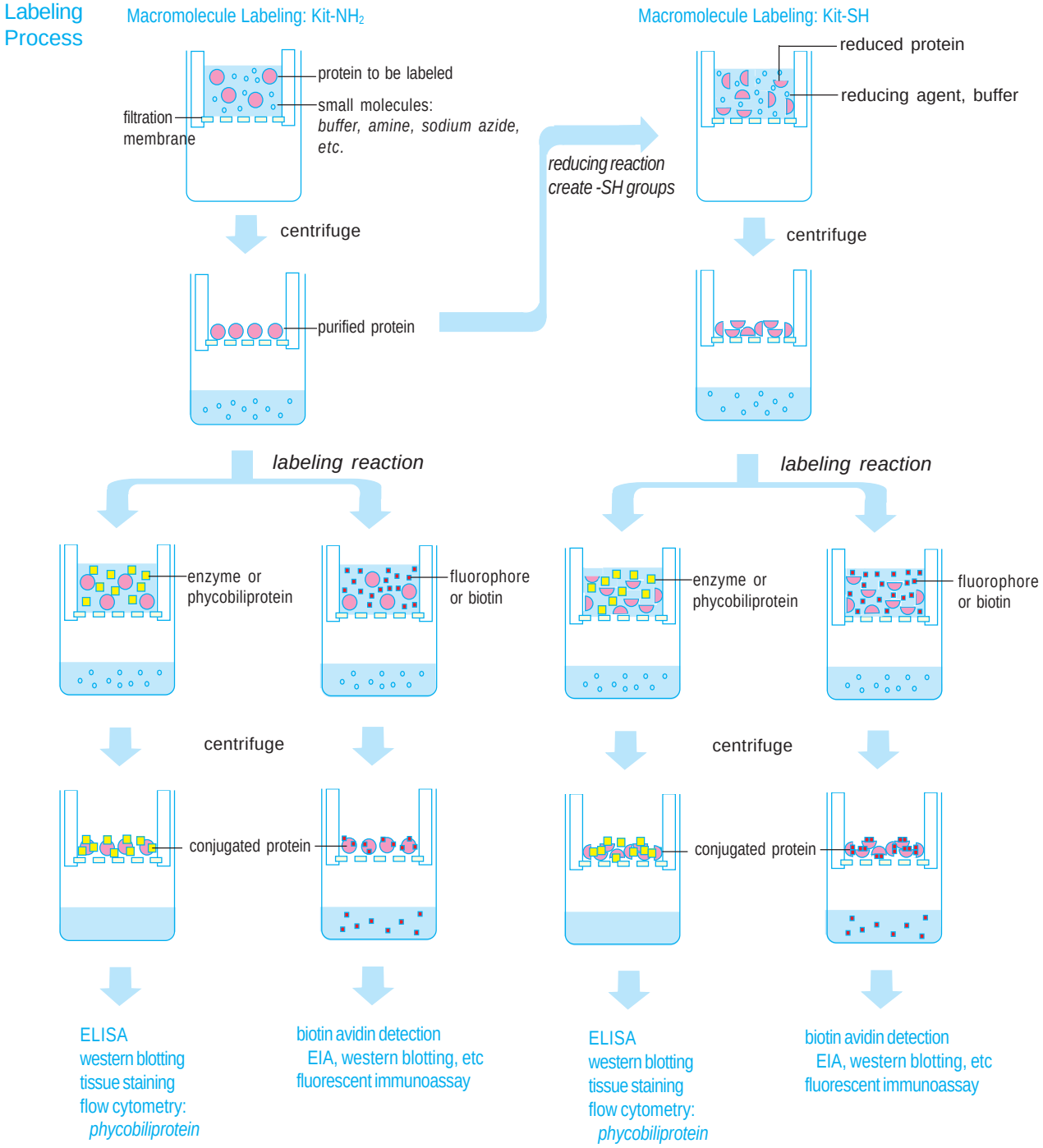


Fig. 1-3 Macromolecule labeling processes

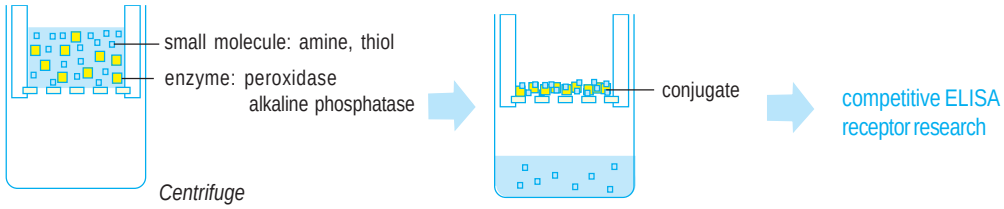


Fig. 1-4 Small molecule labeling processes

I. Protein Labeling

Enzyme Labeling Kits

Peroxidase and alkaline phosphatase are the most frequently used enzymes for enzyme immunoassays (EIA) because of their high sensitivity, high stability, little activity loss by chemical modifications, and wide selection of colorimetric, fluorometric and chemiluminometric substrates. Peroxidase-labeled protein is generally prepared using a reactive periodate-treated peroxidase. An aldehyde group of peroxidase derived from a sugar chain by the periodate oxidation reaction forms Shiff's base with an amine group of the protein to create a covalent bond between the protein and peroxidase. After the conjugation reaction, a reducing agent is usually required to convert the Schiff's base to a C-N single bond, stabilizing the peroxidase conjugate. Dojindo's Peroxidase Labeling Kit-NH_2 and Alkaline phosphatase Labeling Kit-NH_2 can be used to simply and rapidly label both macromolecules (Fig. 1-5) and small molecules with amino groups. Conjugates prepared with this kit have higher enzyme activity and are more stable than other commercially available peroxidase or alkaline phosphatase-labeled antibodies or streptavidins. Peroxidase Labeling Kit-SH and Alkaline phosphatase Labeling Kit-SH are for labeling of molecules with sulfhydryl groups. The overall procedures for the labeling are the same for both, except for the reduction of the protein prior to the labeling of SH groups. Enzyme-labeled IgGs prepared using Labeling Kit-SH sometimes gives higher sensitivity than the conjugates prepared by Labeling Kit-NH_2 due to the site specific labeling with Kit-SH.

A) Enzyme Labeling Kit-NH_2 B) Enzyme Labeling Kit-SH

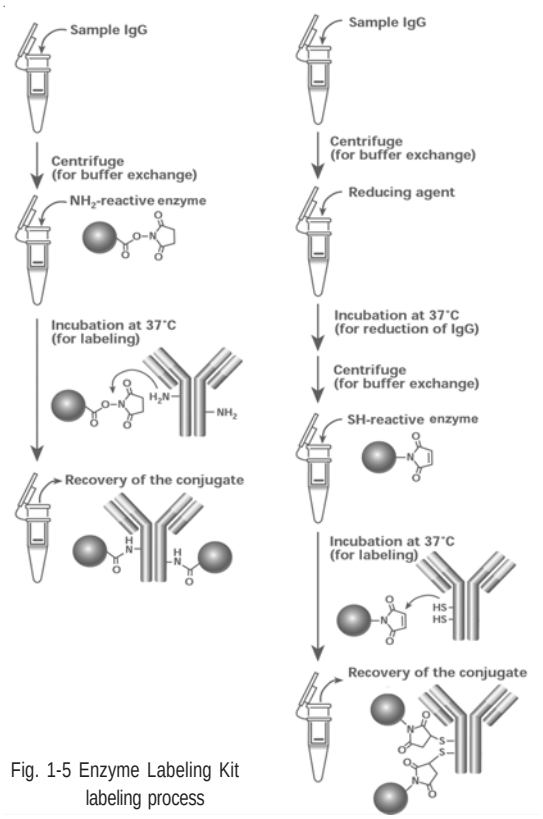


Fig. 1-5 Enzyme Labeling Kit labeling process

Peroxidase Labeling Kits and Alkaline phosphatase Labeling Kits are utilized for small molecule labeling as well as macromolecule labeling. Small molecules with amino group(s) or sulfhydryl group(s) can be labeled with Labeling Kit-NH_2 and Labeling Kit-SH, respectively. The labeling process is indicated in Fig. 1-4. The average number of the small molecules conjugated to an enzyme is 2 per enzyme molecule. The conjugate can be used for competitive ELISA, receptor research, and other assays.

Fluorescent Labeling Kits

Fluorescent compounds such as fluorescein, rhodamine, cyanine dyes, and phycobiliproteins are commonly used for the labeling of biological molecules, especially proteins and DNA. Detection methods using these fluorescent materials are routine and well-established. However, labeling conditions need to be individually determined depending on the type of molecules, reactivity of the labeling agents, and the type of the functional groups to be labeled. Sometimes, pre-treatment of the sample may be necessary to remove molecules and reagents that interfere with the labeling reaction. Fluorophore labeling kits, such as Phycoerythrin, Allophycocyanin, Fluorescein, Rhodamine, and HiLyte dyes are for labeling macromolecules with amino groups or sulfhydryl groups. Labeling processes of Kit-NH_2 and Kit-SH are indicated in Fig. 1-6. The fluorescent compounds in these kits are water soluble and each vial of fluorescent compound can prepare a labeled IgG with four to seven fluorescent molecules. The average number of phycobiliproteins per IgG is about 1-2. These kits include a purification system for the proteins to be labeled so that even protein solutions containing materials which interfere with the reaction such as sodium azide, Tris buffer and thiols, can be used with these kits. The fluorescent spectra of these reagents are shown on page 5.

A) Fluorophore or Biotin Labeling Kit-NH_2 B) Fluorophore or Biotin Labeling Kit-SH

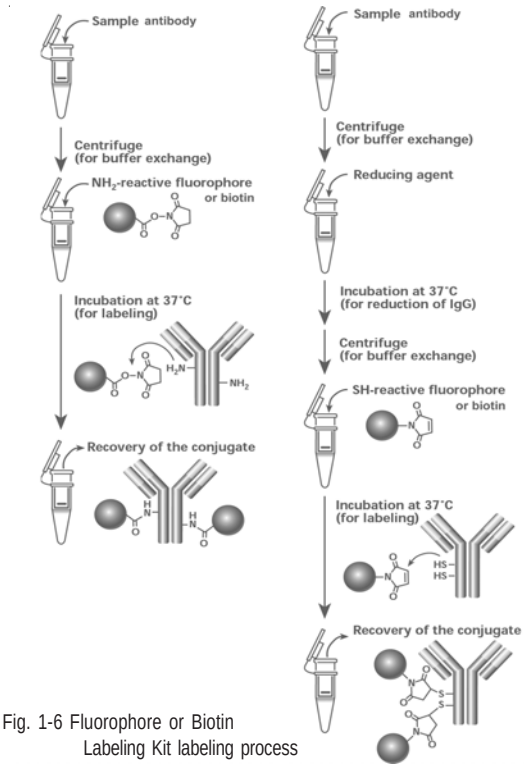


Fig. 1-6 Fluorophore or Biotin Labeling Kit labeling process

I. Proten Labeling

Biotin Labeling Kits and Reagents

Peroxidase-labeled streptavidin or alkaline phosphatase-labeled streptavidin are used for the detection of biotin-tagged macromolecules in EIA. There reactive biotins, and aldehyde reactive biotins. Different lengths of spacers between the biotin molecule and the reactive group are also available. Biotin Labeling Kit-NH_2 is a ready-to-use kit for labeling amino groups of proteins. Since there is a longer spacer between the biotin and succinimidyl ester (NHS) in this kit's biotin labeling reagent, the binding ability of streptavidin with a conjugated biotin molecule is almost identical to that of a free biotin molecule. Biotin Labeling Kit-SH uses Biotin-PE-maleimide for the biotin labeling of sulthydryl groups. Labeling processes of Kit-NH_2 and Kit-SH are indicated in Fig. 1-6.

IgG Purification Kits

Commercially available antibody solutions sometimes contain a stabilizer, such as bovine, serum, albumin, or gelatin. Those stabilizers interfere with a labeling reaction. Dojindo's IgG Purification Kits are useful for preparing a small amount of a purified antibody which can be applied directly to Dojindo's labeling kits. IgG Purification Kit-A and Kit-G contain protein-A and protein-G coated silicagel, respectively. There are four steps in purifying IgG using these kits; 1) binding IgG to the gel, 2) removing unbounded materials, 3) washing, and 4) recovery of IgG from gel (Fig. 1-8). Most IgG molecules bind to protein A and protein G in two minutes. Since all solution is filtered off from the silica gel by centrifuging, the sample solution can be recovered without dilution. If the recovery of IgG is not sufficient by the first purification process, the same sample solution can be processed once more to recover additional IgG. Protein A gel and Protein G gel in the kit are capable of isolating IgG from serum with 80 to 90% purity. The gels can be used at least 20 times with the same performance of IgG recovery.

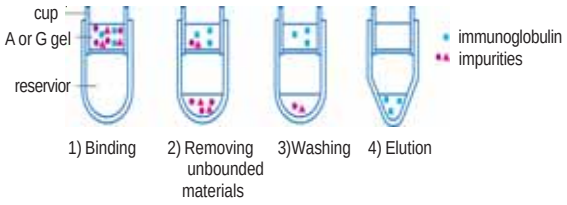


Fig. 1-8 IgG purification process

Chelate Labeling Reagents

Chelate labeling reagents are used for the binding of metal ions to a macromolecule. Macromolecules labeled using radioactive metal ions such as ^{99}Tc and ^{111}In are used for radioimaging with a scintillation camera to investigate macromolecule distribution in the body. Meares' reagents are chelate labeling reagents that have an EDTA structure and a reactive functional group such as isothiocyanate, maleimide, and bromoacetamide. Bromoacetamide-type Meares' reagent, called BABE (4-bromoacetamidobenzyl-EDTA), is used for the analysis of protein-protein or protein-DNA interaction sites.

Bifunctional Cross-Linking Reagents

Cross-linking reagents are used for the conjugation of two or more macromolecules. They are classified into two major groups, hetero-bifunctional cross-linking reagent, and homo-bifunctional cross-linking reagent. Hetero-bifunctional cross-linking reagents have two types of reactive groups in their molecules, succinimide and maleimide. This makes it possible for the biological materials to have different functions, such as amine reactive and thiol reactive, through modification by hetero-bifunctional reagents. These modified materials may then react with other materials through the attached functional groups. In general, conjugated enzymes and monoclonal antibodies for EIA are prepared using these hetero-bifunctional cross-linking reagents. Of the reagents that have succinimide and maleimide as their functional groups, aliphatic compounds such as GMBS and EMCS are superior to aromatic compounds in the stability of maleimide in basic conditions. Four types of maleimide/succinimide cross-linking reagents are available for conjugation, which alters the space length between an enzyme and an antibody. Water-soluble cross-linking reagents are also available. Water-soluble cross-linking reagents do not require organic solvents such as DMF or DMSO to prepare a working solution. Therefore, these water-soluble cross-linking agents are useful for organic solvent sensitive proteins.

I. Protein Labeling

Spectra Data

Fig. 1-9 a) and 1-9b) indicate excitation spectra and emission spectra of IgG conjugates prepared by Fluorophore Labeling Kit-NH_2, respectively. The maximum wavelength of the conjugate prepared by Fluorophore Labeling Kit-SH is almost identical to that of the corresponding conjugate prepared by Fluorophore Labeling Kit-NH_2. Dojindo will increase the number of fluorophore labeling kits and expand the applicable wavelength according to the customer's needs. Access our website for the latest labeling product information at www.dojindo.com.

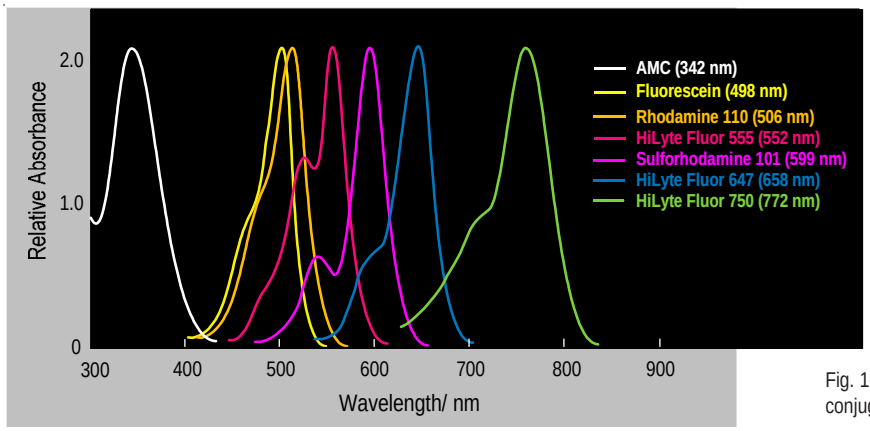


Fig. 1-9a) Excitation spectra of Fluorphore-conjugated IgG

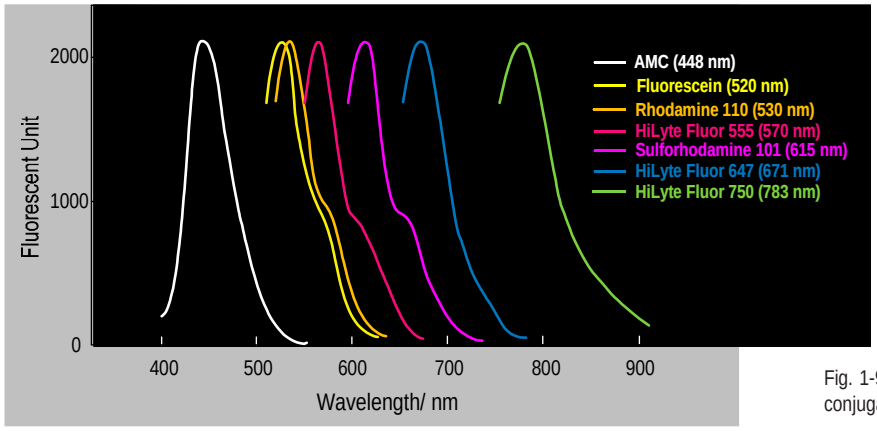


Fig. 1-9b) Emission spectra of Fluorphore-conjugated IgG

Peroxidase Labeling Kit-NH_2

Application: Peroxidase labeling of proteins or amine compounds

Features: Only 3 hours to get conjugates
All processes in a single Filtration tube
High recovery of conjugates

Ordering Information

Product code	Unit
LK11-10	3 samples[a)]
LK51-10	1 sample[b)]
LK52-10	1 sample[c)]

a) Based on 100 µg IgG sample labeling.
b) Based on 1 mg IgG sample labeling.
c) Based on 10 mg IgG sample labeling.

Contents of the Kit

LK11-10

NH_2-reactive peroxidase ..... 100 µg x 3 tubes
Reaction buffer ..... 200 µl x 1 tube
Filtration tube ..... 3 tubes
Washing buffer .....4 ml x 1 bottle
Storage buffer .....4 ml x 1 bottle

LK51-10

NH_2-reactive peroxidase ..... 1 mg x 1 tube
Reaction buffer ..... 1.2 ml x 1 tube
Filtration tube ..... 1 tube
Washing buffer ..... 10 ml x 1 bottle
Storage buffer ..... 10 ml x 1 bottle
15 ml tube.....1 tube

LK52-10

NH_2-reactive peroxidase ..... 5 mg x 2 tubes
Reaction buffer .....12 ml x 1 tube
Filtration tube ..... 2 tubes
Washing buffer .....50 ml x 1 bottle
Storage buffer ..... 50 ml x 1 bottle

Storage Condition	Shipping Condition
0-5 °C	with blue ice

Product Description

Peroxidase Labeling Kit-NH_2 is mainly used for the preparation of peroxidase-labeled IgG for enzyme immunoassay (EIA) and for the preparation of peroxidase-labeled antigen for competitive EIA. NH_2-reactive peroxidase, a component of this kit, has succinimidyl groups (NHS) and reacts with proteins or other molecules that have an amino group in their structures (Fig. 1-10). This kit contains all of the necessary reagents for the labeling process, including Storage buffer. The labeling process is simple; just mix IgG with NH_2-reactive peroxidase and incubate at 37 °C for 2 hours. The NH_2-reactive peroxidase forms a covalent link with the target molecule without any activation process.

The distance of the NHS from peroxidase is about 1.2 nm, half of the radius of the peroxidase molecule. Therefore, the labeling efficiency of the NH_2-reactive peroxidase is high enough to eliminate the purification process after labeling when the peroxidase-labeled IgG is used for EIA. Also, peroxidase labeling will not affect the affinity of the target molecule. If a high purity conjugate is required after labeling, simply use an affinity column or a gel permeation column. When labeling small molecules, excess molecules can be removed by using the Filtration tubes included in this kit. Since the amino groups of NH_2-reactive peroxidase are blocked, no self-conjugation is possible.

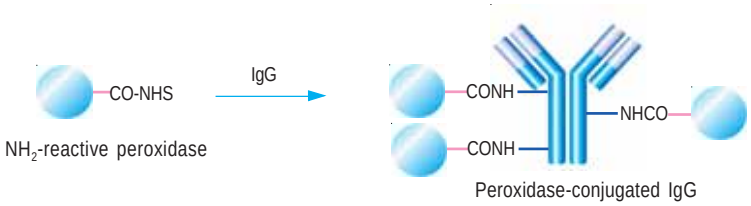


Fig. 1-10 IgG labeling reaction of NH_2-reactive peroxidase

Precaution

⌘ The molecular weight of the protein to be labeled with this kit should be over 50,000.
⌘ The molecular weight of the small amine compound to be labeled with this kit should be smaller than 5,000.
⌘ IgG or peroxidase-conjugated IgG is always on the filter membrane of the Filtration tube during the labeling process.
⌘ If the IgG solution contains other proteins with a molecular weight larger than 10,000, such as BSA or gelatin, purify the IgG solution prior to labeling peroxidase with this kit. IgG solution can be purified by IgG Purification Kits (not included in this kit).
⌘ If the IgG solution contains small insoluble materials, centrifuge the solution and use the supernatant for the labeling.

Required Equipment and Materials

LK11-10: microcentrifuge, 10 µl and 50-200 µl adjustable pipettes, 37 °C incubator, 0.5 ml microtubes
LK51-10: centrifuge, rotor for 15 ml centrifuge tube, 50-200 µl and 1 ml adjustable pipettes, 37 °C incubator, microtubes
LK52-10: centrifuge, rotor for 50 ml centrifuge tube, 1 ml adjustable pipette, 37 °C incubator, 15 ml tubes

I-1. Protein Labeling: Enzyme

General Protocol for IgG Labeling (LK11-10)

Add 100 µl Washing buffer and the sample solution containing 50-200 µg IgG to a Filtration tube.[a)]

Centrifuge at 8,000-10,000 g for 10 min. Add 100 µl Washing buffer and centrifuge once more.[b)]

Add 10 µl Reaction buffer to NH_2-reactive peroxidase and dissolve with pipetting.

Transfer the solution containing NH_2-reactive peroxidase onto the membrane of the Filtration tube where IgG is concentrated.

Rinse the entire surface of the membrane with the solution by pipetting and incubate the tube at 37 °C for 2 hrs.

Add 100 µl Washing buffer to the tube. If the volume of the filtrate is 300µl or more, discard the filtrate prior to going to step 7.

Centrifuge at 8,000-10,000 g for 10 min.[b)]

Add 200 µl Storage buffer and pipette 10 to 15 times to recover the conjugate.[c)] Transfer the solution to a 0.5 ml tube, and store the solution at 0-5 °C.[d)]

a) The recommended amount of IgG is 100 µg. The volume of sample solution should be 100 µl or less. If the antibody concentration is lower than 0.5 mg per ml, repeat steps 1 and 2 until the total IgG accumulation becomes 50-200 µg. If the volume of the filtrate becomes 400 µl or more during the accumulation process, discard the filtrate prior to going to the next centrifuge step.
b) If the solution still remains on the membrane after the centrifugation, spin another 5 min or increase the centrifuge speed.
c) The concentration of the conjugate is 0.5-1.3 mg per ml. Dilute the peroxidase-labeled IgG to prepare a solution with an appropriate concentration prior to using it for enzyme immunoassay, immunoblotting, or immunostaining. One to three molecules of peroxidase should be introduced onto one IgG molecule. Unconjugated peroxidase should not interfere with normal immunoassays. If purification is necessary, use a gel permeation column or an affinity column for IgG.
d) Generally, the peroxidase-labeled IgG in Storage buffer is stable for at least 2 months at 0-5 °C. For longer storage, add glycerol (final concentration: 50%), aliquot, and store at -20 °C. However, it is important to note that the stability will depend on the sample itself.

General Protocol for Small Molecule Labeling (LK11-10)

Prepare 50 µl of 1 mM amine compound solution with Reaction buffer,[a)] and add the solution to a tube of NH_2-reactive peroxidase. Pipette several times to mix and incubate at 37 °C for 1 hr.

Add 100 µl Washing buffer to the reaction solution and transfer the entire solution to a Filtration tube.

Centrifuge at 8,000-10,000 g for 10 min,[b)] discard the filtrate, and add 200 µl Washing buffer to the tube. Repeat this procedure once more. Centrifuge at 8,000-10,000 g for 10 min again.[b)]

Add 200 µl Storage buffer and pipette 10 to 15 times to recover the conjugate.[c)] Transfer the solution to a 0.5 ml tube and store the solution at 0-5 °C.[d)]

a) If the amine compound does not dissolve in aqueous solution, dissolve it with DMSO to prepare 10 mM solution and mix 5 µl of this solution with 45 µl Reaction buffer.
b) If the solution still remains on the membrane after the centrifugation, spin for another 5 min or increase the centrifuge speed.
c) The concentration of the conjugate is about 400-500 µg per ml. One to two target molecules should be conjugated with one peroxidase molecule.
d) The peroxidase-labeled small molecule should be stable for at least 6 months at 0-5 °C.

References

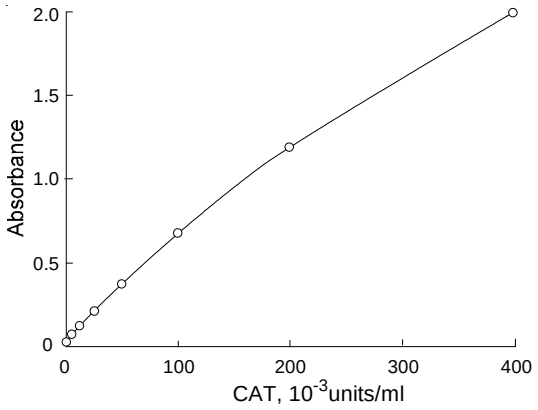
D. Kamimura, *et al.*, *J. Immunol.*, **177**, 306 (2006); K. Sumi, *et al.*, *Mol. Cell. Biol.*, **27**, 4248 (2007); R. Ohgaki, *et al.*, *J. Biol. Chem.*, **283**, 4417 (2008); T. Soeda, *et al.*, *J. Biol. Chem.*, **284**, 3379 (2009).

I-1. Protein Labeling: Enzyme

Experimental Examples

Fig. 1-11 Sandwich ELISA of CAT (chloramphenicol acetyl transferase) assay.

Plate: 2 ug/ml anti-CAT antibody (rabbit anti sera)-coated high binding plate
CAT: 0-400 x 10^{-3}units/ml PBST
Peroxidase-conjugated anti-CAT antibody:
Prepared by Peroxidase Labeling Kit-NH_2.1ug/ml PBST+blocking reagent
Substrate: TMB peroxidase substrate



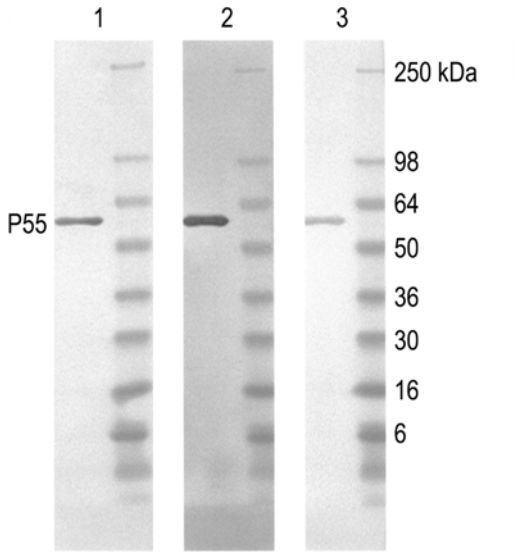


Fig. 1-12 Western blot using peroxidase-labeled monoclonal antibody to SIV p24 Gag(2F12). SIV P55 and molecular weight markers were analyzed in blot 1, 2, and 3.

Blot 1: conjugate prepared using Peroxidase Labeling Kit-NH_2
Blot 2: conjugate prepared using Peroxidase Labeling Kit-SH
Blot 3: primary antibody and peroxidase-conjugated secondary antibody (commercially available).

The western blotting using peroxidase-labeled primary antibody gives a better result than using peroxidase-labeled secondary antibody. In most cases, the sensitivity of the conjugate prepared with Peroxidase/ Alkaline phosphatase Labeling Kit-SH is higher than Labeling Kit-NH_2 due to the site specific conjugation on the antibody.

FAQ

- **Can I use this kit for Fab or Fab' labeling?**
  Yes, you can label Fab or Fab' using this kit. The recovery of the conjugate should be over 80%.
- **Can I use this kit for other proteins?**
  Yes, if the molecular weight is greater than 50,000 or less than 5,000 and it has a reactive primary or secondary amino group. If the molecular weight is higher than 50,000, follow the labeling protocol for IgG and use 0.5-1 nmol of sample protein for LK11-10. If the molecular weight is less than 5,000, follow the labeling protocol for small molecules. If the molecular weight is higher than 5,000 but lower than 50,000, contact our customer service at info@dojindo.com or 1-877-987-2667 for more information.
- **Can I use this kit to label an oligonucleotide or oligopeptide?**
  Yes, if the molecular weight is less than 5,000 and it has a reactive primary or secondary amino group. Follow the labeling protocol for small molecules.
- **What is the minimum amount of IgG that can be labeled with LK11-10?**
  The minimum amount is 50 µg. There is no significant difference in sensitivity and background between 50 µg and 200 µg of IgG. Though 10 µg IgG can still be labeled using this kit, the background will be higher
- **How many peroxidase molecules per IgG are introduced?**
  The average number of peroxidase molecule per IgG is 1 to 3.
- **Does unconjugated NH_2-reactive peroxidase still have an activated ester after the labeling reaction to IgG?**
  No. It is completely hydrolyzed during the reaction.
- **Does NH_2-reactive peroxidase form an oligomer during the labeling reaction?**
  No. Since all amino groups of NH_2-reactive peroxidase are blocked, no oligomerization is possible.
- **Do I have to use Storage buffer included with the kit?**
  No, you do not have to use Storage buffer from the kit. You can choose any kind of buffer appropriate for your experiment. However, the Storage buffer helps to increase the stability of the peroxidase conjugate.
- **Does Storage buffer contain animal products or polymers?**
  No, Storage buffer does not contain any animal products , polymers, or heavy metal ions.

Peroxidase Labeling Kit-SH

Application: Peroxidase labeling of proteins or thiol compounds

Features: Only 3 hours to get conjugates
All processes in a single Filtration tube
High recovery of conjugates

Ordering Information

Product code	Unit
LK09-10	3 samples[a)]
LK53-10	1 sample[b)]
LK54-10	1 sample[c)]

a) Based on 100 µg IgG sample labeling.
b) Based on 1 mg IgG sample labeling.
c) Based on 10 mg IgG sample labeling.

Contents of the Kit

LK09-10

SH-reactive peroxidase ..... 100 µg x 3 tubes
Solution A ..... 4 µl x 1 bottle
Reaction buffer ..... 200 µl x 1 tube
Filtration tube ..... 3 tubes
Reducing agent ..... 3 tubes
Solution B ..... 1 ml x 1 tube
Storage buffer ..... 4 ml x 1 bottle

LK53-10

SH-reactive peroxidase ..... 1 mg x 1 tube
Solution A ..... 10 ml x 1 bottle
Reaction buffer ..... 0.6 ml x 1 tube
Filtration tube ..... 1 tube
Reducing agent ..... 1 tube
Solution B ..... 4 ml x 1 bottle
Storage buffer ..... 10 ml x 1 bottle
15 ml tube ..... 1 tube

LK54-10

SH-reactive peroxidase ..... 5 mg x 2 tubes
Solution A ..... 50 ml x 1 bottle
Reaction buffer ..... 6 ml x 1 bottle
Filtration tube ..... 2 tubes
Reducing agent ..... 2 tubes
Solution B ..... 12 ml x 1 bottle
Storage buffer ..... 50 ml x 1 bottle

Storage Condition	Shipping Condition
0-5 °C	ambient temperature

Product Description

Peroxidase Labeling Kit-SH is mainly used for the preparation of peroxidase-labeled IgG for enzyme immunoassay (EIA) and for the preparation of peroxidase-labeled antigen for competitive EIA. SH-reactive peroxidase, a component of this kit, can react with the thiol groups of proteins or other molecules (Fig. 1-13). This kit contains all of the necessary reagents for the labeling process, including Reducing agent and Storage buffer. SH-reactive peroxidase forms a covalent link with the target molecule. Reducing agent can create free thiol groups in the IgG molecule. The labeling efficiency of the SH-reactive peroxidase is high enough to eliminate any purification process after labeling when the peroxidase-labeled IgG is used for EIA. If a high purity conjugate is required after labeling, simply use an affinity column or a gel permeation column. When labeling small molecules, excess molecules can be removed by using the Filtration tubes included in this kit.

Fig. 1-13 IgG labeling reaction of SH-reactive peroxidase

Precaution

⌘ *The molecular weight of the reduced protein with this kit should be over 50,000.*
⌘ *The molecular weight of the small thiol compound to be labeled with this kit should be smaller than 5,000.*
⌘ *IgG or peroxidase-conjugated IgG is always on the filter membrane of the Filtration tube during the labeling process.*
⌘ *If the IgG solution contains other proteins with molecular weight larger than 10,000, such as BSA or gelatin, purify the IgG solution prior to labeling peroxidase with this kit. IgG solution can be purified by IgG Purification Kits (not included in this kit).*
⌘ *If the IgG solution contains small insoluble materials, centrifuge the solution and use the supernatant for the labeling.*

Required Equipment and Materials

LK09-10: microcentrifuge, 50-200 µl adjustable pipette, 37 °C incubator, 0.5 ml microtubes
LK53-10: centrifuge, rotor for 15 ml centrifuge tube, 50-200 µl and 1 ml adjustable pipettes, 37 °C incubator, microtubes
LK54-10: centrifuge, rotor for 50 ml centrifuge tube, 1 ml adjustable pipette, 37 °C incubator, 15 ml tubes

I-1. Protein Labeling: Enzyme

General Protocol for IgG Labeling (LK09-10)

Add 100 µl Solution A and the sample solution containing 50-200 µg IgG and to a Filtration tube.[a)]

Mix the solution with pipetting several times and centrifuge at 8,000-10,000 g for 10 min.[b)]

Add 150 µl Solution A to Reducing agent and dissolve it with pipetting several times.

Transfer 100 µl the solution from step 3 onto the membrane of the Filtration tube where IgG is concentrated.

Pipette several times and incubate the tube at 37 °C for 30 min.

Add 100 µl Solution B to the tube and centrifuge at 8,000-10,000 g for 10 min. Discard the filtrate, add 200 µl Solution B, and centrifuge again.[b)]

Add 50 µl Reaction buffer to SH-reactive peroxidase and dissolve it with pipetting.

Transfer the SH-reactive peroxidase solution onto the membrane of the Filtration tube where reduced IgG is concentrated.

Pipette several times and incubate the tube at 37 °C for 1 hr.

Add 100 µl Solution A to the tube and centrifuge at 8,000-10,000 g for 10 min.[b)]

Add 200 µl Storage buffer and pipette 10 to 15 times to recover the conjugate.[c)] Transfer the solution to a 0.5 ml tube and store the solution at 0-5 °C.[d)]

a) The recommended amount of IgG is 100 µg. The volume of the sample solution should be 100 µl or less. If the antibody concentration is lower than 0.5 mg per ml, repeat steps 1 and 2 until the total IgG accumulation becomes 50-200 µg. If the volume of the filtrate becomes 400 µl or more during the accumulation process, discard the filtrate prior to going to the next centrifuge step.

b) If the solution still remains on the membrane after the centrifugation, spin another 5 min or increase the centrifuge speed.

c) The concentration of the conjugate is 0.5-1.3 mg per ml. Dilute the peroxidase-labeled reduced IgG to prepare a solution with an appropriate concentration prior to using it for enzyme immunoassay, immunoblotting, or immunostaining. One to two molecules of peroxidase should be introduced onto one reduced IgG molecule. Unconjugated peroxidase should not interfere with normal immunoassays. If purification is necessary, use a gel permeation column or an affinity column for IgG.

d) Generally, the peroxidase-labeled reduced IgG in Storage buffer is stable for at least 2 months at 0-5 °C. For longer storage, add glycerol (final concentration: 50%), aliquot, and store at -20 °C. However, it is important to note that the stability will depend on the sample itself.

General Protocol for Small Molecule Labeling (LK09-10)

Prepare 50 µl of 1 mM thiol compound solution with Reaction buffer,[a)] and add the solution to a tube of SH-reactive peroxidase. Pipette several times to mix and incubate at 37 °C for 1 hr.

Add 100 µl Solution A to the reaction solution and transfer the entire solution to a Filtration tube.

Centrifuge at 8,000-10,000 g for 10 min,[b)] discard the filtrate and add 200 µl Solution A to the tube. Repeat this procedure once more. Centrifuge at 8,000 - 10,000 g for 10 min again.[b)]

Add 200 µl Storage buffer and pipette 10 to 15 times to recover the conjugate.[c)] Transfer the solution to a 0.5 ml tube and store the solution at 0-5 °C.[d)]

a) If the thiol compound does not dissolve in aqueous solution, dissolve it with DMSO to prepare 10 mM solution and mix 5 µl of this solution with 45 µl Reaction buffer.
b) If the solution still remains on the membrane after the centrifugation, spin for another 5 min or increase the centrifuge speed.
c) The concentration of the conjugate is about 400-500 µg per ml. One to two target molecules should be conjugated with one peroxidase molecule.
d) The peroxidase-labeled small molecule should be stable for at least 6 months at 0-5 °C.

Experimental Example

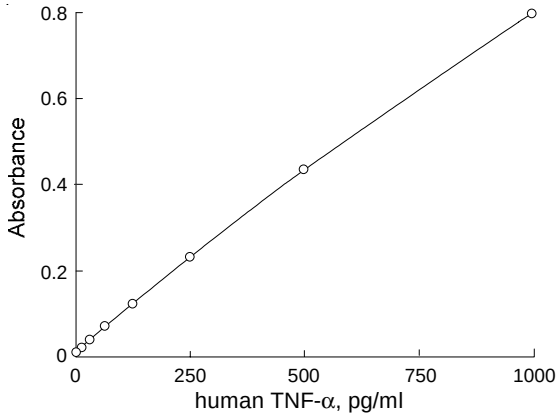


Fig. 1-14 Sandwich ELISA of human TNF-α detection

Plate: 2 µg/ml anti-human TNF-α antibody (rabbit, polyclonal)-coated high binding plate
recombinant human TNF-α:
0-1000 pg/ml PBST
Peroxidase-conjugated anti-human TNF-α antibody:
Prepared by Peroxidase Labeling Kit-SH.1µg/ml PBST+blocking reagent
Substrate: TMB peroxidase substrate

FAQ

- **Can I use this kit for F(ab')$_2$?**
  Yes, please follow the labeling protocol for IgG. The recovery of the conjugate should be over 80%.
- **Can I use this kit for other proteins or peptides?**
  Yes, if the molecular weight of the reduced form is greater than 50,000 or less than 5,000, and it has a reactive SH group, or a disulfide group that can be reduced without losing activity. If the molecular weight is greater than 50,000, follow the labeling protocol for IgG, and use 0.5-1 nmol of sample protein for LK09-10. If the molecular weight is less than 5,000, follow the labeling protocol for small molecules. If the molecular weight is between 5,000 and 50,000, contact our customer service at info@dojindo.com or 1-877-987-2667 for more information.
- **Can I use this kit to label oligopeptides or oligonucleotides?**
  Yes, if the molecular weights of the oligonucleotide or the oligopeptide are less than 5,000 and they have at least one SH group. Follow the labeling protocol for small molecule.
- **What is the minimum amount of IgG that can be labeled with LK09-10?**
  The minimum amount is 50 µg. There is no significant difference in sensitivity and background between 50 µg and 200 µg of IgG. However, even 10 µg IgG can be labeled using 1/5 volume of SH-reactive peroxidase solution at Step 8.
- **How many peroxidase molecules per reduced IgG are introduced?**
  The average number of peroxidase molecule per reduced IgG is 1 to 2.
- **Do I have to use a Filtration tube prior to labeling the protein?**
  If the protein solution does not contain small molecules with reactive SH groups and the concentration of the protein is 10 mg per ml, or about 70 µM, there is no need to use the Filtration tube. Just mix the sample solution with Solution B and add the mixture to a vial of the SH-reactive peroxidase.
- **Do I have to use Storage buffer included with the kit?**
  No, you don't have to use Storage buffer from the kit. You can choose any kind of buffer appropriate for your experiment. However, the Storage buffer helps to increase the stability of the peroxidase conjugate.
- **My sample contains small insoluble material. What should I do?**
  Spin the sample and use the supernatant for labeling.
- **Does unconjugated SH reactive peroxidase still have a reactive maleimide after the labeling reaction to IgG?**
  No. Nearly 100% of SH reactive peroxidase is used for the IgG labeling or the small molecule labeling.
- **Does Storage buffer contain animal products or polymers?**
  No, Storage buffer does not contain any animal products, polymers, or heavy metal ions.

References

K. Sumi, *et al.*, *Mol. Cell. Biol.*, **27**, 4248 (2007); K. Inoue, *et al.*, *Arterioscler. Thromb. Vasc. Biol.*, **27**, 161 (2007).

Alkaline Phosphatase Labeling Kit-NH_2

Application: Alkaline phosphatase labeling of proteins or amine compounds

Features: Only 3 hours to get conjugates
All processes in a single Filtration tube
High recovery of conjugates

Ordering Information

Product code	Unit
LK12-10	3 samples[a)]
LK59-10	1 sample[b)]

a) Based on 100 µg IgG sample labeling.
b) Based on 1 mg IgG sample labeling.

Contents of the Kit

LK12-10

NH_2-reactive ALP ..... 100 µg x 3 tubes
Reaction buffer ..... 200 µl x 1 tube
Filtration tube ..... 3 tubes
Washing buffer ..... 4 ml x 1 bottle
Storage buffer ..... 4 ml x 1 bottle

LK59-10

NH_2-reactive ALP ..... 1 mg x 1 tube
Reaction buffer ..... 1.2 ml x 1 tube
Filtration tube ..... 1 tube
Washing buffer ..... 10 ml x 1 bottle
Storage buffer ..... 10 ml x 1 bottle
15 ml tube ..... 1 tube

* *ALP: alkaline phosphatase*

Storage Condition	Shipping Condition
0-5 °C	with blue ice

Product Description

Alkaline Phosphatase Labeling Kit-NH_2 is mainly used for the preparation of alkaline phosphatase-labeled IgG for enzyme immunoassay (EIA) and for the preparation of alkaline phosphatase-labeled antigen for competitive EIA. NH_2-reactive ALP, a component of this kit, can react with amino groups of proteins or other molecules (Fig. 1-15). This kit contains all of the necessary reagents for the labeling process including Storage buffer. NH_2-reactive ALP forms a covalent link with the target molecule without any activation process. The labeling efficiency of the NH_2-reactive ALP is high enough to eliminate any purification process after labeling when the alkaline phosphatase-labeled IgG is used for EIA. If a high purity conjugate is required after labeling, simply use an affinity column or a gel permeation column. When labeling small molecules, excess molecules can be removed by using the Filtration tubes included in this kit.

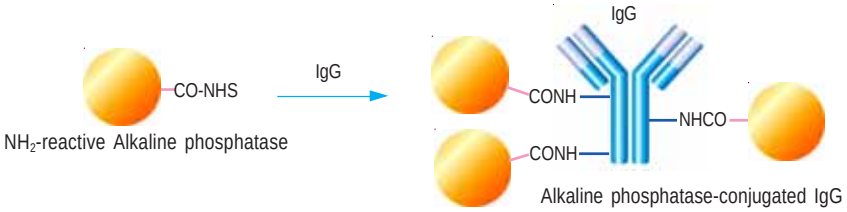


Fig. 1-15 IgG labeling reaction of NH_2-reactive peroxidase

Precaution

⌘ *The molecular weight of the protein to be labeled with this kit should be over 50,000.*
⌘ *The molecular weight of the small amine compound to be labeled with this kit should be smaller than 5,000.*
⌘ *IgG or alkaline phosphatase-conjugated IgG is always on the filter membrane of the Filtration tube during the labeling process.*
⌘ *If the IgG solution contains other proteins with molecular weight larger than 10,000, such as BSA or gelatin, purify the IgG solution prior to labeling alkaline phosphatase with this kit. IgG solution can be purified by IgG Purification Kits (not included in this kit).*
⌘ *If the IgG solution contains small insoluble materials, centrifuge the solution and use the supernatant for the labeling.*

Required Equipment and Materials

LK12-10: microcentrifuge, 10 µl and 50-200 µl adjustable pipettes, 37 °C incubator, 0.5 ml microtubes
LK59-10: centrifuge, rotor for 15 ml centrifuge tube, 50-200 µl and 1 ml adjustable pipettes, 37 °C incubator, microtubes

I-1. Protein Labeling: Enzyme

General Protocol for IgG Labeling (LK12-10)

Add 100 µl Washing buffer and the sample solution containing 50-200 µg IgG to a Filtration tube.[a)]

Centrifuge at 8,000-10,000 g for 10 min. Add 100 µl Washing buffer and centrifuge once more.[b)]

After finishing the centrifuge, add 10 µl Reaction buffer to NH_2-reactive ALP and dissolve with pipetting.

Transfer the solution containing NH_2-reactive ALP onto the membrane of the Filtration tube where IgG is concentrated.

Rinse the entire surface of the membrane with the solution by pipetting and incubate the tube at 37 °C for 2 hrs.

Add 190 µl Storage buffer and pipette 10 to 15 times to recover the conjugate.[c)] Transfer the solution to a 0.5 ml tube and store the solution at 0-5 °C.[d)]

a) The recommended amount of IgG is 100 µg. The volume of sample solution should be 100 µl or less. If the antibody concentration is lower than 0.5 mg per ml, repeat steps 1 and 2 until the total IgG accumulation becomes 50-200 µg.
b) If the solution still remains on the membrane after the centrifugation, spin another 5 min or increase the centrifuge speed.
c) The concentration of the conjugate is 0.5-1.3 mg per ml. Dilute the alkaline phosphatase-labeled IgG to prepare a solution with an appropriate concentration prior to using it for enzyme immunoassay, immunoblotting, or immunostaining. One to three molecules of alkaline phosphatase should be introduced onto one IgG molecule. Unconjugated alkaline phosphatase should not interfere with normal immunoassays. If purification is necessary, use a gel permeation column or an affinity column for IgG.
d) Generally, the alkaline phosphatase-labeled IgG in Storage buffer is stable for at least 2 months at 0-5 °C. For longer storage, store at -20 °C. However, it is important to note that the stability will depend on the sample itself.

General Protocol for Small Molecule Labeling (LK12-10)

Prepare 50 µl of 1 mM amine compound solution with Reaction buffer,[a)] and add the solution to a tube of NH_2-reactive ALP. Pipette several times to mix and incubate at 37 °C for 1 hr.

Add 100 µl Washing buffer to the reaction solution, and transfer the entire solution to a Filtration tube.

Centrifuge at 8,000-10,000 g for 10 min,[b)] discard the filtrate and add 200 µl Washing buffer to the tube. Repeat this procedure once more. Centrifuge at 8,000-10,000 g for 10 min again.[b)]

Add 200 µl Storage buffer, and pipette 10 to 15 times to recover the conjugate.[c)] Transfer the solution to a 0.5 ml tube and store the solution at 0-5 °C.[d)]

a) If the amine compound does not dissolve in aqueous solution, dissolve it with DMSO to prepare 10 mM solution and mix 5 µl of this solution with 45 µl Reaction buffer.
b) If the solution still remains on the membrane after the centrifugation, spin for another 5 min or increase the centrifuge speed.
c) The concentration of the conjugate is about 400-500 µg per ml. One to two target molecules should be conjugated with one alkaline phosphatase molecule.
d) The alkaline phosphatase-labeled small molecule should be stable for at least 6 months at 0-5 °C.

I-1. Protein Labeling: Enzyme

Experimental example

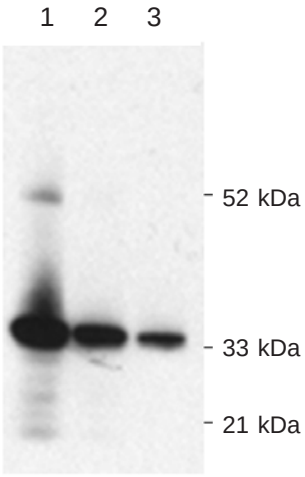


Fig. 1-16 Western blotting of Alkaline phosphatase-conjugated antibody prepared by Alkaline phosphatase Labeling Kit-NH_2.

1: antigen 50 ng
2: antigen 10 ng
3: antigen 2 ng

A target protein (antigen) was detected with ALP-labeled antibody prepared by Alkaline Phosphatase Labeling Kit-NH_2 after it was run with SDS-PAGE and transferred to a nitrocellulose membrane. A target protein was detected with a chemiluminescence substrate for alkaline phosphatase after the treatment with 25,000 times dilution of ALP-labeled primary antibody.

FAQ

◆ Can I use this kit for Fab or Fab' labeling?
Yes, you can label Fab and Fab' using this kit. The recovery of the conjugate should be over 80%.

◆ Can I use this kit for other proteins or peptides?
Yes, if the molecular weight is higher than 50,000 or lower than 5,000, and it has a reactive primary or secondary amino group. If the molecular weight is higher than 50,000, follow the labeling protocol for IgG, and use 0.5-1 nmol of sample protein for LK12-10. If the molecular weight is lower than 5,000, follow the labeling protocol for small molecules. If the molecular weight is lower than 50,000 but higher than 5,000, contact our customer service at info@dojindo.com or 1-877-987-2667 for more information.

◆ Can I use this kit to label an oligonucleotide or oligopeptide?
Yes, if the molecular weight is less than 5,000, and it has a reactive primary or secondary amino group. Follow the labeling protocol for small molecules.

◆ What is the minimum amount of IgG that can be labeled with LK12-10?
The minimum amount is 50 µg. There is no significant difference in sensitivity and background between 50 µg and 200 µg of IgG. Though 10 µg IgG can still be labeled using this kit, the background will be higher.

◆ How many alkaline phosphatase molecules are introduced per IgG?
The average number of alkakine phosphatase molecule per IgG is 1 to 3.

◆ Does unconjugated NH_2-reactive alkaline phosphatase still have an activated ester after the labeling reaction with IgG?
No. NHS is completely hydrolyzed during the reaction.

◆ Does NH_2-reactive alkaline phosphatase form an oligomer during the labeling reaction?
No. Since all reactive amino groups of NH_2-reactive alkaline phosphatase are blocked, no oligomerization is possible.

◆ Do I have to use Storage buffer included with the kit?
No, you do not have to use Storage buffer from the kit. You can choose any kind of buffer appropriate for your experiment.

◆ Does Storage buffer contain animal products or polymers?
No, Storage buffer does not contain any animal products , polymers, or heavy metal ions.

Alkaline Phosphatase Labeling Kit-SH

Application: Alkaline phosphatase labeling of proteins or thiol compounds

Features: Only 3 hours to get conjugates
All processes in a single Filtration tube
High recovery of conjugates

Ordering Information

Product code	Unit
LK13-10	3 samples[a)]
LK61-10	1 sample[b)]

a) Based on 100 µg IgG sample labeling.
b) Based on 1 mg IgG sample labeling.

Contents of the Kit

LK13-10

SH-reactive ALP .....100 µg x 3 tubes
Solution A ..... 4 ml x 1 bottle
Reaction buffer ..... 200 µl x 1 tube
Filtration tube .....3 tubes
Reducing agent .....3 tubes
Solution B .....1 ml x 1 tube
Storage buffer ..... 4 ml x 1 bottle

LK61-10

SH-reactive ALP .....1 mg x 1 tube
Solution A ..... 10 ml x 1 bottle
Reaction buffer ..... 0.6 ml x 1 tube
Filtration tube .....1 tube
Reducing agent .....1 tube
Solution B .....4 ml x 1 bottle
Storage buffer .....10 ml x 1 bottle
15 ml tube ..... 1 tube

** ALP: alkaline phosphatase*

Storage Condition	Shipping Condition
0-5 °C	ambient temperature

Product Description

Alkaline Phosphatase Labeling Kit-SH is mainly used for the preparation of alkaline phosphatase-labeled IgG for enzyme immunoassay (EIA) and for the preparation of alkaline phosphatase-labeled antigen for competitive EIA. SH-reactive ALP, a component of this kit, can react with the thiol groups of proteins or other molecules (Fig. 1-17). This kit contains all of the necessary reagents for the labeling process including Reducing agent and Storage buffer. SH-reactive ALP forms a covalent link with the target molecule. Reducing agent can create free thiol groups in the IgG molecule. The labeling efficiency of the SH-reactive ALP is high enough to eliminate any purification process after labeling when the alkaline phosphatase-labeled IgG is used for EIA. If a high purity conjugate is required after labeling, simply use an affinity column or a gel permeation column. When labeling small molecules, excess molecules can be removed by using the Filtration tubes included in this kit.

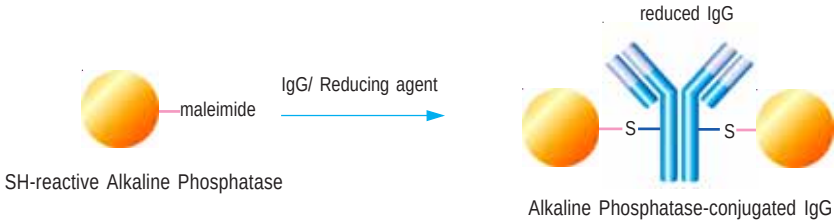


Fig. 1-17 IgG labeling reaction of SH-reactive alkaline phosphatase

Precaution

⌘ The molecular weight of the reduced protein to be labeled with this kit should be over 50,000.
⌘ The molecular weight of the small amine compound to be labeled with this kit should be smaller than 5,000.
⌘ IgG or alkaline phosphatase-conjugated IgG is always on the filter membrane of the Filtration tube during the labeling process.
⌘ If the IgG solution contains other proteins with molecular weight larger than 10,000, such as BSA or gelatin, purify the IgG solution prior to labeling alkaline phosphatase with this kit. IgG solution can be purified by IgG Purification Kits (not included in this kit).
⌘ If the IgG solution contains small insoluble materials, centrifuge the solution and use the supernatant for the labeling.

Required Equipment and Materials

LK13-10: microcentrifuge, 50-200 µl adjustable pipette, 37 °C incubator, 0.5 ml microtubes
LK61-10: centrifuge, rotor for 15 ml centrifuge tube, 50-200 µl and 1 ml adjustable pipettes, 37 °C incubator, microtubes

I-1. Protein Labeling: Enzyme

General Protocol for IgG Labeling (LK13-10)

Add 100 µl Solution A and the sample solution containing 50-200 µg IgG to a Filtration tube.[a)]

Mix the solution with pipetting several times and centrifuge at 8,000-10,000 g for 10 min.[b)]

Add 150 µl Solution A to Reducing agent and dissolve it with pipetting several times.

Transfer 100 µl the solution from step 3 onto the membrane of the Filtration tube where IgG is concentrated.

Pipette several times and incubate the tube at 37 °C for 30 min.

Add 100 µl Solution B to the tube and centrifuge at 8,000-10,000 g for 10 min. Discard the filtrate, add 200 µl Solution B, and centrifuge again.[b)]

Add 50 µl Reaction buffer to SH-reactive ALP and dissolve it with pipetting.

Transfer the SH-reactive ALP solution onto the membrane of the the Filtration tube where reduced IgG is concentrated.

Pipette several times and incubate the tube at 37 °C for 1 hr.

Add 150 µl Storage buffer and pipette 10 to 15 times to recover the conjugate.[c)] Transfer the solution to a 0.5 ml tube and store the solution at 0-5 °C.[d)]

a) The recommended amount of IgG is 100 µg. The volume of sample solution should be 100 µl or less. If the antibody concentration is lower than 0.5 mg per ml, repeat steps 1 and 2 until the total IgG accumulation becomes 50-200 µg.
b) If the solution still remains on the membrane after the centrifugation, spin another 5 min or increase the centrifuge speed.
c) The concentration of the conjugate is 0.5-1.3 mg per ml. Dilute the alkaline phosphatase-labeled reduced IgG to prepare a solution with an appropriate concentration prior to using it for enzyme immunoassay, immunoblotting, or immunostaining. One to two molecules of alkaline phosphatase should be introduced onto one reduced IgG molecule. Unconjugated alkaline phosphatase should not interfere with normal immunoassays. If purification is necessary, use a gel permeation column or an affinity column for IgG.
d) Generally, the alkaline phosphatase-labeled reduced IgG in Storage buffer is stable for at least 2 months at 0-5 °C. For longer storage, store at -20 °C. However, it is important to note that the stability will depend on the sample itself.

General Protocol for Small Molecule Labeling (LK13-10)

Prepare 50 µl of 1 mM thiol compound solution with Reaction buffer,[a)] and add the solution to a tube of SH-reactive ALP. Pipette several times to mix and incubate at 37 °C for 1 hr.

Add 100 µl Solution A to the reaction solution, and transfer the entire solution to a Filtration tube.

Centrifuge at 8,000 - 10,000 g for 10 min,[b)] discard the filtrate, and add 200 µl Solution A to the tube. Repeat this procedure once more. Centrifuge at 8,000-10,000 g for 10 min again.[b)]

Add 200 µl Storage buffer and pipette 10 to 15 times to recover the conjugate.[c)] Transfer the solution to a 0.5 ml tube, and store the solution at 0-5 °C.[d)]

I-1. Protein Labeling: Enzyme

a) If the thiol compound does not dissolve in aqueous solution, dissolve it with DMSO to prepare 10 mM solution, and mix 5 µl of this solution with 45 µl Reaction buffer.
b) If the solution still remains on the membrane after the centrifugation, spin for another 5 min or increase the centrifuge speed.
c) The concentration of the conjugate is about 400-500 µg per ml. One to two target molecules should be conjugated with one alkaline phosphatase molecule.
d) The alkaline phosphatase-labeled small molecule should be stable for at least 6 months at 0-5 °C.

Experimental Example

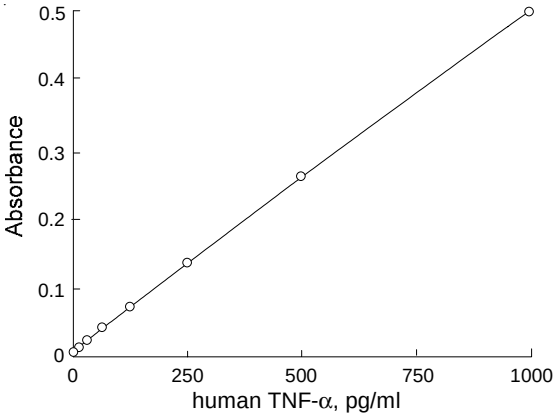


Fig. 1-18 Sandwich ELISA of human TNF-α detection

Plate: 2ug/ml anti-human TNF-α antibody (rabbit, polyclonal)-coated high binding plate
recombinant human TNF-α:
0-1000 pg/ml PBST
ALP-conjugated anti-human TNF-α antibody:
Prepared by Alkaline phosphatase Labeling Kit-SH .1ug/ml PBST+blocking reagent
Substrate: *p*-Nitrophenylphosphate, ALP substrate

FAQ

- **Can I use this kit for F(ab')$_2$?**
  Yes, please follow the labeling protocol for IgG. The recovery of the conjugate should be over 80%.
- **Can I use this kit for other proteins or peptides?**
  Yes, if the molecular weight of the reduced form is greater than 50,000 or less than 5,000, and it has a reactive SH group or a disulfide group that can be reduced without losing activity. If the molecular weight is greater than 50,000, follow the labeling protocol for IgG and use 0.5-1 nmol of sample protein for LK13-10. If the molecular weight is less than 5,000, follow the labeling protocol for small molecules. If the molecular weight is between 5,000 and 50,000, contact our customer service at info@dojindo.com or 1-877-987-2667 for more information.
- **Can I use this kit to label oligopeptides or oligonucleotides?**
  Yes, if the molecular weights of the oligonucleotide or the oligopeptide are less than 5,000 and they have at least one SH group. Follow the labeling protocol for small molecule.
- **What is the minimum amount of IgG that can be labeled with LK13-10?**
  The minimum amount is 50 µg. There is no significant difference in sensitivity and background between 50 µg and 200 µg of IgG. However, even 10 µg IgG can be labeled, using 1/5 volume of SH-reactive ALP solution at step 8.
- **How many alkaline phosphatase molecules per reduced IgG are introduced?**
  The average number of alkaline phosphatase molecule per reduced IgG is 1 to 2.
- **Do I have to use a Filtration tube prior to labeling the protein?**
  If the protein solution does not contain small molecules with reactive SH groups and the concentration of the protein is 10 mg per ml, or about 70 µM, there is no need to use the Filtration tube. Just mix 10 µl of the sample solution with Solution B and add the mixture to a vial of the SH-reactive peroxidase.
- **Do I have to use Storage buffer included with the kit?**
  No, you don't have to use Storage buffer from the kit. You can choose any kind of buffer appropriate for your experiment.
- **My sample contains small insoluble material. What should I do?**
  Spin the sample and use the supernatant for labeling.
- **Does unconjugated SH-reactive ALP still have a reactive maleimide after the labeling reaction to IgG?**
  No. Nearly 100% of SH-reactive ALP is used for the IgG labeling or the small molecule labeling.
- **Does Storage buffer contain animal products or polymers?**
  No, Storage buffer does not contain any animal products , polymers, or heavy metal ions.

I-2. Protein Labeling: Phycobiliprotein

R-Phycoerythrin Labeling Kit-NH_2

Application: R-Phycoerythrin labeling of proteins or amine compounds

Features: Only 3 hours to get conjugates
All processes in a single Filtration tube
High recovery of conjugates
Applicable for 50-200 µg IgG

Ordering Information

Product code	Unit
LK23-10	3 samples*

* Based on 100 µg IgG labeling.

Contents of the Kit

NH_2-reactive R-PE ..... 350 µg x 3 tubes
Reaction buffer ..... 200 µl x 1 tube
WS buffer ..... 4 ml x 1 bottle
Filtration tube ..... 3 tubes

**R-PE: R-phycoerythrin*

Storage Condition	Shipping Condition
0-5 °C	with blue ice

Product Description

Phycobiliproteins are fluorescent proteins derived from cyanobacteria and eukaryotic algae. Their fluorescence is much higher than chemical fluorescent probes such as fluorescein and rhodamine. R-Phycoerythrin (R-PE) is one of the phycobiliproteins and has a red fluorescence at around 578 nm, and it can be excited at 488nm (Fig. 1-19). Because of this high fluorescence, phycobiliprotein labeled antibodies, and other molecules can give greater sensitivity in flow cytometry and immunostaining (Fig. 1-20). R-Phycoerythrin Labeling Kit-NH_2 is for simple and rapid preparation of R-PE-labeled IgG (Fig. 1-21). NH_2-reactive R-PE (a component of this kit) has an activated ester group and can easily make a covalent bond with an amino group of the target molecule without any activation process. The Filtration tube in this kit allows a quick buffer exchange and concentration of sample IgG solution. This kit contains all of the necessary reagents for R-PE labeling, including the Storage buffer for conjugates.

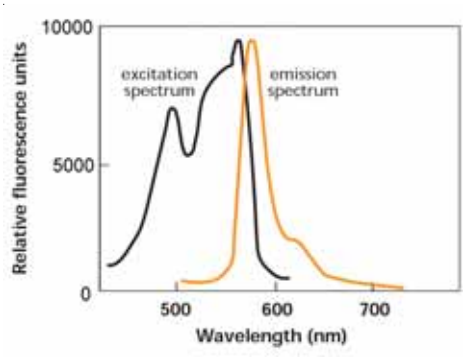


Fig. 1-19 Fluorescence spectrum of R-PE

Excitation wavelength: 566 nm
Emission wavelength: 578 nm

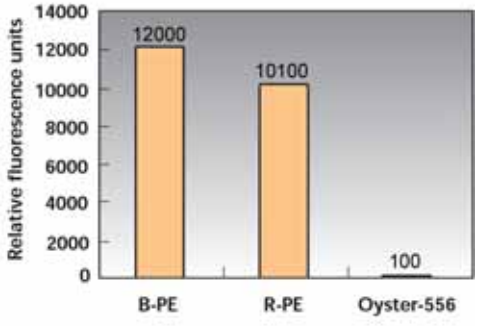
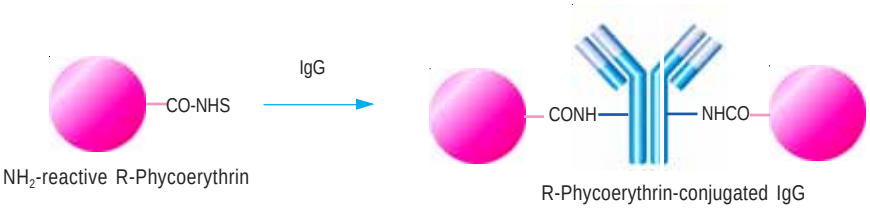


Fig. 1-20 Comparison of fluorescence intensity of Phycobiliproteins and cyanine dye

The 575 nm fluorescence of Phycoerythrin excited at 565 nm was compared with the 578 nm fluorescence of Oyster-556* excited at 556 nm.
**Oyster-556 is a cyanine dye. Oyster is a trademark of denovoBiolables GmbH.*



NH_2-reactive R-Phycoerythrin

R-Phycoerythrin-conjugated IgG

Fig. 1-21 IgG labeling reaction of NH_2-reactive R-PE

Required Equipment and Materials

microcentrifuge, 10 µl and 100-200 µl adjustable pipettes, 37 °C incubator, 0.5 ml microtubes

I-2. Protein Labeling: Phycobiliprotein

Precaution

⌘ The molecular weight of the protein to be labeled with this kit should be over 50,000.
⌘ IgG or R-Phycoerythrin-conjugated IgG is always on the filter membrane of the Filtration tube during the labeling process.
⌘ If the IgG solution contains other proteins with molecular weight larger than 10,000, such as BSA or gelatin, purify the IgG solution prior to labeling R-Phycoerythrin with this kit. IgG solution can be purified by IgG Purification Kits (not included in this kit).
⌘ If the IgG solution contains small insoluble materials, centrifuge the solution and use the supernatant for the labeling.

General Protocol for IgG Labeling

Add 100 µl WS buffer and the sample solution containing 100 µg IgG to a Filtration tube.[a)]

Centrifuge at 8,000-10,000 g for 10 min. Add 100 µl WS buffer and centrifuge once more.[b)]

After finishing the centrifuge, add 10 µl Reaction buffer to NH_2-reactive R-PE and dissolve with pipetting.

Transfer the solution containing NH_2-reactive R-PE onto the membrane of the Filtration tube where IgG is concentrated.

Rinse the entire surface of the membrane with the solution by pipetting, and incubate the tube at 37 °C for 2 hrs.

Add 190 µl WS buffer and pipette 10 to 15 times to recover the conjugate.[c)] Transfer the solution to a 0.5 ml tube, and store the solution at 0-5 °C.[d)]

a) The volume of sample solution should be 100 µl or less. If the IgG concentration is lower than 0.5 mg per ml, repeat steps 1 and 2 until the total IgG accumulation becomes 100 µg.
b) If the solution still remains on the membrane after the centrifugation, centrifuge for another 5 min or increase the centrifuge speed.
c) The concentration of the conjugate is 1.4-1.8 mg per ml. Dilute the R-PE-labeled IgG to prepare a solution with an appropriate concentration prior to using it for flow cytometry, immunoblotting, or immunostaining. One to two of R-PE molecules should be introduced onto one IgG molecule. Unconjugated R-PE should not interfere with a normal assay. If purification is necessary, use a gel permeation column or an affinity column for IgG.
d) Generally, the R-PE-labeled IgG in Storage buffer is stable for at least 2 months at 0-5 °C. For longer storage, add glycerol (final concentration: 50%), aliquot, and store at -20 °C. However, it is important to note that the stability will depend on the sample itself.

FAQ

◆ Can I use this kit for other proteins?
Yes, if the molecular weight is higher than 50,000, and it has a reactive primary or secondary amino group. Follow the protocol for IgG labeling with 0.5-1 nmol of sample protein.

◆ How many R-PE molecules per IgG are introduced?
The average number of R-PE molecule per IgG is 1 to 2.

◆ Does unconjugated NH_2-reactive R-PE still have an activated ester after the labeling reaction to IgG?
No. It is completely hydrolyzed during the reaction.

◆ Does NH_2-reactive R-PE form an oligomer during the labeling reaction?
No. Since all amino groups of NH_2-reactive R-PE are blocked, no oligomerization is possible.

◆ What is the minimum amount of IgG that can be labeled with LK23-10?
The minimum amount is 50 µg. There is no significant difference in sensitivity and background between 50 µg and 200 µg of IgG.

◆ Do I have to use WS buffer included with the kit?
Yes. Use the WS buffer to prepare a stock solution of the conjugate. However, you can choose any kind of buffer appropriate to dilute the conjugate stock solution for your experiment.

◆ How long is the conjugate stable?
If you store at 4 °C, it is stable for over 2 months. For longer storage, add 100% volume of glycerol, aliquot, and store at -20 °C. However, please note that the stability depends on the protein itself.

References

K. Nagao, *et al.*, *PNAS*, **106**, 3312 (2009).

I-2. Protein Labeling: Phycobiliprotein

R-Phycoerythrin Labeling Kit-SH

Application: R-Phycoerythrin labeling of proteins

Features: Only 3 hours to get conjugates
All processes in a single Filtration tube
High recovery of conjugates
Applicable for 50-200 µg IgG

Ordering Information

Product code	Unit
LK26-10	3 samples*

* Based on 100 µg IgG labeling.

Contents of the Kit

SH-reactive R-PE ..... 350 µg x 3 tubes
WS buffer ..... 4 ml x 1 bottle
Filtration tube ..... 3 tubes
Reducing agent ..... 3 tubes
Reaction buffer ..... 200 µl x 1 tube
RA solution .....1 ml x 1 tube

**R-PE: R-Phycoerythrin*

Storage Condition	Shipping Condition
0-5 °C	ambient temperature

Product Description

Phycobiliproteins are fluorescent proteins derived from cyanobacteria and eukaryotic algae. Their fluorescence is much higher than chemical fluorescent probes such as fluorescein and rhodamine. R-Phycoerythrin (R-PE) is one of the phycobiliproteins and has an orange fluorescence at around 578 nm, and it can be excited at 488 nm (Fig. 1-22). Because of this high fluorescence, phycobiliprotein labeled antibodies or other molecules can give greater sensitivity in flow cytometry and immunostaining (Fig. 1-23). R-Phycoerythrin Labeling Kit-SH is for simple and rapid preparation of R-PE-labeled IgG (Fig. 1-24). SH-reactive R-PE(a component of this kit) has a maleimide group and can easily make a covalent bond with a sulfhydryl group of the target molecule without any activation process. The Filtration tube in this kit allows a quick buffer exchange and concentration of sample IgG solution. This kit contains all of the necessary reagents for R-PE labeling, including the Reducing agent for preparation of reduced IgG that has a SH group and the Storage buffer for conjugates.

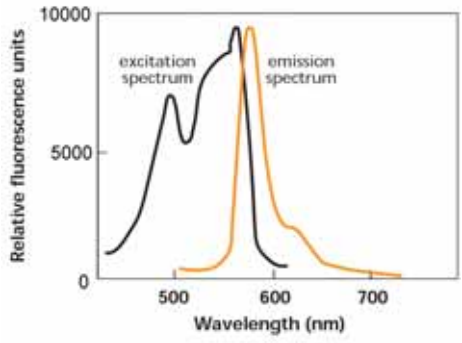


Fig. 1-22 Fluorescence spectrum of R-PE
Excitation wavelength: 566 nm
Emission wavelength: 578 nm

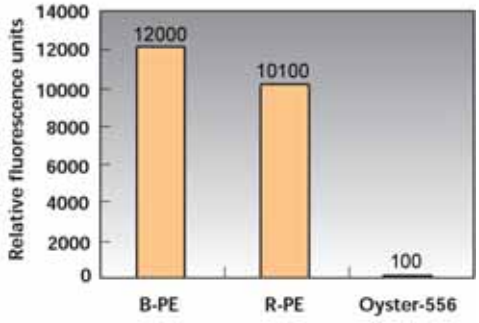
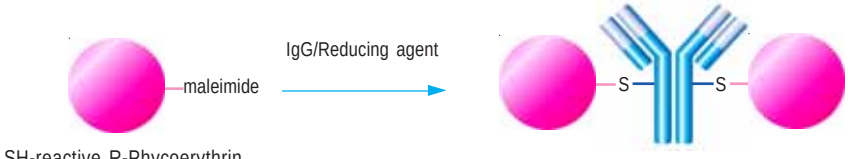


Fig. 1-23 Comparison of fluorescence intensity of Phycobiliproteins and cyanine dye

The 575 nm fluorescence of Phycoerythrin excited at 565 nm was compared with the 578 nm fluorescence of Oyster-556* excited at 556 nm.
**Oyster-556 is a cyanine dye. Oyster is a trademark of denovoBiolables GmbH.*



SH-reactive R-Phycoerythrin
R-Phycoerythrin-conjugated IgG

Fig. 1-24 IgG labeling reaction of SH-reactive R-PE

Required Equipment and Materials

microcentrifuge, 10 µl and 100-200 µl adjustable pipettes, 37 °C incubator, 0.5 ml microtubes

I-2. Protein Labeling: Phycobiliprotein

Precaution

⌘ The molecular weight of the reduced protein to be labeled with this kit should be over 50,000.
⌘ IgG or R-Phycoerythrin-conjugated IgG is always on the filter membrane of the Filtration tube during the labeling process.
⌘ If the IgG solution contains other proteins with molecular weight larger than 10,000, such as BSA or gelatin, purify the IgG solution prior to labeling R-Phycoerythrin with this kit. IgG solution can be purified by IgG Purification Kits (not included in this kit).
⌘ If the IgG solution contains small insoluble materials, centrifuge the solution and use the supernatant for the labeling.

General Protocol for IgG Labeling

Add 100 µl WS buffer and the sample solution containing 100 µg IgG and to a Filtration tube.[a)]

Mix the solution with pipetting several times and centrifuge at 8,000-10,000 g for 10 min.[b)]

Add 150 µl WS buffer to Reducing agent and dissolve it with pipetting several times.

Transfer 100 µl the solution from step 3 onto the membrane of the Filtration tube where IgG is concentrated.

Pipette several times and incubate the tube at 37 °C for 30 min.

Add 100 µl RA solution to the tube and centrifuge at 8,000-10,000 g for 10 min. Discard the filtrate add 200 µl RA solution and centrifuge again.[b)]

Add 50 µl Reaction buffer to SH-reactive R-PE and dissolve it with pipetting.

Transfer the SH-reactive R-PE solution onto the membrane of the Filtration tube where reduced IgG is concentrated.

Pipette several times and incubate the tube at 37 °C for 1 hr.

Add 150 µl WS buffer and pipette 10 to 15 times to recover the conjugate.[c)] Transfer the solution to a 0.5 ml tube and store the solution at 0-5 °C.[d)]

a) The volume of sample solution should be 100 µl or less. If the antibody concentration is lower than 0.5 mg per ml, repeat steps 1 and 2 until the total IgG accumulation becomes 100 µg.
b) If the solution still remains on the membrane after the centrifugation, centrifuge for another 5 min or increase the centrifuge speed.
c) The concentration of the conjugate is 1.4-1.8 mg per ml. Dilute the R-PE-labeled reduced IgG to prepare a solution with an appropriate concentration prior to using it for flow cytometry, immunoblotting, or immunostaining. One to two R-PE molecules should be introduced onto one reduced IgG molecule. Unconjugated R-PE should not interfere with a normal assay. If purification is necessary, use a gel permeation column or an affinity column for IgG.
d) Generally, the R-PE-labeled reduced IgG in Storage buffer is stable for at least 2 months at 0-5 °C. For longer storage, add glycerol (final concentration: 50%), aliquot, and store at -20 °C. However, it is important to note that the stability will depend on the sample itself.

FAQ

◆ Can I use this kit for F(ab')$_2$?
Yes, please follow the labeling protocol for IgG. The recovery of the conjugate should be over 80%.

◆ Can I use this kit for other proteins?
Yes, if the molecular weight of the reduced form is greater than 50,000 and it has a reactive SH group, or a disulfide group that can be reduced without losing activity. Follow the labeling protocol for IgG, and use 0.5-1 nmol of sample protein.

◆ How many R-PE molecules per reduced IgG are introduced?
The average number of R-PE molecule per reduced IgG is 1 to 2.

◆ Do I have to use the Filtration tube prior to labeling the protein?
If the protein solution does not contain small molecules with reactive SH groups and the concentration of the protein is 10 mg per ml, or about 70 µM, there is no need to use the Filtration tube. Just mix 10 µl of the sample solution with Reaction buffer and add the mixture to a vial of the SH-reactive R-PE.

◆ Do I have to use WS buffer included with the kit?
Yes. Use the WS buffer to prepare a stock solution of the conjugate. However, you can choose any kind of buffer appropriate to dilute the conjugate stock solution for your experiment.

References

K. Nagao, *et al.*, *PNAS*, **106**, 3312 (2009).

Allophycocyanin Labeling Kit-NH_2

Application: Allophycocyanin labeling of proteins

Features: Only 3 hours to get conjugates
All processes in a single Filtration tube
High recovery of conjugates
Applicable for 50-200 µg IgG

Ordering Information

Product code	Unit
LK21-10	3 samples*

* Based on 100 µg IgG labeling.

Contents of the Kit

NH_2-reactive APC ..... 250 µg x 3 tubes
Reaction buffer ..... 200 µl x 1 tube
WS buffer ..... 4 ml x 1 bottle
Filtration tube ..... 3 tubes

**APC: Allophycocyanin*

Storage Condition: 0-5 °C

Shipping Condition: with blue ice

Product Description

Phycobiliproteins are fluorescent proteins derived from cyanobacteria and eukaryotic algae. Their fluorescence is much higher than chemical fluorescent probes such as fluorescein and rhodamine. Allophycocyanin (APC) is one of the phycobiliproteins, and it has a blue fluorescence at around 660 nm (Fig. 1-25). Because of this high fluorescence, phycobiliprotein labeled antibodies and other molecules can give greater sensitivity in flow cytometry and immunostaining (Fig. 1-26).

Allophycocyanin Labeling Kit-NH_2 is for simple and rapid preparation of APC-labeled IgG (Fig. 1-27). NH_2-reactive APC (a component of this kit) has an activated ester group and can easily make a covalent bond with an amino group of the target molecule without any activation process. The Filtration tube in this kit is used for buffer exchange and concentration of sample IgG solution. This kit contains all of the necessary reagents for APC labeling including the Storage buffer for conjugates.

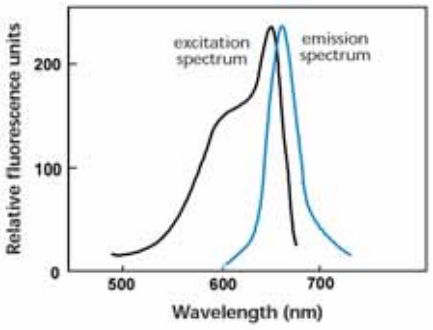


Fig. 1-25 Fluorescence spectrum of APC
Excitation wavelength: 650 nm
Emission wavelength: 660 nm

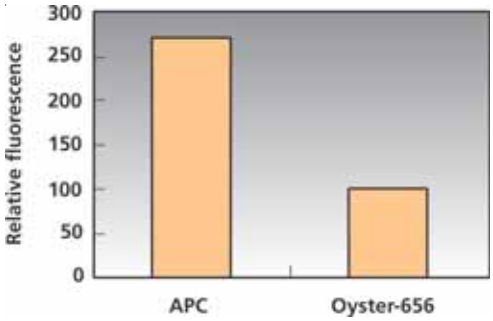
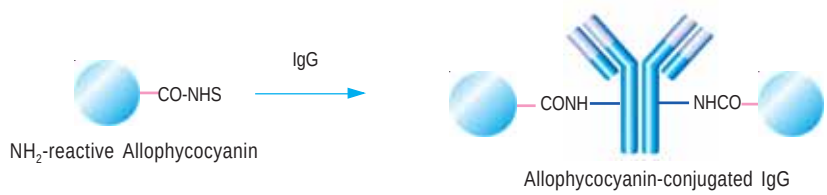


Fig. 1-26 Comparison of fluorescence intensity of Phycobiliproteins and cyanine dye

The 660 nm fluorescence of allophycocyanin excited at 650 nm was compared with the 660 nm fluorescence of Oyster-656* excited at 656 nm.
**Oyster-656 is a cyanine dye. Oyster is a trademark of denovoBiolables GmbH.*



NH_2-reactive Allophycocyanin

Allophycocyanin-conjugated IgG

Fig. 1-27 IgG labeling reaction of NH_2-reactive APC

Required Equipment and Materials

microcentrifuge, 10 µl and 100-200 µl adjustable pipettes, 37 °C incubator, 0.5 ml microtubes

I-2. Protein Labeling: Phycobiliprotein

Precaution

⌘ The molecular weight of the protein to be labeled with this kit should be over 50,000.
⌘ IgG or Allophycocyanin-conjugated IgG is always on the filter membrane of the Filtration tube during the labeling process.
⌘ If the IgG solution contains other proteins with molecular weight larger than 10,000, such as BSA or gelatin, purify the IgG solution prior to labeling Allophycocyanin with this kit. IgG solution can be purified by IgG Purification Kits (not included in this kit).
⌘ If the IgG solution contains small insoluble materials, centrifuge the solution and use the supernatant for the labeling.

General Protocol for IgG Labeling

Add 100 µl WS buffer and the sample solution containing 100 µg IgG to a Filtration tube.[a)]

Centrifuge at 8,000-10,000 g for 10 min. Add 100 µl WS buffer and centrifuge once more.[b)]

After finishing the centrifuge, add 10 µl Reaction buffer to NH_2-reactive APC and dissolve with pipetting.

Transfer the solution containing NH_2-reactive APC onto the membrane of the Filtration tube where IgG is concentrated.

Rinse the entire surface of the membrane with the solution by pipetting and incubate the tube at 37 °C for 2 hrs.

Add 190 µl WS buffer and pipette 10 to 15 times to recover the conjugate.[c)] Transfer the solution to a 0.5 ml tube and store the solution at 0-5 °C.[d)]

a) The volume of sample solution should be 100 µl or less. If the IgG concentration is lower than 0.5 mg per ml, repeat steps 1 and 2 until the total IgG accumulation becomes 100 µg.
b) If the solution still remains on the membrane after the centrifugation, centrifuge for another 5 min or increase the centrifuge speed.
c) The concentration of the conjugate is 1.4-1.8 mg per ml. Dilute the APC-labeled IgG to prepare a solution with an appropriate concentration prior to using it for flow cytometry, immunoblotting, or immunostaining. One to two of APC molecules should be introduced onto one IgG molecule. Unconjugated APC should not interfere with a normal assay. If purification is necessary, use a gel permeation column or an affinity column for IgG.
d) Generally, the APC-labeled IgG in Storage buffer is stable for at least 2 months at 0-5 °C. For longer storage, add glycerol (final concentration: 50%), aliquot, and store at -20 °C. However, it is important to note that the stability will depend on the sample itself.

FAQ

◆ Can I use this kit for other proteins?
Yes, if the molecular weight is higher than 50,000 and it has a reactive primary or secondary amino group. Follow the protocol for IgG labeling with 0.5-1 nmol of sample protein.

◆ How many APC molecules per IgG are introduced?
The average number of APC molecule per IgG is 1 to 2.

◆ Does unconjugated NH_2-reactive APC still have an activated ester after the labeling reaction to IgG?
No. It is completely hydrolyzed during the reaction.

◆ Does NH_2-reactive APC form an oligomer during the labeling reaction?
No. Since all amino groups of NH_2-reactive APC are blocked, no oligomerization is possible.

◆ What is the minimum amount of IgG that can be labeled with LK21-10?
The minimum amount is 50 µg. There is no significant difference in sensitivity and background between 50 µg and 200 µg of IgG.

◆ Do I have to use the WS buffer included with the kit?
Yes. Use the WS buffer to prepare a stock solution of the conjugate. However, you can choose any kind of buffer appropriate to dilute the conjugate stock solution for your experiment.

◆ How long is the conjugate stable?
If you store at 4 °C, it is stable for over 2 months. For longer storage, add 100% volume of glycerol, aliquot and store at -20 °C. However, please note that the stability depends on the protein itself.

I-2. Protein Labeling: Phycobiliprotein

Allophycocyanin Labeling Kit-SH

Application: Allophycocyanin labeling of proteins

Features: Only 3 hours to get conjugates
All processes in a single Filtration tube
High recovery of conjugates
Applicable for 50-200 µg IgG

Ordering Information

Product code	Unit
LK24-10	3 samples*

* Based on 100 µg IgG labeling.

Contents of the Kit

SH-reactive APC ..... 200 µg x 3 tubes
WS buffer ..... 4 ml x 1 bottle
Filtration tube ..... 3 tubes
Reducing agent ..... 3 tubes
Reaction buffer.....200 µl x 1 tube
RA solution .....1 ml x 1 tube

**APC: Allophycocyanin*

Storage Condition	Shipping Condition
0-5 °C	ambient temperature

Product Description

Phycobiliproteins are fluorescent proteins derived from cyanobacteria and eukaryotic algae. Their fluorescence is much higher than chemical fluorescent probes such as fluorescein and rhodamine. Allophycocyanin (APC) is one of the phycobiliproteins, and it has a blue fluorescence at around 660 nm (Fig. 1-28). Because of this high fluorescence, phycobiliprotein labeled antibodies and other molecules can give greater sensitivity in flow cytometry and immunostaining (Fig. 1-29). Allophycocyanin Labeling Kit-SH is for simple and rapid preparation of APC-labeled IgG (Fig. 1-30). SH-reactive APC (a component of this kit) has a maleimide group and can easily make a covalent bond with a sulfhydryl group of the target molecule without any activation process. The Filtration tube in this kit allows a quick buffer exchange and concentration of sample IgG solution. This kit contains all of the necessary reagents for APC labeling, including a Reducing agent for preparation of reduced IgG that has a SH group and a Storage buffer for conjugates.

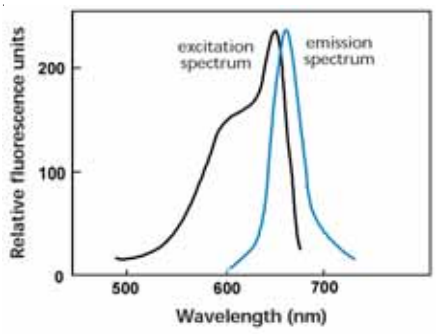


Fig. 1-28 Fluorescence spectrum of APC
Excitation wavelength: 650 nm
Emission wavelength: 660 nm

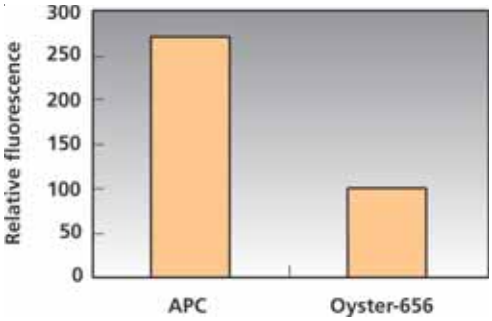
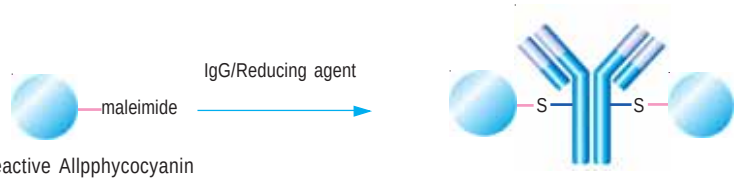


Fig. 1-29 Comparison of fluorescence intensity of Phycobiliproteins and cyanine dye

The 660 nm fluorescence of allophycocyanin excited at 650 nm was compared with the 660 nm fluorescence of Oyster-656* excited at 656 nm.
**Oyster-656 is a cyanine dye. Oyster is a trademark of denovoBiolables GmbH.*



SH-reactive Allpphycocyanin

Allophycocyanin-conjugated IgG

Fig. 1-30 IgG labeling reaction of SH-reactive APC

Required Equipment and Materials

microcentrifuge, 10 µl and 100-200 µl adjustable pipettes, 37 °C incubator, 0.5 ml microtubes

I-2. Protein Labeling: Phycobiliprotein

Precaution

⌘ The molecular weight of the reduced protein to be labeled with this kit should be over 50,000.
⌘ IgG or Allophycocyanin-conjugated IgG is always on the filter membrane of the Filtration tube during the labeling process.
⌘ If the IgG solution contains other proteins with molecular weight larger than 10,000, such as BSA or gelatin, purify the IgG solution prior to labeling Allophycocyanin with this kit. IgG solution can be purified by IgG Purification Kits (not included in this kit).
⌘ If the IgG solution contains small insoluble materials, centrifuge the solution and use the supernatant for the labeling.

General Protocol for IgG Labeling

Add 100 µl WS buffer and the sample solution containing 100 µg IgG and to a Filtration tube.[a)]

Mix the solution with pipetting several times and centrifuge at 8,000-10,000 g for 10 min.[b)]

Add 150 µl WS buffer to Reducing agent and dissolve it with pipetting several times.

Transfer 100 µl the solution from step 3 onto the membrane of the Filtration tube where IgG is concentrated.

Pipette several times and incubate the tube at 37 °C for 30 min.

Add 100 µl RA solution to the tube and centrifuge at 8,000-10,000 g for 10 min. Discard the filtrate, add 200 µl RA solution, and centrifuge again.[b)]

Add 50 µl Reaction buffer to SH-reactive APC and dissolve it with pipetting.

Transfer the SH-reactive APC solution onto the membrane of the Filtration tube where reduced IgG is concentrated.

Pipette several times and incubate the tube at 37 °C for 1 hr.

Add 150 µl WS buffer and pipette 10 to 15 times to recover the conjugate.[c)] Transfer the solution to a 0.5 ml tube, and store the solution at 0-5 °C.[d)]

a) The volume of sample solution should be 100 µl or less. If the antibody concentration is lower than 0.5 mg per ml, repeat steps 1 and 2 until the total IgG accumulation becomes 100 µg.
b) If the solution still remains on the membrane after the centrifugation, centrifuge for another 5 min or increase the centrifuge speed.
c) The concentration of the conjugate is 1.4-1.8 mg per ml. Dilute the APC-labeled reduced IgG to prepare a solution with an appropriate concentration prior to using it for flow cytometry, immunoblotting, or immunostaining. One to two APC molecules should be introduced onto one reduced IgG molecule. Unconjugated APC should not interfere with a normal assay. If purification is necessary, use a gel permeation column or an affinity column for IgG.
d) Generally, the APC-labeled reduced IgG in Storage buffer is stable for at least 2 months at 0-5 °C. For longer storage, add glycerol (final concentration: 50%), aliquot, and store at -20 °C. However, it is important to note that the stability will depend on the sample itself.

FAQ

◆ Can I use this kit for $F(ab')_2$?
Yes, please follow the labeling protocol for IgG. The recovery of the conjugate should be over 80%.

◆ Can I use this kit for other proteins?
Yes, if the molecular weight of the reduced form is greater than 50,000 and it has a reactive SH group, or a disulfide group that can be reduced without losing activity. Follow the labeling protocol for IgG, and use 0.5-1 nmol of sample protein.

◆ How many APC molecules per reduced IgG are introduced?
The average number of APC molecule per reduced IgG is 1 to 2.

◆ Do I have to use the Filtration tube prior to labeling the protein?
If the protein solution does not contain small molecules with reactive SH groups and the concentration of the protein is 10 mg per ml, or about 70 µM, there is no need to use the Filtration tube. Just mix 10 µl of the sample solution with Reaction buffer and add the mixture to a vial of the SH-reactive APC.

◆ Do I have to use WS buffer included with the kit?
Yes. Use the WS buffer to prepare a stock solution of the conjugate. However, you can choose any kind of buffer appropriate to dilute the conjugate stock solution for your experiment.

I-3. Protein Labeling: Fluorophore

Fluorescein Labeling Kit-NH_2

Application: Fluorescein labeling of proteins

Features: Only 1 hour to get conjugates
All processes in a single Filtration tube
High recovery of conjugates
Applicable for 50-200 µg IgG

Ordering Information

Product code	Unit
LK01-10	3 samples*
LK63-10	1 sample**

* Based on 100 ug IgG labeling.
* * Based on 1mg IgG labeling

Contents of the Kit

LK01-10

NH_2-reactive fluorescein ..... 3 tubes
Reaction buffer ..... 0.5 ml x 1 tube
WS buffer ..... 4 ml x 1 bottle
Filtration tube ..... 3 tubes

LK63-10

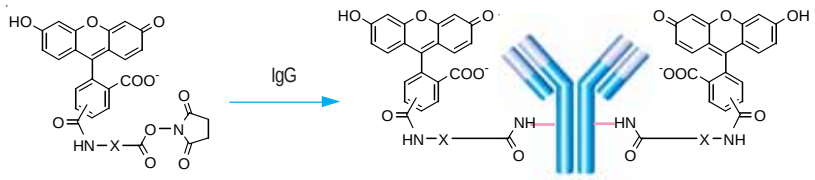
NH_2-reactive fluorescein ..... 1 tubes
Reaction buffer ..... 2 ml x 1 tube
15 ml tube .....1 tube
WS buffer ..... 15 ml x 1 bottle
Filtration tube ..... 1 tubes

Storage Condition
0-5 °C

Shipping Condition
ambient temperature

Product Description

Fluorescein Labeling Kit-NH_2 is mainly used for the preparation of fluorescein-labeled proteins such as IgG for immunostaining and cellular proteins for tracing. Amine reactive fluorescein, a component of this kit, has succinimidyl groups (NHS) that react with the amino groups of proteins or other molecules (Fig. 1-31). This kit contains all of the necessary reagents for labeling, including storage buffer. Each vial of fluorescein can label up to 200 µg of IgG, conjugating about 4 to 6 fluorescein molecules per IgG molecule. Since this kit also includes a buffer exchange system, a sample containing amine base buffer can be labeled. Though membrane filtration sometimes causes IgG aggregation, the buffer system used in this kit prevents aggregation during the concentration of IgG or fluorescein-labeled IgG solution. A fluorescein-labeled IgG solution prepared using this kit is stable for more than 2 months at 4 °C. The excitation and emission wavelengths of the fluorescein-labeled IgG are 495 nm and 520 nm, respectively (Fig. 1-32).



NH_2-reactive fluorescein — Fluorescein-conjugated IgG

Fig. 1-31 IgG labeling reaction of NH_2-reactive fluorescein

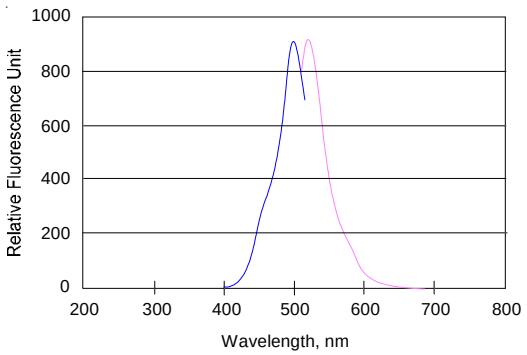


Fig. 1-32 Fluorescence spectrum of fluorescein-conjugated IgG
excitation spectrum
emission spectrum

Required Equipment and Materials

LK01-10: microcentrifuge, 10 µl and 100-200 µl adjustable pipettes, 37 °C incubator, 0.5 ml microtubes, DMSO or ethanol
LK63-10:centrifuge, rotor for 15 ml centrifuge tube, 50-200 µl and 1 ml adjustable pipettes, 37 °C incubator, microtubes

I-3. Protein Labeling: Fluorophore

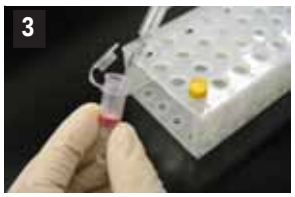
Precaution

⌘ The molecular weight of the protein to be labeled with this kit should be over 50,000.
⌘ IgG or fluorescein-conjugated IgG is always on the filter membrane of the Filtration tube during the labeling process.
⌘ If the IgG solution contains other proteins with molecular weight larger than 10,000, such as BSA or gelatin, purify the IgG solution prior to labeling fluorescein with this kit. IgG solution can be purified by IgG Purification Kits (not included in this kit).
⌘ If the IgG solution contains small insoluble material, centrifuge the solution and use the supernatant for the labeling.

General Protocol for IgG Labeling

Add 100 µl WS buffer and the sample solution containing 100 µg IgG to a Filtration tube.[a)]

Centrifuge at 8,000-10,000 g for 10 min.[b)]



Add 10 µl DMSO to NH_2-reactive fluorescein and dissolve with pipetting.[c)]

Add 100 µl Reaction buffer and 8 µl NH_2-reactive fluorescein solution to the Filtration tube and pipette to mix.[d)]

Incubate the tube at 37 °C for 10 min.

Add 100 µl WS buffer to the Filtration tube and centrifuge at 8,000-10,000 g for 10 min.[b)] Discard the filtrate.

Add 200 µl WS buffer to the Filtration tube and centrifuge at 8,000-10,000 g for 10 min.[b)] Repeat this step.

Add 200 µl WS buffer and pipette 10 to 15 times to recover the conjugate.[e)] Transfer the solution to a 0.5 ml tube, and store at 0-5 °C.

a) The volume of IgG solution should be less than 100 µl. If the antibody concentration is less than 1 mg per ml, repeat steps 1 and 2 until the total IgG accumulation becomes 100 µg. If the volume of the filtrate becomes 400 µl or more during the accumulation process, discard the filtrate prior to going to the next centrifuge step.
b) If solution still remains on the filter after the centrifugation, spin for another 5 min or increase the centrifuge speed.
c) NH_2-reactive fluorescein is on the bottom of the tube. Add 10 µl DMSO to the bottom of the tube, and pipette several times to dissolve. If DMSO is not available, you may use ethanol.
d) If the amount of IgG is 200 µg, add the entire NH_2-reactive fluorescein solution at step 4.
e) You do not have to use WS buffer to recover fluorescein-conjugated IgG. You can choose any kind of buffer appropriate for your experiment.

Determination of Fluorescein/IgG Ratio

Measure the absorbance of the fluorescein-labeled IgG solution at 280 nm and 500 nm. Calculate the ratio using the following equation:

$$\text{Ratio (fluorescein molecules per IgG molecule)} = \frac{3 \times A_{500}}{A_{280} - 0.2 \times A_{500}}$$

A_{500}: absorbance at 500 nm
A_{280}: absorbance at 280 nm

FAQ

◆ Can I use this kit for other proteins?
Yes, if the molecular weight is greater than 50,000.

◆ Do I have to use a Filtration tube prior to labeling the protein?
If the protein solution does not contain small molecules with an amino group and the concentration of the protein is 10 mg per ml, or about 70 µM, there is no need to use the Filtration tube. Just mix 10 µl of the sample solution with 90 µl of Reaction buffer and add 8 µl NH_2-reactive fluorescein (prepared at step 3) to the mixture, and follow the protocol starting at step 4.

◆ How long is the fluorescein-labeled protein stable?
If you store at 4 °C, it is stable for over 2 months. For longer storage, add 100% volume of glycerol, aliquot, and store at -20 °C. However, please note that stability depends on the protein itself.

◆ What is the minimum amount of IgG that can be labeled by this kit?
The minumum amount of IgG is 10 µg; simply follow the protocol. The labeling ratio remains the same for 10 µg to 100 µg of IgG.

◆ Can I use this kit to label oligonucleotides or peptides?
No. Oligonucleotides and peptides may be too small to retain on the membrane filter of the Filtration tube.

References

M. Hiyoshi, *et al.*, *Blood*, **111**, 243 (2008).

I-3. Protein Labeling: Fluorophore

HiLyte Fluor 555 Labeling Kit-NH_2

Application: HiLyte Fluor 555 labeling of proteins

Features: Only 1 hour to get conjugates
All processes in a single Filtration tube
High recovery of conjugates
Applicable for 50-200 µg IgG

Ordering Information

Product code	Unit
LK14-10	3 samples*

* Based on 100 µg IgG labeling.

Contents of the Kit

NH_2-reactive HiLyte Fluor 555 ..... 3 tubes
Reaction buffer ..... 0.5 ml x 1 tube
WS buffer .....4 ml x 1 bottle
Filtration tube .....3 tubes

Storage Condition
0-5 °C

Shipping Condition
ambient temperature

Product Description

HiLyte Fluor* 555 Labeling Kit-NH_2 is mainly used for the preparation of red color fluorescence-labeled proteins, such as IgG, for immunostaining and cellular proteins for tracing. NH_2-reactive HiLyte Fluor 555, a component of this kit, has a succinimidyl group (NHS) that reacts with an amino group of proteins or other molecules (Fig. 1-33). This kit contains all of the necessary reagents for labeling. Each tube of HiLyte Fluor 555 can label up to 200 µg of IgG, conjugating about 4 to 6 HiLyte Fluor 555 molecules per IgG molecule. The labeling process is simple. Add the NH_2-reactive HiLyte Fluor 555 to IgG solution on a filter membrane and incubate at 37 °C for 10 min. The excess HiLyte Fluor 555 molecules can be removed by a Filtration tube. The excitation and emission wavelengths of the HiLyte Fluor 555-labeled IgG are 555 nm and 570 nm, respectively (Fig. 1-34).

** HiLyte Fluor is a trademark of AnaSpec, Inc.*

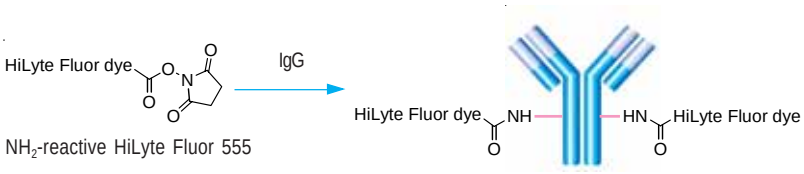


Fig. 1-33 IgG labeling reaction of NH_2-reactive Hilyte Fluor 555

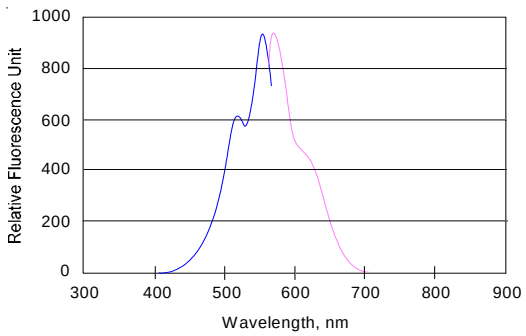


Fig. 1-34 Fluorescence spectrum of HiLyte Fluor 555-conjugated IgG
excitation spectrum
emission spectrum

Required Equipment and Materials

microcentrifuge, 10 µl and 100-200 µl adjustable pipettes, 37 °C incubator, 0.5 ml microtubes, DMSO

Precaution

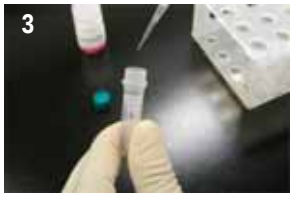
⌘ The molecular weight of the protein to be labeled with this kit should be over 50,000.
⌘ IgG or HiLyte Fluor 555-conjugated IgG is always on the filter membrane of the Filtration tube during the labeling process.
⌘ If the IgG solution contains other proteins with molecular weights larger than 10,000, such as BSA or gelatin, purify the IgG solution prior to labeling HiLyte Fluor 555 with this kit. IgG solution can be purified by IgG Purification Kits (not included in this kit).
⌘ If the IgG solution contains small insoluble materials, centrifuge the solution and use the supernatant for the labeling.

I-3. Protein Labeling: Fluorophore

General Protocol for IgG Labeling

Add 100 µl WS buffer and the sample solution containing 100 µg IgG to a Filtration tube.[a)]

Centrifuge at 8,000-10,000 g for 10 min.[b)]

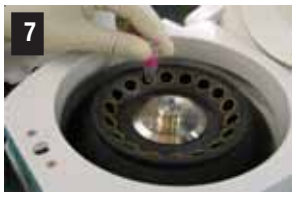


Add 10 µl DMSO to NH_2-reactive HiLyte Fluor 555 and dissolve with pipetting.[c)]

Add 100 µl Reaction buffer and 8 µl NH_2-reactive HiLyte Fluor 555 solution to the Filtration tube and pipette to mix.[d)]

Incubate the tube at 37 °C for 10 min.

Add 100 µl WS buffer to the Filtration tube and centrifuge at 8,000-10,000 g for 10 min.[b)] Discard the filtrate.



Add 200 µl WS buffer to the Filtration tube and centrifuge at 8,000 g for 10 min.[b)] Repeat this step.

Add 200 µl WS buffer per and pipette 10 to 15 times to recover the conjugate.[e)] Transfer the solution to a 0.5 ml tube and store at 0-5 °C.

a) The volume of IgG solution should be less than 100 µl. If the antibody concentration is less than 1 mg per ml, repeat steps 1 and 2 until the total IgG accumulation becomes 100 µg. If the volume of the filtrate becomes 400 µl or more during the accumulation process, discard the filtrate prior to going to the next centrifuge step.
b) If solution still remains on the filter after the centrifugation, spin for another 5 min or increase the centrifuge speed.
c) NH_2-reactive HiLyte Fluor 555 is on the bottom of the tube. Add 10 µl DMSO to the bottom of the tube, and pipette several times to dissolve.
d) If the amount of IgG is 200 µg, add the entire NH_2-reactive HiLyte Fluor 555 solution.
e) You do not have to use WS buffer to recover HiLyte Fluor 555-conjugated IgG. You can choose any kind of buffer appropriate for your experiment.

Determination of HiLyte Fluor 555 /IgG Ratio

Measure the absorbance of the HiLyte Fluor 555-labeled IgG solution at 280 nm and 555 nm. Calculate the ratio using the following equation:

$$\text{Ratio (HiLyte Fluor 555 molecules per IgG molecule)} = \frac{1.4 \times A_{555}}{A_{280} - 0.1 \times A_{555}}$$

A_{555}: absorbance at 555 nm
A_{280}: absorbance at 280 nm

FAQ

- **Can I use this kit for other proteins?**
  Yes, if the molecular weight is greater than 50,000.
- **Do I have to use a Filtration tube prior to labeling the protein?**
  If the protein solution does not contain small molecules with an amino group and the concentration of the protein is 10 mg per ml, or about 70 µM, there is no need to use the Filtration tube. Mix 10 µl of the sample solution with 90 µl of Reaction buffer and add 8 µl NH_2-reactive HiLyte Fluor 555 (prepared at step 3) to the mixture, and follow the protocol starting at step 4.
- **How long is the HiLyte Fluor 555-labeled protein stable?**
  If you store at 4 °C, it is stable for over 2 months. For longer storage, add 100% volume of glycerol, aliquot, and store at -20 °C. However, please note that stability depends on the protein itself.
- **What is the minimum amount of IgG that can be labeled by this kit?**
  The minumum amount of IgG is 10 µg. For less than 20 µg, follow the manual and add 4 µl NH_2-reactive HiLyte Fluor 555 instead of 8 µl at step 4.
- **Can I use this kit to label oligonucleotides or peptides?**
  No. Oligonucleotides and peptides may be too small to retain on the membrane filter of the Filtration tube.

References

K. M. Nishida, *et al.*, *RNA*, **13**, 1911 (2007); M. Nagaoka, *et al.*, *J. Biol. Chem.*, **283**, 26468 (2008).

I-3. Protein Labeling: Fluorophore

HiLyte Fluor 647 Labeling Kit-NH_2

Application: HiLyte Fluor 647 labeling of proteins

Features:
- Only 1 hour to get conjugates
- All processes in a single Filtration tube
- High recovery of conjugates
- Applicable for 50-200 µg IgG

Ordering Information

Product code	Unit
LK15-10	3 samples*

* Based on 100 µg IgG labeling.

Contents of the Kit

NH_2-reactive HiLyte Fluor 647 ..... 3 tubes
Reaction buffer ..... 0.5 ml x 1 tube
WS buffer ..... 4 ml x 1 bottle
Filtration tube ..... 3 tubes

Storage Condition

0-5 °C

Shipping Condition

ambient temperature

Product Description

HiLyte Fluor* 647 Labeling Kit-NH_2 is mainly used for the preparation of blue color fluorescence-labeled proteins, such as IgG, for immunostaining and cellular proteins for tracing. NH_2-reactive HiLyte Fluor 647, a component of this kit, has a succinimidyl group (NHS) that reacts with an amino group of proteins or other molecules (Fig. 1-35). This kit contains all of the necessary reagents for labeling. Each tube of HiLyte Fluor 647 can label up to 200 µg of IgG, conjugating about 4 to 6 HiLyte Fluor 647 molecules per IgG molecule. The labeling process is simple. Add the NH_2-reactive HiLyte Fluor 647 to IgG solution on a filter membrane and incubate at 37 °C for 10 min. The excess HiLyte Fluor 647 molecules can be removed by a Filtration tube. The excitation and emission wavelengths of the HiLyte Fluor 647-labeled IgG are 652 nm and 673 nm, respectively (Fig. 1-36). **HiLyte Fluor is a trademark of AnaSpec, Inc.*

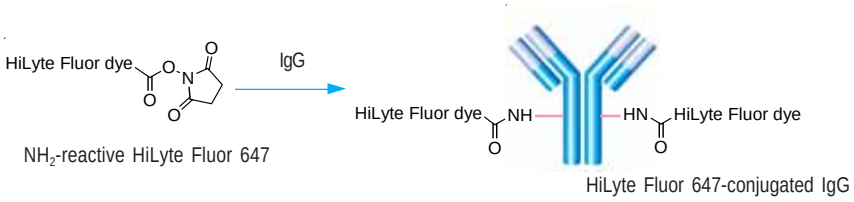


Fig. 1-35 IgG labeling reaction of NH_2-reactive Hilyte Fluor 647

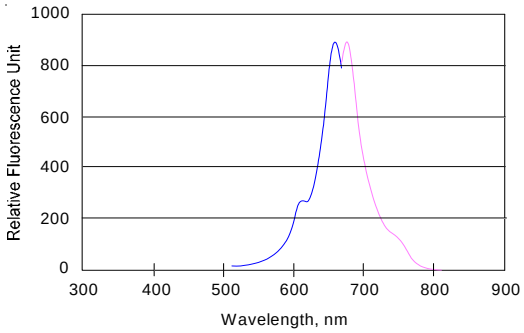


Fig. 1-36 Fluorescence spectrum of HiLyte Fluor 647-conjugated IgG
excitation spectrum
emission spectrum

Required Equipment and Materials

microcentrifuge, 10 µl and 100-200 µl adjustable pipettes, 37 °C incubator, 0.5 ml microtubes, DMSO

Precaution

- *The molecular weight of the protein to be labeled with this kit should be over 50,000.*
- *IgG or HiLyte Fluor 647-conjugated IgG is always on the filter membrane of the Filtration tube during the labeling process.*
- *If the IgG solution contains other proteins with molecular weights larger than 10,000, such as BSA or gelatin, purify the IgG solution prior to labeling HiLyte Fluor 647 with this kit. IgG solution can be purified by IgG Purification Kits (not included in this kit).*
- *If the IgG solution contains small insoluble materials, centrifuge the solution and use the supernatant for the labeling.*

I-3. Protein Labeling: Fluorophore

General Protocol for IgG Labeling

Add 100 µl WS buffer and the sample solution containing 100 µg IgG to a Filtration tube.[a)]

Centrifuge at 8,000-10,000 g for 10 min.[b)]

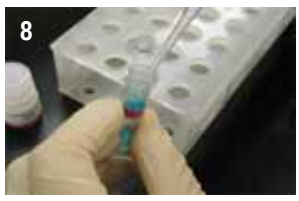
Add 10 µl DMSO to NH_2-reactive HiLyte Fluor 647 and dissolve with pipetting.[c)]

Add 100 µl Reaction buffer and 8 µl NH_2-reactive HiLyte Fluor 647 solution to the Filtration tube and pipette to mix.[d)]

Incubate the tube at 37 °C for 10 min.

Add 100 µl WS buffer to the Filtration tube and centrifuge at 8,000-10,000 g for 10 min.[b)] Discard the filtrate.

Add 200 µl WS buffer to the Filtration tube and centrifuge at 8,000 g for 10 min.[b)] Repeat this step.



Add 200 µl WS buffer and pipette 10 to 15 times to recover the conjugate.[e)] Transfer the solution to a 0.5 ml tube and store at 0-5 °C.

a) The volume of IgG solution should be less than 100 µl. If the antibody concentration is lower than 1 mg per ml, repeat steps 1 and 2 until the total IgG accumulation becomes 100 µg. If the volume of the filtrate becomes 400 µl or more during the accumulation process, discard the filtrate prior to going to the next centrifuge step.

b) If solution still remains on the filter after the centrifugation, spin for another 5 min, or increase the centrifuge speed.

c) NH_2-reactive HiLyte Fluor 647 is on the bottom of the tube. Add 10 µl DMSO to the bottom of the tube and pipette several times to dissolve.

d) If the amount of IgG is 200 µg, add entire NH_2-reactive HiLyte Fluor 647 solution.

e) You do not have to use WS buffer to recover HiLyte Fluor 647-conjugated IgG. You can choose any kind of buffer appropriate for your experiment.

Determination of HiLyte Fluor 647/IgG Ratio

Measure the absorbance of the HiLyte Fluor 647-labeled IgG solution at 280 nm and 652 nm. Calculate the ratio using the following equation:

$$\text{Ratio (HiLyte Fluor 647 molecules per IgG molecule)} = \frac{0.85 \times A_{652}}{A_{280} - 0.08 \times A_{652}}$$

A_{652}: absorbance at 652 nm
A_{280}: absorbance at 280 nm

FAQ

- **Can I use this kit for other proteins?**
  Yes, if the molecular weight is greater than 50,000.
- **Do I have to use a Filtration tube prior to labeling the protein?**
  If the protein solution does not contain small molecules with an amino group and the concentration of the protein is 10 mg per ml, or about 70 µM, there is no need to use the Filtration tube. Mix 10 µl of the sample solution with 90 µl of Reaction buffer and add 8 µl NH_2-reactive HiLyte Fluor 647 (prepared at step 3) to the mixture, and follow the protocol starting at step 4.
- **How long is the HiLyte Fluor 647-labeled protein stable?**
  If you store at 4 °C, it is stable for over 2 months. For longer storage, add 100% volume of glycerol, aliquot, and store at -20 °C. However, please note that stability depends on the protein itself.
- **What is the minimum amount of IgG that can be labeled by this kit?**
  The minumum amount of IgG is 10 µg. For less than 20 µg, follow the manual and add 4 µl NH_2-reactive HiLyte Fluor 647 instead of 8 µl at step 4.
- **Can I use this kit to label oligonucleotides or peptides?**
  No. Oligonucleotides and peptides may be too small to retain on the membrane filter of the Filtration tube.

I-3. Protein Labeling: Fluorophore

HiLyte Fluor 750 Labeling Kit-NH_2

Application: HiLyte Fluor 750 labeling of proteins

Features:
- Only 1 hour to get conjugates
- All processes in a single Filtration tube
- High recovery of conjugates
- Applicable for 50-200 µg IgG

Ordering Information

Product code	Unit
LK16-10	3 samples*

* Based on 100 µg IgG labeling.

Contents of the Kit

- NH_2-reactive HiLyte Fluor 750 ..... 3 tubes
- Reaction buffer ..... 1.5 ml x 1 tube
- WS buffer ..... 4 ml x 1 bottle
- Filtration tube ..... 3 tubes

Storage Condition
0-5 °C

Shipping Condition
ambient temperature

Product Description

HiLyte Fluor* 750 Labeling Kit-NH_2 is mainly used for the preparation of near infrared fluorescence-labeled proteins, such as IgG, for immunostaining and cellular proteins for tracing. NH_2-reactive HiLyte Fluor 750, a component of this kit, has a succinimidyl group (NHS) that reacts with an amino group of proteins or other molecules (Fig. 1-37). This kit contains all of the necessary reagents for labeling. Each tube of HiLyte Fluor 750 can label up to 200 µg of IgG, conjugating about 3 to 5 HiLyte Fluor 750 molecules per IgG molecule. The labeling process is simple. Add the NH_2-reactive HiLyte Fluor 750 to IgG solution on a filter membrane and incubate at 37 °C for 10 min. The excess HiLyte Fluor 750 molecules can be removed by a Filtration tube. The excitation and emission wavelengths of the HiLyte Fluor 750-labeled IgG are 762 nm and 777 nm, respectively (Fig. 1-38). **HiLyte Fluor is a trademark of AnaSpec, Inc.*

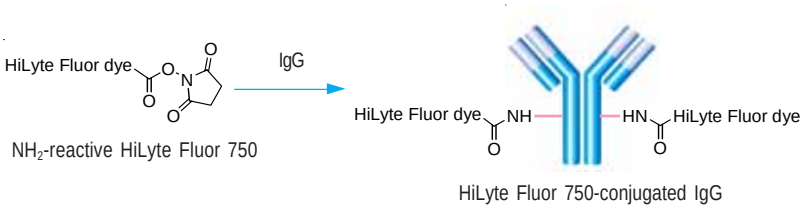


Fig. 1-37 IgG labeling reaction of NH_2-reactive Hilyte Fluor 750

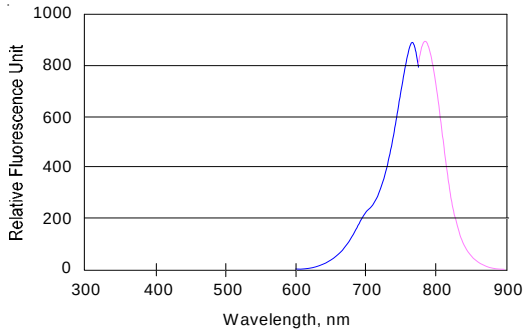


Fig. 1-38 Fluorescence spectrum of HiLyte Fluor 750-conjugated IgG
- excitation spectrum
- emission spectrum

Required Equipment and Materials

microcentrifuge, 10 µl and 100-200 µl adjustable pipettes, 37 °C incubator, 0.5 ml microtubes, DMSO

Precaution

- *The molecular weight of the protein to be labeled with this kit should be over 50,000.*
- *IgG or HiLyte Fluor 750-conjugated IgG is always on the filter membrane of the Filtration tube during the labeling process.*
- *If the IgG solution contains other proteins with molecular weights larger than 10,000, such as BSA or gelatin, purify the IgG solution prior to labeling HiLyte Fluor 750 with this kit. IgG solution can be purified by IgG Purification Kits (not included in this kit).*
- *If the IgG solution contains small insoluble materials, centrifuge the solution and use the supernatant for the labeling.*

I-3. Protein Labeling: Fluorophore

General Protocol for IgG Labeling

Add 100 µl WS buffer and the sample solution containing 100 µg IgG to a Ffiltration tube.[a)]

Centrifuge at 8,000-10,000 g for 10 min.[b)]

Add 10 µl DMSO to NH_2-reactive HiLyte Fluor 750 and dissolve with pipetting.[c)]

Add 400 µl Reaction buffer and 8 µl NH_2-reactive HiLyte Fluor 750 solution to the Filtration tube and pipette to mix.[d)]

Incubate the tube at 37 °C for 10 min.

Centrifuge at 8,000-10,000 g for 15 min.[b)] Discard the filtrate.

Add 400 µl WS buffer to the Filtration tube and centrifuge at 8,000 g for 15 min.[b)]

Add 200 µl WS buffer, and pipette 10 to 15 times to recover the conjugate.[e)] Transfer the solution to a 0.5 ml tube and store at 0-5 °C.

a) The volume of IgG solution should be less than 100 µl. If the antibody concentration is lower than 1 mg per ml, repeat steps 1 and 2 until the total IgG accumulation becomes 100 µg. If the volume of the filtrate becomes 400 µl or more during the accumulation process, discard the filtrate prior to going to the next centrifuge step.
b) If solution still remains on the filter after the centrifugation, spin for another 5 min, or increase the centrifuge speed.
c) NH_2-reactive HiLyte Fluor 750 is on the bottom of the tube. Add 10 µl DMSO to the bottom of the tube and pipette several times to dissolve.
d) If the amount of IgG is 200 µg, add entire NH_2-reactive HiLyte Fluor 750 solution.
e) You do not have to use WS buffer to recover HiLyte Fluor 750-conjugated IgG. You can choose any kind of buffer appropriate for your experiment.

Determination of HiLyte Fluor 750/IgG Ratio

Measure the absorbance of the HiLyte Fluor 750-labeled IgG solution at 280 nm and 760 nm. Calculate the ratio using the following equation:

$$\text{Ratio (HiLyte Fluor 750 molecules per IgG molecule)} = \frac{0.8 \times A_{760}}{A_{280} - 0.05 \times A_{760}}$$

A_{760}: absorbance at 760 nm
A_{280}: absorbance at 280 nm

FAQ

- **Can I use this kit for other proteins?**
  Yes, if the molecular weight is greater than 50,000.
- **Do I have to use a Filtration tube prior to labeling the protein?**
  If the protein solution does not contain small molecules with an amino group and the concentration of the protein is 10 mg per ml, or about 70 µM, there is no need to use the Filtration tube. Mix 10 µl of the sample solution with 390 µl of Reaction buffer and add 8 µl NH_2-reactive HiLyte Fluor 750 (prepared at step 3) to the mixture, and follow the protocol starting at step 4.
- **How long is the HiLyte Fluor 750-labeled protein stable?**
  If you store at 4 °C, it is stable for over 2 months. For longer storage, add 100% volume of glycerol, aliquot, and store at -20 °C. However, please note that stability depends on the protein itself.
- **What is the minimum amount of IgG that can be labeled by this kit?**
  The minumum amount of IgG is 10 µg. There is no significant difference in sensitivity and background between 10 and 200 µg of IgG.
- **Can I use this kit to label oligonucleotides or peptides?**
  No. Oligonucleotides and peptides may be too small to retain on the membrane filter of the Filtration tube.

I-3. Protein Labeling: Fluorophore

IC3-OSu special packaging

N-Ethyl-*N'*-[5-(*N'*-succinimidyloxycarbonyl)pentyl]indocarobcyanine chloride

Application: Indocyanine labeling of proteins

Appearance: dark red powder
Purity: >80% (HPLC)
MW: 604.18, $C_{35}H_{42}ClN_3O_4$

Storage Condition	Shipping Condition
-20 °C	with dry ice or blue ice

Ordering Information

Product code	Unit*
I271-10	20 ug x 3

IC5-OSu special packaging

N-Ethyl-*N'*-[5-(*N'*-succinimidyloxycarbonyl)pentyl]-3,3,3",3"-tetramethyl-2,2'-indocarobcyanine chloride

Application: Indocyanine labeling of proteins

Appearance: dark blue powder
Purity: >80% (HPLC)
MW: 630.23, $C_{37}H_{44}ClN_3O_4$

Storage Condition	Shipping Condition
-20 °C	with dry ice or blue ice

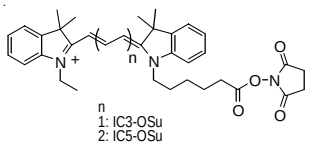
Ordering Information

Product code	Unit*
I272-10	20 ug x 3

Product Description

IC3 and IC5 are indocyanine dyes which can react with aminegroups on proteins, peptides, and amine-modified oligonucleotides. Since both IC3 and IC5 do not have a sulfonate group in the structure, water-solubility of the molecules is very low. An organic solvent is required to dissolve the compound. Over labeling of proteins with these compounds may precipitate the labeled protein from aqueous solutions due to the poor water-solubility. However, since no negative charge is added to the protein, the total number of charges of the modified protein is the same before and after the labeling with these compounds. The IC dye package contains 100 ug dye. This amount is sufficient to modify 1-2 mg proteins or more depending on the purpose of the downstream experiments.

Structural Formula



Reference

K. Zhao, *et al.*, *J Biol. Chem.*, **280**, 17758 (2005).

ICG-Sulfo-OSu

2-[7-[1,3-Dihydro-1,1-dimethyl-3-(4-sulfobutyl)-2H-benzo[e]indol-2-ylidene]-1,3,5-heptatrienyl]-1,1-dimethyl-3-[5-(3-sulfosuccinimidyl)oxycarbonylpentyl]-1H-benzo[e]indolium, inner salt, sodium salt

Application: Indocyanine green labeling of proteins

Appearance: dark green powder
Purity: >95% (HPLC)
MW: 930.07, $C_{49}H_{52}N_3O_{10}S_2$

Ordering Information

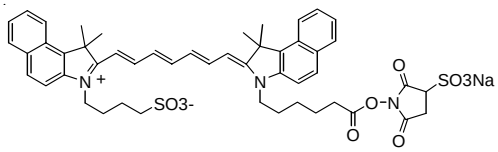
Product code	Unit*
I254-10	1 mg

Storage Condition	Shipping Condition
-20 °C	ambient temperature

Product Description

ICG is one of the dyes which is used for determining cardiac output, hepatic function, and liver blood flow, as well as for ophthalmic angiography. It has a long excitation wavelength and emission wavelength of about 780 nm and 800 nm, respectively. Due to such a long wavelength near the infrared region and low cytotoxicity, ICG is used to label antibodies for in vivo assay. However, the fluorescent intensity after the conjugation with protein is quite low due to the formation of H-dimer or an energy transfer to antibody moleculse after excitation. Dr. Kobayashi and others reported that the use of SDS and beta-mecraptoethanol to the conjugate increases fluorescent intensity dramatically by diminishing hydrophobic π–π interactions and separation of IgG chains. They applied a treated ICG-conjugated daclizumab (Dac.. humanized monoclonal antibody) and humanized anti-HER IgG2 monoclonal antibody for in vivo assay to specifically visualized tumors.

Structural Formula



Labeling procedure for IgG

1. Prepare 6.8 nmol antibody solution with pH 8.5 carbonate buffer or Bicine buffer (Good's buffer).
2. Add 6.8-68 nmol of ICG-Sulfo-OSu/DMSO solution to the antibody solution and incubate at room temperature for 30 min.
3. Purify the reaction mixture with a Sephadex G50 column.

Reference

M. Ogawa, *et al.*, *Cancer Res.*, **69**, 1268 (2009).

I-4. Protein Labeling: Biotin

Biotin Labeling Kit-NH_2

Application: Biotin labeling of protein, amine reactive

Features: Only 1 hour to get conjugates
All processes in a single Filtration tube
High recovery of conjugates
Applicable for 50-200 µg IgG

Ordering Information

Product code	Unit
LK03-10	3 samples*
LK55-10	1 sample**

* Based on 100 µg IgG labeling.
** Based on 1 mg IgG labeling.

Contents of the Kit

LK03-10

NH_2-reactive biotin ..... 3 tubes
Reaction buffer ..... 0.5 ml x 1 tube
WS buffer ..... 4 ml x1 bottle
Filtration tube ..... 3 tubes

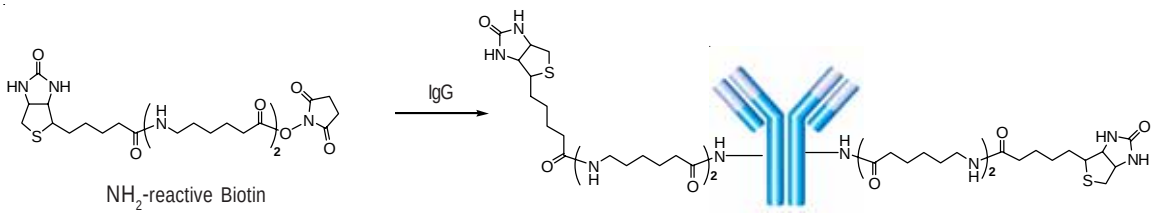
LK55-10

NH_2-reactive biotin ..... 1 tube
Reaction buffer ..... 2 ml x 1 bottle
15 ml tube ..... 1 tube
WS buffer .....15 ml x 1bottle
Filtration tube .....1 tube

Storage Condition	Shipping Condition
-20 °C	ambient temperature

Product Description

Biotin Labeling Kit-NH_2 is mainly used for the preparation of biotin-labeled IgG for enzyme immunoassay (EIA). NH_2-reactive biotin, a component of this kit, has succinimidyl groups (NHS) that react with the amino groups of proteins or other molecules (Fig. 1-39). This kit contains all of the necessary reagents for the labeling. The labeling process is very simple. Just add the NH_2- reactive Biotin to IgG solution and incubate at 37 °C for 10 min. An average of 5 to 8 biotin molecules conjugates to each IgG molecule. The number of biotin molecule per protein can be determined by HABA assay(page 37). Excess biotin molecules can be removed by a Filtration tube.



NH_2-reactive Biotin

Biotin-conjugated IgG

Fig. 1-39 IgG labeling reaction with NH_2-reactive biotin

Required Equipment and Materials

LK03-10: microcentrifuge, 10 µl and 100-200 µl adjustable pipettes, 37 °C incubator, 0.5 ml microtubes, DMSO
LK55-10: centrifuge, rotor for 15 ml centrifuge tube, 100-200 µl and 1 ml adjustable pipettes, 37 °C incubator, microtubes, DMSO

Precaution

- *The molecular weight of the protein to be labeled with this kit should be over 50,000.*
- *IgG or biotin-conjugated IgG is always on the filter membrane of the Filtration tube during the labeling process.*
- *If the IgG solution contains other proteins with molecular weights larger than 10,000, such as BSA or gelatin, purify the IgG solution prior to labeling biotin with this kit. IgG solution can be purified by IgG Purification Kits (not included in this kit).*
- *If the IgG solution contains small insoluble materials, centrifuge the solution and use the supernatant for the labeling.*

General Protocol for IgG Labeling

Add 100 µl WS buffer and the sample solution containing 100 µg IgG to a Filtration tube.[a)]

Centrifuge at 8,000-10,000 g for 10 min.[b)]

Add 10 µl DMSO to NH_2-reactive Biotin and dissolve with pipetting.[c)]

Add 100 µl Reaction buffer to the Filtration tube, 8 µl NH_2-reactive Biotin solution to the Filtration tube and pipette to mix.[d)]

Incubate the tube at 37 °C for 10 min.

Add 100 µl WS buffer to the Filtration tube and centrifuge at 8,000-10,000 g for 10 min.[b] Discard the filtrate.

Add 200 µl WS buffer to the Filtration tube and centrifuge at 8,000-10,000 g for 10 min.[b] Repeat this step.

Add 200 µl WS buffer and pipette 10 to 15 times to recover the conjugate.[e] Transfer the solution to a 0.5 ml tube and store at 0-4 °C.

a) The volume of IgG solution should be less than 100 µl. If the antibody concentration is lower than 1 mg per ml, repeat steps 1 and 2 until the total IgG accumulation becomes 100 µg. If the volume of the filtrate becomes 400 µl or more during the accumulation process, discard the filtrate prior to going to the next centrifuge step.
b) If the solution still remains on the filter after the centrifugation, spin for another 5 min, or increase the centrifuge speed.
c) NH_2-reactive Biotin is on the bottom of the tube. Add 10 µl DMSO to the bottom of the tube and pipette several times to dissolve.
d) If the amount of IgG is 200 µg, add entire NH_2-reactive Biotin solution.
e) You do not have to use WS buffer to recover biotin-conjugated IgG. You can choose any kind of buffer appropriate for your experiment.

Determination of Biotin/ Protein Ratio

The average number of biotin per IgG should be in the range of 5 and 8. If you need to determine the precise number of biotin molecules per protein, use HABA assay. The following is a HABA assay protocol.

Reagent solution:

200 µM HABA (4-hydroxyazobenzene-2-carboxylic acid) solution prepared with PBS, pH 7.4 ..... 1 ml
0.5 mg avidin/ml solution prepared with PBS, pH 7.4 ..... 1 ml
diluted sample solution (55 µl biotinylated protein solution + 110 µl PBS, pH 7.4)
25 µM biotin prepared with a mixed solution (2 volumes of PBS, pH 7.4 + 1 volume of WS buffer) ..... 200 µl
Prepare various concentration solutions (12.5 µM, 6.25 µM, 3.13 µM, 1.56 µM) with serial dilution ..... 200 µl each

1. Mix HABA solution and avidin solution in a plastic tube.
2. Add 100 µl of the HABA-avidin solution to 15 wells for multiple assays (n=3).
3. Add 50 µl biotin solution (12.5 µM, 6.25 µM, 3.13 µM, and 1.56 µM) to 3 wells each and 50 µl of diluted sample solution to the rest of the 3 wells.
4. Read the O.D. at 405 nm with a reference at 492 nm and prepare a calibration curve using the O.D. of various concentration of biotin solution. Read the O.D. at 280 nm to determine the protein concentration. (*e.g.* molar absorptivity of IgG at 280 nm: 216,000).
5. Determine the concentration of biotin in the sample solution and calculate the number of biotin molecule per protein.

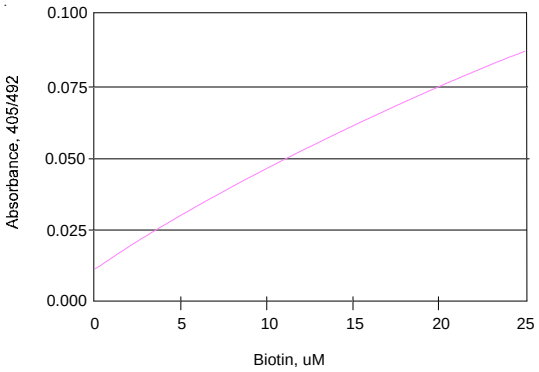


Fig. 1-40 Typical calibration curve of HABA assay.

FAQ

◆ **Can I use this kit for other proteins?**
Yes, if the molecular weight is greater than 50,000.

◆ **Do I have to use a Filtration tube prior to labeling the protein?**
If the protein solution does not contain small molecules with amino groups and the concentration of the protein is 10 mg per ml or about 70 µM, there is no need to use the Filtration tube. Just mix 10 µl of the sample solution with 90 µl of Reaction buffer and add the mixture to a vial of NH_2-reactive Biotin. After the reaction, transfer all of the reaction mixture to a Filtration tube, and then follow the protocol starting at step 6.

◆ **Do I have to use WS buffer to store the biotin-labeled protein?**
You don't have to use WS buffer. You can choose any kind of buffer according to your experiment.

◆ **My sample contains small insoluble material. What should I do?**
Spin the sample and use the supernatant for the labeling.

◆ **How long is the biotin-labeled protein stable?**
If you store the biotin-labeled protein at 0-5 °C, it is stable for 2 months. For longer storage, add 100% volume of glycerol, aliquot, and store at -20 °C (if the protein can be frozen). However, please note that stability depends on the protein itself.

◆ **What is the minimum amount of IgG that can be labeled by this kit?**
The minimum amount is 10 µg IgG; simply follow the protocol. The labeling ratio remains the same for 10 µg to 100 µg of IgG.

References

T. Yamabuki, *et al.*, *Cancer Res.*, **67**, 2517 (2007); N. Ishikawa, *et al.*, *Cancer Res.*, **67**, 11601 (2007); H. Kohara, *et al.*, *Blood*, **110**, 4153 (2007); T. Yoshida, *et al.*, *Genes Cells*, **13**, 667 (2008); K. Miyado, *et al.*, *PNAS*, **105**, 12921 (2008); I. Huang, *et al.*, *J. Virol.*, **82**, 4834 (2008); Y. C. Kim, *et al.*, *Cancer Res.*, **68**, 10145 (2008); T. Sakurai, *et al.*, *J. Cell Biol.*, **183**, 339 (2008).

Biotin Labeling Kit-SH

Application: Biotin labeling of protein, sulfhydryl reactive

Features: Only 3 hours to get conjugates
All processes in a single Filtration tube
High recovery of conjugates
Applicable for 50-200 µg IgG

Ordering Information

Product code	Unit
LK10-10	3 samples*
LK57-10	1 sample **

* Based on 100 µg IgG labeling.
**Based on 1mg IgG labeling.

Contents of the Kit

LK10-10

SH-reactive biotin ..... 3 tubes
Reducing agent ..... 3 tubes
Filtration tube ..... 3 tubes
WS buffer ..... 4 ml x 1 bottle
Reaction buffer ..... 1 ml x 1 tube

LK57-10

SH-reactive biotin ..... 1 tube
Reducing agent ..... 1 tube
Filtration tube ..... 1 tube
WS buffer ..... 15 ml x 1 bottle
Reaction buffer ..... 4 ml x 1 tube
15 ml tube ..... 1 tube

Storage Condition: 0-5 °C
Shipping Condition: ambient temperature

Product Description

Biotin Labeling Kit-SH is mainly used for the preparation of biotin-labeled IgG for enzyme immunoassay (EIA). SH reactive biotin, a component of this kit, reacts with sulfhydryl groups of proteins or other molecules (Fig. 1-41). The kit contains all of the necessary reagents for the labeling. The Reducing agent included in this kit creates sulfhydryl groups in the IgG molecule. Since the Reducing agent is a water-soluble phosphine compound, it can be removed by a Filtration tube with a simple process. An average of 5 to 8 biotin molecules conjugates to each IgG molecule. The number of biotin molecules per protein can be determined by HABA assay (page 37) Excess biotin molecules can be removed by a Filtration tube.

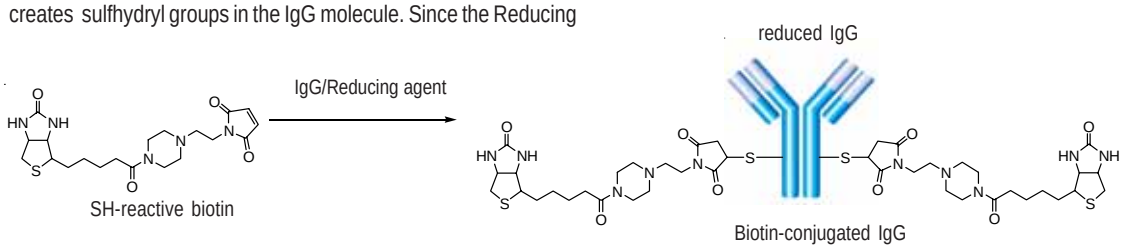


Fig. 1-41 IgG labeling reaction with SH-reactive biotin

Required Equipment and Materials

microcentrifuge, 10 µl and 100-200 µl adjustable pipettes, 37 °C incubator, 0.5 ml microtubes, DMSO

Precaution

⌘ *The molecular weight of the reduced protein to be labeled with this kit should be over 50,000.*
⌘ *IgG or biotin-conjugated IgG is always on the filter membrane of the Filtration tube during the labeling process.*
⌘ *If the IgG solution contains other proteins with molecular weights larger than 10,000, such as BSA or gelatin, purify the IgG solution prior to labeling biotin with this kit. IgG solution can be purified by IgG Purification Kits (not included in this kit).*
⌘ *If the IgG solution contains small insoluble materials, centrifuge the solution and use the supernatant for the labeling.*

General Protocol for IgG Labeling (LK10)

Add 100 µl WS buffer and the sample solution containing 100 µg IgG to a Filtration tube.[a)]

Centrifuge at 8,000-10,000 g for 10 min.[b)]

Add 150 µl WS buffer to a tube of Reducing agent and dissolve with pipetting.

Transfer 100 µl of the Reducing agent solution onto the membrane of the Filtration tube, and pipette to dissolve the IgG on the membrane.

I-4. Protein Labeling: Biotin

Incubate the tube at 37 °C for 30 min. Add 100 µl Reaction buffer and centrifuge at 8,000-10,000 g for 10 min.[b)]

Add 10 µl DMSO to SH-reactive biotin and dissolve with pipetting.[c)]

Add 100 µl Reaction buffer and 8 µl SH-reactive Biotin solution to the Filtration tube and pipette to mix.[d)]

Incubate the tube at 37 °C for 30 min. Add 100 µl WS buffer to the Filtration tube and centrifuge at 8,000-10,000 g for 10 min.[b)]

Add 200 µl WS buffer and centrifuge at 8,000-10,000 g for 10 min.[b)] Repeat this step.

Add 200 µl WS buffer and pipette 10 to 15 times to recover the conjugate.[e)] Transfer the solution to a 0.5 ml tube and store at 0-5 °C .

a) The volume of IgG solution should be less than 100 µl. If the antibody concentration is lower than 1 mg per ml, repeat steps 1 and 2 until the total IgG accumulation becomes 100 µg. If the volume of the filtrate becomes 400 µl or more during the accumulation process, discard the filtrate prior to going to the next centrifuge step.
b) If solution still remains on the filter after the centrifugation, spin for another 5 min, or increase the centrifuge speed.
c) SH-reactive biotin is on the bottom of the tube. Add 10 µl DMSO to the bottom of the tube and pipette several times to dissolve.
d) If the amount of IgG is 200 µg, add entire SH-reactive Biotin solution.
e) You do not have to use WS buffer to recover biotin-conjugated IgG; you can choose any kind of buffer appropriate for your experiment.

FAQ

- **Can I use this kit for other proteins?**
  Yes, if the molecular weight is greater than 50,000.
- **Do I have to use a Filtration tube prior to labeling the protein?**
  There is no need to use the Filtration tube if the following conditions are met.
  a) The protein has disulfide groups and its solution does not contain small molecules with SH groups or disulfide groups. The concentration of the protein is 10 mg per ml or about 70 µM: Mix 10 µl protein solution and 100 µl Reducing agent solution, and follow the protocol starting at step 4.
  b) The protein has SH groups and its solution does not contain small molecules with SH groups. The concentration of the protein is 10 mg per ml or about 70 µM: Mix 10 µl protein solution and 90 µl Reaction buffer, and add 8 µl SH-reactive biotin solution. Then follow the protocol starting at step 7.
- **Do I have to use WS buffer to store the biotin-labeled protein?**
  You don't have to use WS buffer. You can choose any kind of buffer according to your experiment.
- **My sample contains small, insoluble material. What should I do?**
  Spin the sample and use the supernatant for the labeling.
- **How long is the biotin-labeled protein stable?**
  If you store the biotin-labeled protein at 4 °C, it is stable for 2 months. For longer storage, add 100% volume of glycerol, aliquot, and store at -20 °C (if the protein can be frozen). However, please note that stability depends on the protein itself.
- **What is the minimum amount of IgG that can be labeled by this kit?**
  The minimum amount is 10 µg. There is no significant difference in sensitivity and background between 10 and 100 µg of IgG.

References

Y. Terasaki, *et al.*, *Am. J. Respir. Crit. Care Med.*, **174**, 665 (2006); H. Kohara, *et al.*, *Blood*, **110**, 4153 (2007); T. Into, *et al.*, *Mol. Cell. Biol.*, **28**, 1338 (2008); K. Miyado, *et al.*, *PNAS*, **105**, 12921 (2008); I. Huang, *et al.*, *J. Virol.*, **82**, 4834 (2008); Y. C. Kim, *et al.*, *Cancer Res.*, **68**, 10145 (2008).

I-4. Protein Labeling: Biotin

Biotin-OSu

Biotin *N*-hydroxysuccinimide ester [CAS: 35013-72-0]

Application: Biotin labeling of molecules with primary and secondary amines

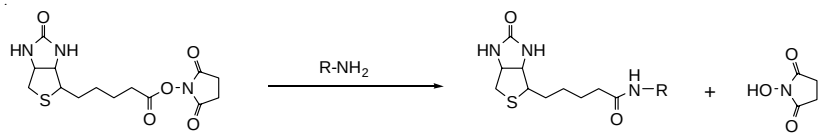
Appearance: white or slightly yellow powder
Purity: >95.0% (HPLC)
MW: 341.38, $C_{14}H_{19}N_3O_5S$

Storage Condition	Shipping Condition
-20 °C	ambient temperature

Ordering Information:

Product code	Unit
B304-10	10 mg

Reaction Scheme



Biotin-OSu

Biotin-AC5-OSu

6-(Biotinylamino)hexanoic acid *N*-hydroxysuccinimide ester [CAS: 72040-63-2]

Application: Biotin labeling of molecules with primary and secondary amines

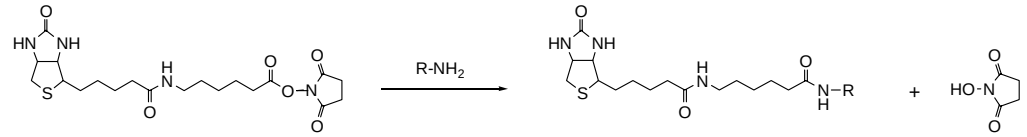
Appearance: white or slightly yellow powder
Purity: >95.0% (HPLC)
MW: 454.54, $C_{20}H_{30}N_4O_6S$

Storage Condition	Shipping Condition
-20 °C	ambient temperature

Ordering Information:

Product code	Unit
B305-10	10 mg

Reaction Scheme



Biotin-AC_5-OSu

Biotin-$(AC_5)_2$-OSu

6-[6-(Biotinylamino)hexanoylamino]hexanoic acid *N*-hydroxysuccinimide ester [CAS: 89889-52-1]

Application: Biotin labeling of molecules with primary and secondary amines

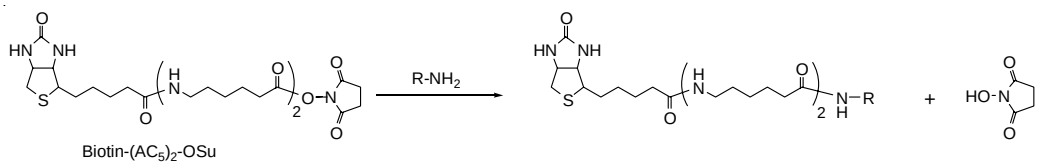
Appearance: white or slightly yellow powder
Purity: >90.0% (HPLC)
MW: 567.70, $C_{26}H_{41}N_5O_7S$

Storage Condition	Shipping Condition
-20 °C	ambient temperature

Ordering Information:

Product code	Unit
B306-10	10 mg

Reaction Scheme



Biotin-$(AC_5)_2$-OSu

Biotin Sulfo-OSu

Biotin *N*-hydroxy-sulfosuccinimide ester [CAS: 119616-38-5]

Application: Biotin labeling of molecules with primary and secondary amines

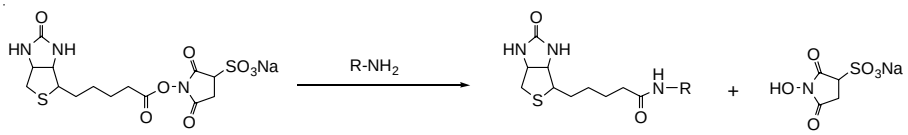
Appearance: white or pale reddish-brown powder
Purity: >95.0% (HPLC)
MW: 443.43, $C_{14}H_{18}N_3NaO_8S_2$

Storage Condition	Shipping Condition
-20 °C	with dry ice or blue ice

Ordering Information:

Product code	Unit
B319-10	10 mg

Reaction Scheme



Biotin-sulfo-OSu

Biotin-AC5 Sulfo-OSu

6-(Biotinylamino)hexanoic acid *N*-hydroxy-sulfosuccinimide ester [CAS: 109940-19-4]

Application: Biotin labeling of molecules with primary and secondary amines

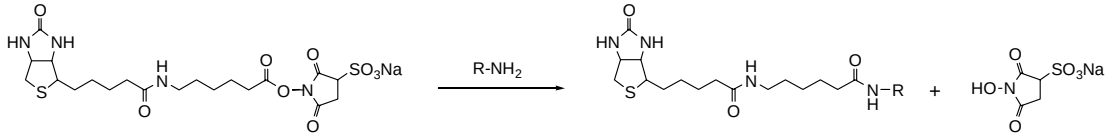
Appearance: white or pale reddish-brown powder
Purity: >95.0% (HPLC)
MW: 556.59, $C_{20}H_{29}N_4NaO_9S_2$

Storage Condition	Shipping Condition
-20 °C	with dry ice or blue ice

Ordering Information:

Product code	Unit
B320-10	10 mg

Reaction Scheme



Biotin-AC_5-sulfo-OSu

Biotin-(AC5)2 Sulfo-OSu

6-[6-(Biotinylamino)hexanoylamino]hexanoic acid *N*-hydroxy-sulfosuccinimide ester [CAS: 180028-78-8(free acid)]

Application: Biotin labeling of molecules with primary and secondary amines

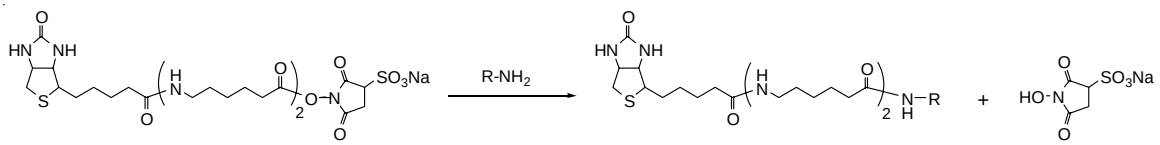
Appearance: white or pale reddish-brown powder
Purity: >95.0% (HPLC)
MW: 669.75, $C_{26}H_{40}N_5NaO_{10}S_2$

Storage Condition	Shipping Condition
-20 °C	with dry ice or blue ice

Ordering Information:

Product code	Unit
B321-10	10 mg

Reaction Scheme



Biotin-$(AC_5)_2$-sulfo-OSu

I-4. Protein Labeling: Biotin

Product Description of Amine-Reactive Biotins

The avidin-biotin system has many applications in immunology and histochemistry. The interaction between avidin and biotin is remarkably strong with a dissociation constant on the order of 10^{-15} M. Biotin is usually added to primary or secondary antibodies such as anti-IgG and anti-IgM. After preparing the antigen-antibody complex with the biotin-labeled antibody, colorimetric, or fluorometric detection of the antigen is performed using enzyme or fluorescein-labeled avidin or streptavidin. Succinimidyl ester biotins react with primary and secondary amines, such as amino acids and proteins, at pH 7-9. Succinimidyl ester reacts with free amine groups to create a stable amide bond. Succinimidyl biotin reagents must be dissolved in DMSO, DMF, or alcohol. Stock solutions prepared with DMSO are stable for several months at -20 °C. Sulfosuccinimidyl biotin reagents are soluble in water, so there is no need to use organic solvents such as DMF or DMSO. IgG prepared using biotin with a longer spacer such as Biotin-$(AC_5)_2$-OSu or Biotin-$(AC_5)_2$-Sulfo-OSu, has a better signal-to-noise ratio. The longer spacer enables streptavidin or anti-biotin IgG to recognize biotin without structural inhibition. Therefore, Biotin-$(AC_5)_2$-OSu is utilized as the biotin labeling agent in the Biotin Labeling Kit-NH_2.

Labeling Procedure for IgG

1. Prepare 10 mM of the biotin labeling reagent using DMSO.
2. Prepare 100 µl of 1 mg per ml IgG buffer solution (pH 7.5-8.5) that does not contain any large molecules with amine compounds.
3. Add 1-5 µl biotin labeling reagent DMSO solution to the IgG buffer solution and incubate at 37 °C for 1 hour.
4. Remove excess biotin labeling reagent using gel filtration or dialysis.
5. Prepare solutions for further experiment using an appropriate buffer such as PBST (0.05% Tween 20/PBS).

References

J. Guesdon, *et al., J. Histochem. Cytochem.*, **27**, 1131 (1979); J. J. Leary, *et al., Proc. Natl. Acad. Sci. USA*, **80**, 4045 (1983); W. T. Lee, *et al., J. Exp. Med.*, **159**, 1790 (1984); M. Shimkus, *et al., Proc. Natl. Acad. Sci. USA*, **82**, 2593 (1985); H. M. Ingalls, *et al., Proc. Natl. Acad. Sci. USA*, **83**, 4779 (1986); W. J. LaRochelle, *et al., J. Immunol. Methods*, **92**, 65 (1986); P. S. R. Anjaneyulu, *et al., Int. J. Peptide Protein Res.*, **30**, 117 (1987); J. Wormmeester, *et al., Methods Enzymol.*, **187**, 314 (1990); H. I. Magazine, *et al., Biochem. Biophys. Res. Commun.*, **181**, 1245 (1991); H. D. Grimes, *J. Plant Physiol.*, **139**, 45 (1991); S. Hase, *J. Biochem.*, 112, 266 (1992); L. N. Amankwa, *et al., Nucleic Acids Res.*, 20, 4831 (1992); C. K. J. Oh, *et al., J. Pharm. Biomed. Anal.*, **10**, 813 (1992); J. Zhang, *et al., Biochemistry*, **32**, 2228 (1993); J. A. Bradburne, *et al., Appl. Environ. Microbiol.*, **59**, 663 (1993); R. B. del Rosario, *et al., J. Nucl. Med.* **34**, 1147 (1993); W. M. Pardridge, *et al., Delivery*, **1**, 43 (1993); V. R. Muzykantov, *et al., Anal. Biochem.*, **223**, 142 (1994); Y. Imai, *et al., J. Immunol. Methods*, **171**, 23 (1994); I. Islam, *et al., J. Med. Chem.*, **37**, 293 (1994); T. Hoshi, *et al., Anal. Sci.*, **11**, 311 (1995); Su-Yau *et al., Methods Mol. Biol.*, **34**, 49 (1994); T. Saga, *et al., Cancer Res.*, **54**, 2160 (1994); M. O. De Jong, *et al., J. Immunol. Methods*, **184**, 101 (1995). D. Bowman, *et al., Neuropharmacology*, **34**, 743 (1995); H. J. Schuberth, *et al., J. Immunol. Methods*, **189**, 89 (1996); K. L. Manning, *et al., Lab. Anim. Sci.*, **46**, 545 (1996); D. Storm, *et al., J. Immunol. Methods*, **199**, 87 (1996); E. K. De La Fuente, *et al., Am. J. Physiol.*, **272**, L461 (1997); G. Hoffmann-Fezer, *et al., Ann. Hematol.*, **74**, 231 (1997); B. T. Miller, *et al., Peptides*, **18**, 1585 (1997). K. J. Wardrop, *et al., Am. J. Vet. Res.*, **59**, 397 (1998); A. von Leoprechting, *et al., Anal. Biochem.*, **262**, 110 (1998); G. Maturi, *et al., Dev, Biol.*, **204**, 210 (1998); P. Malik, *et al., Tissue Antigens*, **53**, 576 (1999); H. Shimosato, *et al., Plant Cell*, **19**, 107 (2007); N. Kawasaki, *et al., J. Biochem.*, **141**, 221 (2007).

Biotin-PE-maleimide

N-Biotinyl-*N'*-[2-(*N*-Maleimido)ethyl]piperazone, hydrochloride

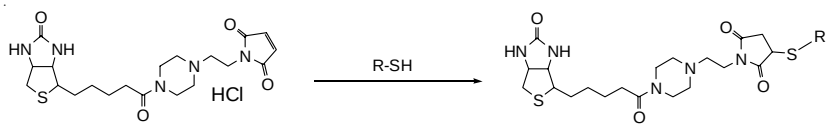
Application: Biotin labeling of molecules with sulfhydryl groups

Appearance: white or slightly yellow powder
Purity: >90.0% (HPLC)
MW: 472.00, $C_{20}H_{30}ClN_5O_4S$

Ordering Information:	
Product code	Unit
B300-10	10 mg

Storage Condition	Shipping Condition
-20 °C	ambient temperature

Reaction Scheme



Biotin-PE-maleimide

Biotin-PEAC5-maleimide

N-6-(Biotinylamino)hexanoyl-*N'*-[2-(*N*-maleimido)ethyl]piperazone, hydrochloride

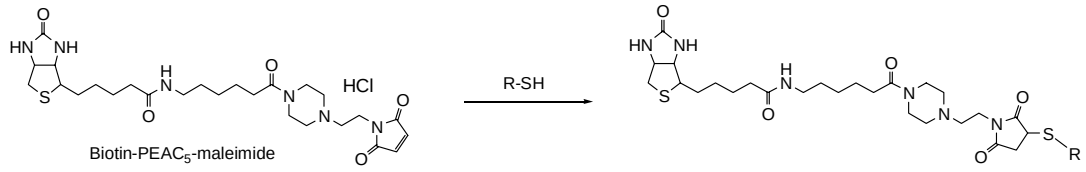
Application: Biotin labeling of molecules with sulfhydryl groups

Appearance: white or slightly yellow powder
Purity: >90.0% (HPLC)
MW: 585.16, $C_{26}H_{41}ClN_6O_5S$

Ordering Information:	
Product code	Unit
B299-10	10 mg

Storage Condition	Shipping Condition
-20 °C	ambient temperature

Reaction Scheme



Biotin-PEAC$_5$-maleimide

Product Description

The avidin-biotin system has many applications in immunology and histochemistry. The interaction between avidin and biotin is remarkably strong with a dissociation constant on the order of the 10^{-15} M. Biotin is usually added to primary or secondary antibodies such as anti-IgG and anti-IgM. After preparing the antigen-antibody complex with the biotin-labeled antibody, colorimetric or fluorometric detection of the antigen is performed using enzyme or fluorophore-labeled avidin or streptavidin. Maleimide biotins react with thiol compounds, such as proteins or peptides with sulfhydryl groups, at pH 7-7.5. Maleimide reacts with sulfhydryl group to create a thioether bond. Though other maleimide biotin reagents must be dissolved in DMSO, DMF, or alcohol, Biotin-PE-maleimide can be solubilized in PBS at pH 7.4 to prepare 2 mM solution without using an organic solvent. The reactivity of maleimide with sulfhydryl groups is higher than that of bromoacetamide, so the required concentration of maleimide biotin is much lower than that of bromoacetamide biotins. Stock solutions of Biotin-PE-maleimide and Biotin-PEAC$_5$-maleimide in DMSO are stable for one year at -20 °C.

Labeling Procedure for Reduced IgG

1. Prepare 10 mM of the biotin labeling reagent using DMSO.
2. Prepare 100 µl of 1 mg per ml reduced IgG/ml buffer solution which does not contain any large molecules with SH groups. Reduced IgG can be prepared by TCEP (tricarboxyethylphosphine), DTT or 2-mercaptoethylamine.
3. Add 1-5 µl of biotin labeling reagent DMSO solution to the IgG buffer solution and incubate at 37 °C for 1 hour.
4. Remove excess biotin labeling reagent using a gel column or a Filtration tube.
5. Prepare solutions for further experiment using an appropriate buffer such as PBST (0.05% Tween 20/PBS).

References

E. Muneyuki, *et al.*, *Biophys. J.*, **92**, 1806 (2007).

I-4. Protein Labeling: Biotin

ARP (Aldehyde Reactive Probe)

N-(Aminooxyacetyl)-*N*'-biotinyl-hydrazine
[CAS: 139585-03-8]

Application: Biotin labeling of molecules with aldehyde or ketone groups

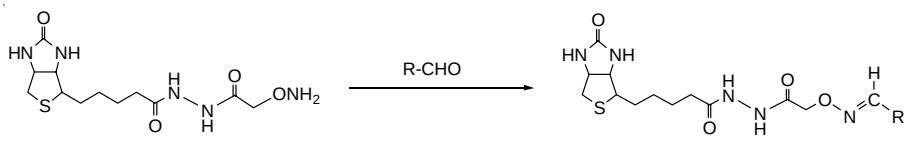
Appearance: white powder
Purity: > 95.0% (HPLC)
MW: 331.39, $C_{12}H_{21}N_5O_4S$

Ordering Information:

Product code	Unit
A305-10	10 mg
A305-12	25 mg

Storage Condition: -20 °C
Shipping Condition: ambient temperature

Reaction Scheme



ARP

Product Description

Aldehyde Reactive Probe (ARP) is utilized for the detection of abasic sites (AP sites, depurine/depyrimidine sites) in DNA because of its aldehyde-specific reactivity. ARP reacts with aldehyde and ketone groups, and it adds biotin to AP sites in DNA. The biotin-tagged DNA is then detected using peroxidase-labeled avidin and oxidative chromogenic dyes. The ARP method has been used to detect less than 1 AP site in $1x10^4$ nucleotides of DNA. Dojindo offers DNA Damage Quantification Kit (Product Code: DK02) containing all the necessary reagents and components for the determination of 1 to 40 AP sites per $1x10^5$ base pairs. ARP is highly soluble in water and the stock solution can be stored at 4 °C for one year without significant loss of reactivity.

Labeling Procedure for DNA Abasic Site

1. Prepare 10 mM ARP with water.
2. Adjust the DNA concentration to 100 µg per ml with TE buffer (pH 7.4).
3. Mix equal volumes of the DNA and ARP solutions and incubate at 37 °C for 1 hour.
4. Isolate the ARP-labeled DNA using either ethanol precipitation or membrane filtration tube.
5. Dissolve the DNA pellet in TE to prepare 10-100 µg per ml solution.

References

K. Kubo, *et al.*, *Biochemistry*, **31**, 3703 (1992); H. Ide, *et al.*, *Biochemistry*, **32**, 8276 (1993); J. Nakamura, *et al.*, *Cancer Res.*, **58**, 222 (1998); A. Asaeda, *et al.*, *Nucleosides Nucleotides*, **17**, 503 (1998); G. Maulik, *et al.*, *Nucleic Acids Res.*, **27**, 1316 (1999); V. J. Melendez-Colon, *et al.*, *Carcinogenesis*, **20**, 1885 (1999); H. Atamna, *et al.*, *J. Biol. Chem.*, **275**, 6741 (2000); H. B. Sun, *et al.*, *Anal. Chem.*, **73,** 2229 (2001); J. Zielinska-Park, *et al.*, *Carcinogenesis*, **25**, 1727 (2004); P. D. Chastain II, *et al.*, *FASEB J.*, **20**, 2127 (2006); L. Yan, *et al.*, *Clin. Cancer Res.*, **13**, 1532 (2007).

AB-NTA free acid

N-(5-Amino-1-carboxypentyl)iminodiacetic acid [CAS: 129179-17-5]

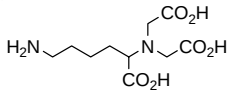
Application: Chelate labeling

Appearance: pale yellowish-white powder
Purity: > 97.0% (HPLC)
MW: 262.26, $C_{10}H_{18}N_2O_6$

Ordering Information:	
Product code	Unit
A459-10	100 mg

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



Product Description

AB-NTA free acid replaces AB-NTA (disodium salt form; former Product Code: A296-10). The new free acid form is less hygroscopic than the disodium salt form. The procedure stays the same as the disodium salt when dissolving it in a buffer. It can also be dissolved in water with sonication (up to 3%). Aminobutyl-NTA was first used by Dr. Hochuli in 1987 for the purification of recombinant proteins (the "His-tag" technique). Since then, the compound has become an indispensable tool for immobilizing proteins with high specificity on solid surfaces, such as glass or the gold electrode. A solid surface is modified by AB-NTA and bio-functionalized via Ni (II) with a genetically expressed protein bearing a hexahistidine extension at its terminus. The His-tag technique has become increasingly important, particularly in surface plasmon resonance and structural analyses of proteins by x-ray interference. Using the His-tag technique, Dr. Noji was able to directly observe the rotation of F1-ATPase by fluorescence microscope.

References

E. Hochuli, *et al.*, *J. Chromatogr*., **411**, 177 (1987); E. Hochuli, *et al.*, *J. Chromatogr*, **444**, 293 (1988); Y. C. Sasaki, *et al.*, *Science*,. **263**, 62 (1994); G. B. Sigal, *et al.*, *Anal Chem*., **68**, 490 (1996); E. L. Schmid, *et al.*, *Anal. Chem*., 69, 1979 (1997); YH. Noji, *et al.*, *Nature*, 386, 299 (1997); S. Sivasankar, *et al.*, *Biophys. J.*, **80**, 1758 (2001).

BABE

1-(p-Bromoacetamidobenzyl) ethylenediamine *N,N,N',N'*-tetraaceticacid [CAS: 81677-64-7]

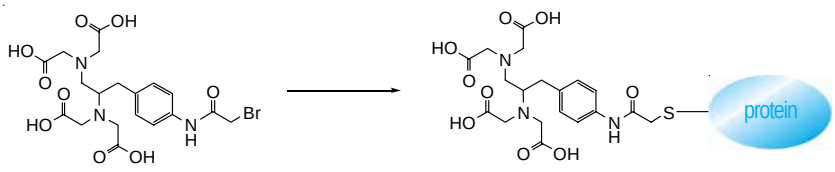
Application: Chelate labeling of molecules with sulfhydryl groups

Appearance: white or light gray powder
Purity: >90.0% (HPLC)
MW: 518.32, $C_{19}H_{24}BrN_3O_9$

Ordering Information:	
Product code	Unit
B437-10	10 mg

Storage Condition: -20 °C
Shipping Condition: ambient temperature

Reaction Scheme



BABE — EDTA-conjugated protein

FeBABE

(*S*)-[1-[[Bis(carboxymethyl)amino]methyl]-2-[4-[(2-bromoacetyl)amino]phenylethyl]-(carboxymethyl)amino]acetic acid iron(III)

Application: Chelate labeling of molecules with sulfhydryl groups

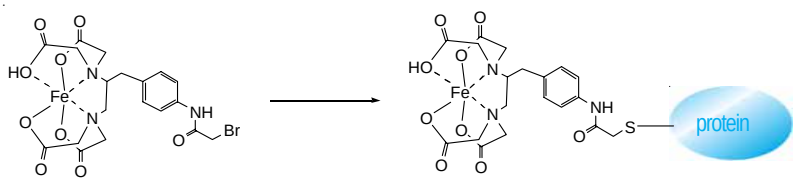
Appearance: yellowish-brown powder
Purity: < 95.0%(HPLC)
MW: 571.14, $C_{19}H_{21}BrFeN_3O_9$

Ordering Information:	
Product code	Unit
F279-10	1 mg

Storage Condition: -20 °C
Shipping Condition: ambient temperature

I-5. Protein Labeling: Chelate

Reaction Scheme



FeBABE — FeBABE-conjugated protein

Product Description of BABEs

Bromoacetamidobenzyl-EDTA (BABE) is a chelate labeling reagent that conjugates with sulfhydryl groups. The iron chelate of BABE (FeBABE) is a unique tool for determining the three-dimensional structure of proteins and the binding structures of protein-protein or protein-DNA complexes. BABE adds EDTA moieties to proteins through their sulfhydryl groups. Once attached to a protein, FeBABE cuts a nearby peptide or DNA chain. The cleavage site is within 12 angstroms of the FeBABE binding site. Iron (II)-chelate cleaves a peptide or DNA chain in the presence of hydrogen peroxide. The cleavage reaction completes very quickly; 10 seconds to 20 minutes of incubation is sufficient. The size of the cleaved fragment is analyzed with gel electrophoresis such as SDS-PAGE.

Labeling Procedure

1. Dialyze the protein solution in conjugation buffer (10-20 mM MOPS, 0.2 M NaCl, 2 mM EDTA, 5% glycerol, pH 8.0) at 4 °C overnight.
2. After dialysis, adjust the protein concentration to 15-30 mM.
3. Add 15 ul of 20 mM FeBABE DMSO solution to 1 ml of the protein solution and incubate it at 37 °C for 1 hour. The final concentration of FeBABE is 0.3 mM (10-20X excess to the protein)
4. Dialyze the reaction mixture in protein storage buffer (10-20 mM Tris, 0.1-0.2 M KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 50% glycerol, pH 7.6) at 4 °C overnight.

References

L. H. DeRiemer, *et al.*, *J. Labelled Compd. Radiopharm.*, **18**, 1517 (1981); T. M. Rana, *et al.*, *Proc. Natl. Acad. Sci. USA*, **88**, 10578 (1991); J. B. Ghaim, *et al.*, *Biochemistry*, **34**, 11311 (1995); K. Murakami, *et al.*, *Proc. Natl. Acad. Sci. USA*, **94**, 1709 (1997); R. Miyake, *et al.*, *Biochemsitry*, **37**, 1344 (1998); J. T. Owens, *et al.*, *Proc. Natl. Acad. Sci. USA*, **95**, 6021 (1998); J. T. Owens, *et al.*, *Biochemistry*, **37**, 7670 (1998); K. B. Hall, *et al.*, *Methods*, **18**, 78 (1999); C. S. Badorrek, *et al.*, *Proc. Natl. Acad. Sci.*, **103**, 13640 (2006).

Isothiocyanobenzyl-EDTA

1-(4-Isothiocyanatobenzyl)ethylenediamine-*N,N,N',N'*-tetraacetic acid [CAS: 105394-74-9]

Application: chelate labeling of proteins

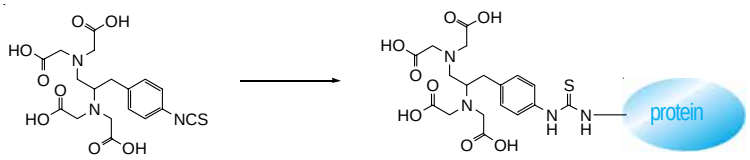
Appearance: slightly yellowish-brown powder
Purity: > 90.0% (HPLC)
MW: 439.44, $C_{18}H_{21}N_3O_8S$

Ordering Information:

Product code	Unit
M030-10	10 mg

Storage Condition: -20 °C
Shipping Condition: ambient temperature

Reaction Scheme



Isothiocyanobenzyl-EDTA — EDTA-conjugated protein

Product Description

Isothiocyanobenzyl-EDTA adds EDTA moieties to proteins through their amine groups. The EDTA-tagged protein can then be labeled with heavy metal ions and radioactive metals, such as ^{111}In and ^{99}Tc, and used for immunoimaging.

References

C. F. Meares, *J. Med. Chem.*, **17**, 1304 (1974); C. F. Meares, *et al.*, *Proc. Natl. Acad. Sci. USA.*, **73**, 3803 (1976); C. S-H. Leung, *et al.*, *Biochem. Biophys. Res. Commun.*, **75**, 149 (1977); S. M. Yeh, *et al.*, *Anal. Biochem.*, **100**, 152 (1979); L. H. DeRiemer, *et al.*, *J. Med. Chem.*, **22**, 1019 (1979); C. F. Meares, *et al.*, *Anal. Biochem.*, **142**, 68 (1984); C. F. Meares, *et al.*, *J. Protein chem.*, **3**, 215 (1984); M. W. Brechbiel, *et al.*, *Inorg. Chem.*, **25**, 2772 (1986); S. V. Deshpande, *et al.*, *J. Nuclear Med.*, **31**, 218 (1990); M. Rana, *et al.*, *J. Am. Chem. Soc.*, **112**, 2457 (1990); M. Studer, *et al.*, *Bioconjug. Chem.*, **3**, 420 (1992); Y. Arano, *et al.*, *Nucl. Med. Biol.*, **22**, 555 (1995); Y. Arano, *et al.*, *Bioconjug Chem.*, **7**, 628 (1996); A. I. Song, *et al.*, *Bioconjug. Chem.*, **8**, 249 (1997); Y. Arano, *et al.*, *Bioconjug. Chem.*, **9**, 497 (1998); T. Mukai, *et al.*, *Nucl. Med. Biol.*, **26**, 281 (1999); N. Watanabe, *et al.*, *Nucl. Med. Biol.*, **26**,239 (1999); Y. Fijibayashi, *et al.*, *Nucl. Med. Biol.*, **26**,17 (1999); T. Iwata *et al.*, *J. Chromatogr. A.*, **859**, 13(1999); C. S. Badorrek, *et al.*, *Proc. Natl. Acad. Sci.*, **103**, 13640 (2006).

DTPA anhydride

Diethylenetriamine-*N,N,N',N",N"*-pentaacetic dianhydride [CAS: 23911-26-4]

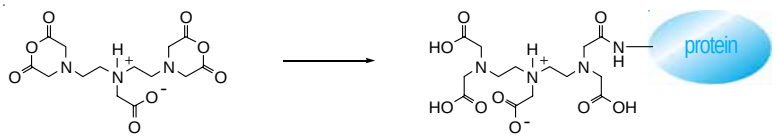
Application: Chelate labeling of proteins

Appearance: slightly yellow or yellowish-brown powder
Purity: > 99.0% (Titration)
MW: 357.32, $C_{14}H_{19}N_3O_8$

Ordering Information:	
Product code	Unit
D033-10	1 g
D033-12	5 g

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Reaction Scheme



DTPA anhydride — DTPA-conjugated protein

Product Description

DTPA anhydride is utilized to add chelate functions to molecules or surfaces with amine groups. After adding chelate functions, metal ions such as radioisotopes can be introduced to the molecule. According to Dr. Hnatowich, IgG may be labeled with radioactive indium using DTPA-anhydride. DTPA is first linked to IgG through a covalent bond, then In(III) is tightly chelated to DTPA. Such radioactive antibodies are known to bind with antigen *in vitro* and *in vivo*.

References

D. J. Hnatowich, *et al., J. Appl. Radiat. Isot.*, **33**, 327 (1982); D. J. Hnatowich, *et al., Science*, **220**, 613 (1983); C. H. Paik, *et al., J. Nucl. Med.*, **26**, 482 (1985); J. C. Saccavini, *et al., Invest. Radiol.*, **23 (Suppl. 1)**, S292 (1988); G. R. Boniface, *et al., J. Nucl. Med.*, **30**, 683 (1989); Y. C. Lee, *et al., Cancer Res.*, **50**, 4546 (1990); I. Virgolini, *et al., J. Nucl. Med.*, **32**, 2132 (1991); R. Reilly, *et al., Int. J. Rad. Appl. Instrum. A*, **43**, 961 (1992); M. Li, *et al., Bioconjug. Chem.*, **8**, 127 (1997); E. R. Wisner, *et al., J. Med. Chem.*, **40**, 3992 (1997); Higuchi, *et al., Anticancer Drugs*, **10**, 89 (1999).

Maleimido-C3-NTA

N-(5-(3-Maleimidopropylamido)-1-carboxy-pentyl)iminodiacetic acid, disodium salt, monohydrate

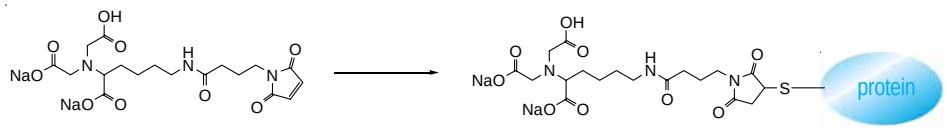
Application: Chelate labeling of proteins or surfaces with sulfhydryl groups

Appearance: pale yellowish-white powder
Purity: >97.0% (HPLC)
MW: 489.38 $C_{18}H_{23}N_3Na_2O_9 \cdot H_2O$

Ordering Information:	
Product code	Unit
M035-10	10 mg

Storage Condition: 0-5 ºC
Shipping Condition: with blue ice or dry ice

Reaction Scheme



Maleimido-C_3-NTA — NTA-conjugated protein

Product Description

Maleimido-C_3-NTA is utilized to modify the surfaces on which thiol groups are attached. Through the NTA moiety attached on the surface, genetically expressed protein, bearing a hexahistidine extension at its terminus, can be immobilized *via* Ni (II) (His-Tag method). Using this technique, Dr. Noji and co-workers were able to directly observe the rotation of F1-ATPase with a fluorescence microscope.

References

L. H. DeRiemer, *et al., J. Labeled Compd. Radiopharm.*, **18**, 1517 (1981); T. M. Rana, *et al., Proc. Natl. Acad. Sci. USA*, **88**, 10578 (1991); J. B. Ghaim, *et al., Biochemistry*, **34**, 11311 (1995); K. Murakami, *et al., Proc. Natl. Acad. Sci. USA*, **94**, 1709 (1997); R. Miyake, *et al., Biochemsitry*, **37**, 1344 (1998); J. T. Owens, *et al., Proc. Natl. Acad. Sci. USA*, **95**, 6021 (1998); J. T. Owens, *et al., Biochemistry*, **37**, 7670 (1998); K. B. Hall, *et al., Methods*, **18**, 78 (1999).

I-6. Protein Labeling: Cross-linking Reagent

EMCS

N-(6-Maleimidocaproyloxy)succinimide [CAS: 55750-63-5]

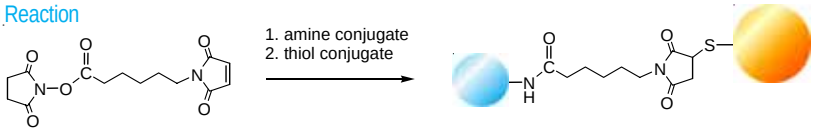
Application: Amine and sulfhydryl group cross-linking

Appearance: white or slightly yellow powder
Purity: >90.0% (HPLC)
MW: 308.29, $C_{14}H_{16}N_2O_6$

Storage Condition	Shipping Condition
0-5 °C	ambient temperature

Ordering Information	
Product code	Unit
E018-10	50 mg
E018-12	100 mg

Cross-linking Reaction



GMBS

N-(4-Maleimidobutyryloxy)succinimide [CAS: 80307-12-6]

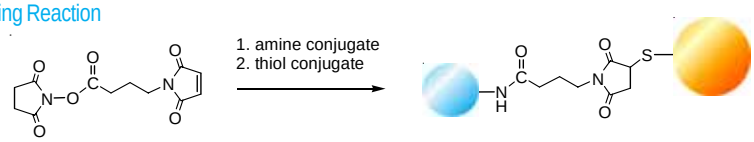
Application: Amine and sulfhydryl group cross-linking

Appearance: white powder
Purity: >95.0% (HPLC)
MW: 280.23, $C_{12}H_{12}N_2O_6$

Storage Condition	Shipping Condition
0-5 °C	ambient temperature

Ordering Information	
Product code	Unit
G005-10	50 mg
G005-12	100 mg

Cross-linking Reaction



HMCS

N-(8-Maleimidocapryloxy)succinimide

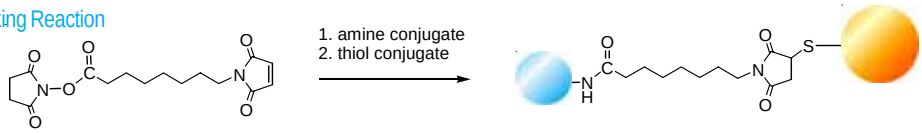
Application: Amine and sulfhydryl group cross-linking

Appearance: colorless or pale yellow oil, or waxy solid or powder
Purity: >90.0% (HPLC)
MW: 336.34, $C_{16}H_{20}N_2O_6$

Storage Condition	Shipping Condition
0-5 °C, protect from moisture	with dry ice or blue ice

Ordering Information	
Product code	Unit
H257-10	50 mg

Cross-linking Reaction



KMUS

N-(11-Maleimidoundecanoyloxy)succinimide [CAS: 87981-04-2]

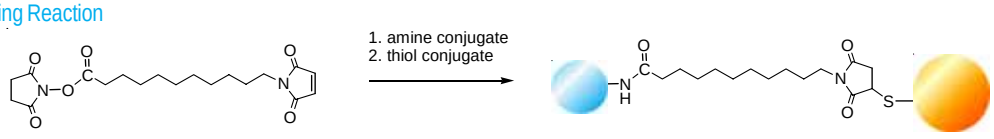
Application: Amine and sulfhydryl group cross-linking

Appearance: colorless or pale yellow oil, or waxy solid
Purity: >90.0% (HPLC)
MW: 378.42, $C_{19}H_{26}N_2O_6$

Storage Condition	Shipping Condition
0-5 °C, protect from moisture	ambient temperature

Ordering Information	
Product code	Unit
K214-10	50 mg

Cross-linking Reaction



Sulfo-EMCS

N-(6-Maleimidocaproyloxy)sulfosuccinimide, sodium salt

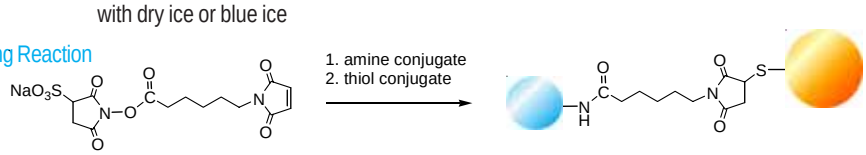
Application: Amine and sulfhydryl group cross-linking, water-soluble

Appearance: white or slightly yellowish-pink powder
Purity: >90.0% (HPLC)
MW: 410.33, $C_{14}H_{15}N_2NaO_9S$

Ordering Information	
Product code	Unit
S024-10	50 mg

Storage Condition: 0-5 °C
Shipping Condition: with dry ice or blue ice

Cross-linking Reaction



Sulfo-GMBS

N-(4-Maleimidobutyryloxy)sulfosuccinimide, sodium salt

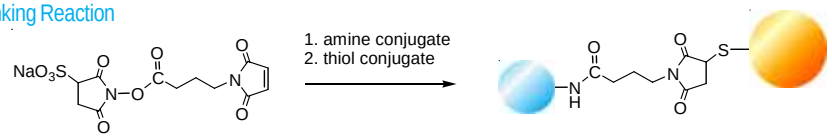
Application: Amine and sulfhydryl group cross-linking, water-soluble

Appearance: white or slightly yellowish-pink powder
Purity: >90.0% (HPLC)
MW: 382.28, $C_{12}H_{11}N_2NaO_9S$

Ordering Information	
Product code	Unit
S025-10	50 mg

Storage Condition: 0-5 °C
Shipping Condition: with dry ice or blue ice

Cross-linking Reaction



Sulfo-HMCS

N-(8-Maleimidocapryloxy)sulfosuccinimide, sodium salt [CAS: 211236-35-0]

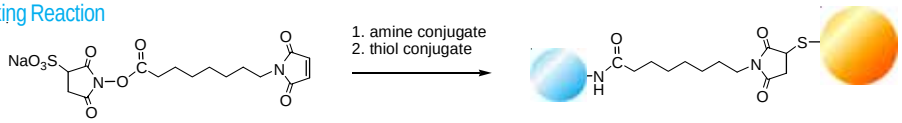
Application: Amine and sulfhydryl group cross-linking, water-soluble

Appearance: white or slightly yellowish-pink powder
Purity: >90.0% (HPLC)
MW: 438.39, $C_{16}H_{19}N_2NaO_9S$

Ordering Information	
Product code	Unit
S026-10	50 mg

Storage Condition: 0-5 °C, protect from moisture
Shipping Condition: with dry ice or blue ice

Cross-linking Reaction



Sulfo-KMUS

N-(11-Maleimidoundecanoyloxy)sulfosuccinimide, sodium salt [CAS: 211236-68-9]

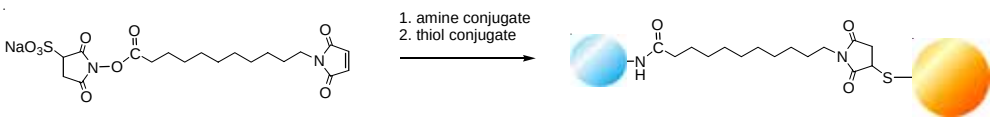
Application: Amine and sulfhydryl group cross-linking, water-soluble

Appearance: white or slightly yellowish-pink powder
Purity: >90.0% (HPLC)
MW: 480.47, $C_{19}H_{25}N_2NaO_9S$

Ordering Information	
Product code	Unit
S250-10	50 mg

Storage Condition: 0-5 °C, protect from moisture
Shipping Condition: with dry ice or blue ice

Cross-linking Reaction

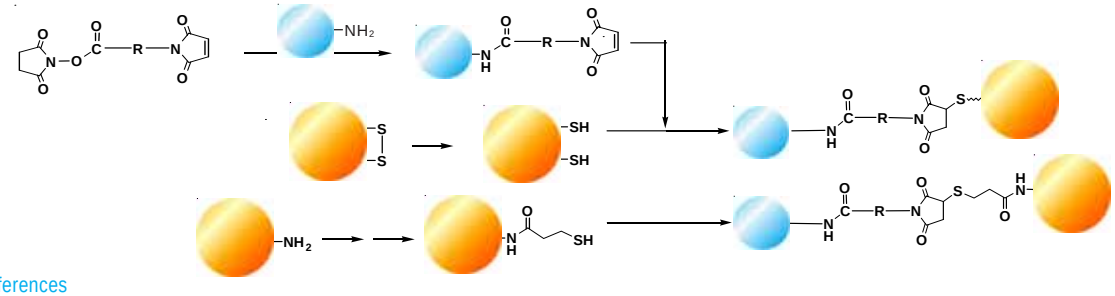


I-6. Protein Labeling: Cross-linking Reagent

Product Discription

Hetero-bifunctional cross-linking reagents have activated esters and maleimide reactive groups. These functional groups react with amines and sulfhydryl groups of proteins, respectively. Enzyme-labeled haptens are prepared using hetero-bifunctional cross-linking reagents such as EMCS or GMBS. The cross-linking reaction requires neutral pH and mild temperature since it is necessary to maintain enzyme activity and antibody titers in the cross-linking reaction. Hetero-bifunctional cross-linking reagents with 3, 5, 7, or 10 linear carbon chains are available. These linear aliphatic chains act as spacers between the two reactive sites and their water-soluble reagents. They are more stable than the aromatic cross-linking reagents such as succinimidyl-4-*N*-maleimidobenzoate in a wider pH range.

Conjugation of Macromolecules with Hetero-Bifunctional Cross-Linking Reagent



References

EMCS: S. Yoshitake, *et al.*, *Eur J. Biochem.*, **101**, 395 (1979); T. Kitagawa, *et al.*, *Chem. Pharm. Bull.*, **29**, 1130 (1981); S. Yoshitake, *et al.*, *J. Biochem.*, **92**, 1413 (1982); E. Ishikawa, *et al.*, *J. Immunol.*, **4**, 209 (1983); I. Weels, *et al.*, *Clin. Chem.*, **29**, 1480 (1983); S. Hashida, *et al.*, *J. Appl. Biochem*, **6**, 56 (1984); S. Inoue, *et al.*, *Anal. Lett.*, **17**, 229 (1984); M. Koizumi, *et al.*, *Cancer Res.*, **48,** 1189 (1988); N. Shinha, *et al.*, *Nucleic Acids Res.*, **16**, 2659 (1988); T. Kohno, *et al.*, *Anal. Lett.*, **21**, 1019 (1988).
GMBS: H. Tanimori, et al., *J. Pharm. Dyn.*, **4** , 812 (1981); K. Fujiwara, et al., *J. Immunological Methods*, **45**, 195 (1981); H. Tanimori, *et al.*, *J. Immunol. Methods*, **83**, 327 (1985); J. Sunamoto, *et al.*, *Biochim. Biophys. Acta*, **898**, 323 (1987); K. Fujiwara, *et al.*, *J. Immunol. Methods*, **110**, 47 (1988); K. Fujiwara, *et al.*, *J. Immunological Methods*, **122**, 77 (1988); B. A. Warden, *et al.*, *J. Immunol. Methods*, **131**, 77 (1990); P. Hermentin, *et al.*, *Bioconjug. Chem.*, **1**, 411 (1990); Fujiwara K, *Histochemistry*, **101**, 287 (1994); K. Fujiwara, *et al.*, *Histochemistry*, **102**, 397 (1994); K. Fujiwara, *et al.*, *J. Biochem.*, **118**, 1211 (1995); M. Matsuzawa, *et al.*, *J. Neurosci. Methods*, **69**, 189 (1996); L. C. Shriver-Lake, *et al.*, *Biosens. Bioelectron.*, **12**, 1101 (1997); K. Fujiwara, *et al.*, *J. Biochem.*, **124**, 244 (1998).
Sulfo type: J. Staros, *et al.*, *Biochemistry*, **21**, 3950 (1982); P. Anjaneyulu, *et al.*, *Int. J. Peptide Protein Res.*, **30**, 117 (1987); Y. Fukami, *et al.*, *J. Biol. Chem.*, **268**, 1132 (1993).

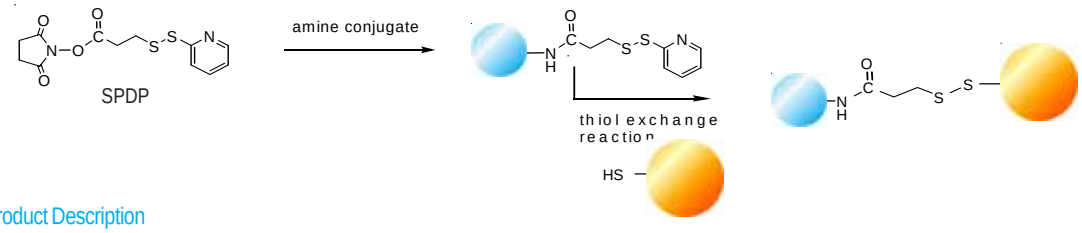
SPDP *N*-Succinimidyl 3-(2-pyridyldithio)propionate [CAS: 68181-17-9]

Application: Amine and sulfhydryl group cross-linking
Appearance: white or slightly yellow powder
Purity: >98.0% (HPLC)
MW: 312.37, $C_{12}H_{12}N_2O_4S_2$

Ordering Information	
Product code	Unit
S291-10	100 mg

Storage Condition	Shipping Condition
0-5 °C	ambient temperature

Cross-linking Reaction



Product Description

SPDP, a hetero-bifunctional reagent, is useful for bridging two different proteins, such as enzymes and antibodies. SPDP first reacts with a protein molecule through its amino groups. SPDP introduces a pyridyl disulfide moiety to the protein, which is then reduced by DTT to form thiol groups. These thiol groups then form a disulfide bond with another protein molecule thus yielding a heterodimer protein.

References

J. Carlsson, *et al.*, *Biochem. J.*, **173**, 723 (1978). A. R. Neurath, *et al.*, *J. Viro. Metth.* , **3**, 155 (1981). A. J. Cumber, *et al.*, *Meth. Enzymol.*, **112**, 207 (1985). Y. Singh, *et al.*, *Mol. Cell. Biochem.* , **120**, 95 (1993). T. S. Moll, *et al.*, *Biochemistry*, **33**, 15469 (1994). E. Harokopakis, *et al.*, *J. Immunol. Methods*, **185**, 31 (1995). K. Mitsui, *et al.*, *FEBS Lett.*, **385**, 29 (1996). D. Delforge, *et al.*, *J. Biol. Chem.*, **272**, 2276 (1997). P. G. Tardi, *et al.*, *J. Immunol. Methods*, **210**, 137 (1997). T. Ueno, *et al.*, *J. Biochem.*, **124**, 485 (1998). D. H. Na, *et al.*, *Bioconjug. Chem.*, **10**, 306 (1999). Y. Nakano, *et al.*, *Int. Arch. Allergy Immunol.*, **120**, 199 (1999).

IgG Purification Kit-A

Application: Immunoglobulin G purification, isolation

Features: All processes are proceeded in one tube
IgG recovery is 70-90%
Purity of the IgG from serum is over 80%
Purified IgG is available in 30 min
High reproducibility and no affinity loss

Ordering Information

Product code	Unit
AP01-10	1 sample

Contents of the Kit

Protein A pack ..... 1 pack
Elution buffer .....4 ml x 1 bottle
Washing buffer ..... 10 ml x 1 bottle
Catching buffer .....1.5 ml x 1 bottle

Storage Condition: 0-5 °C
Shipping Condition: ambient temperature

IgG Purification Kit-G

Application: Immunoglobulin G purification, isolation

Features: All processes are proceeded in one tube
IgG recovery is 70-90%
Purity of the IgG from serum is over 80%
Purified IgG is available in 30 min
High reproducibility and no affinity loss

Ordering Information

Product code	Unit
AP02-10	1 sample

Contents of the Kit

Protein G pack .....1 pack
Elution buffer .....4 ml x 1 bottle
Washing buffer ..... 10 ml x 1 bottle
Catching buffer .....1.5 ml x 1 bottle

Storage Condition: 0-5 °C
Shipping Condition: ambient temperature

Product Description

IgG Purification Kits are used for isolation and purification of immunoglobulin G of goat, mouse, rabbit, and other animals. The kit contains immobilized protein A or G and buffer solutions for maximum recovery of IgG. The total time to isolate and purify IgG from serum or other solutions containing IgG is about 30 min (Fig. 1-42). Since the support of protein A or G is silica-base gel, the volume of the retained solution on the gel after the centrifugation is very small. Therefore, all proteins or other materials that do not bind to protein A can be removed by washing twice. SDS-PAGE of purified IgG from various animals are shown in Fig. 1-43. Additionally, denaturing of IgG during the elution process is minimal because IgGs bound to the gel are released with a very quick process. The gel in this kit can be used repeatedly 20 times or more with an equal performance. Used gel is also stable for one year in Washing buffer at 0-5 °C .

Precaution

⌘ If the IgG solution contains gelatin, enzyme digestion may be required prior to apply the solution to the kit.

Required Equipment and Materials

microcentrifuge, 100-200 µl adjustable pipettes, voltex mixer, 0.5 ml microtubes

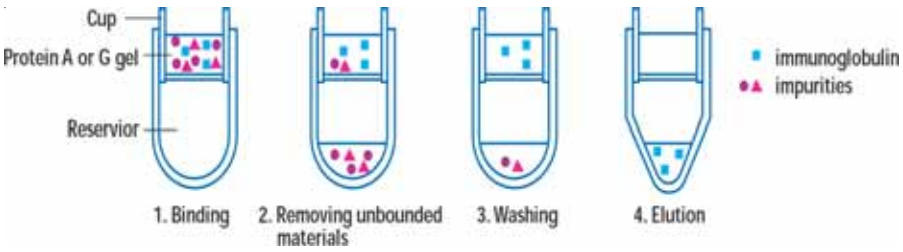


Fig 1-42 IgG isolation process

I-7. Protein Labeling: IgG purification

General Protocol for IgG Isolation

Mix 50 µl sample solution containing IgG and 50 µl Washing buffer.

Add the mixed solution prepared at step 1 to a cup of a cartridge tube in Protein A pack. *Do not close the cap.*

Rotate the cup with a finger several times to mix the gel.[a] Leave the tube at room temperature for 2 min.

Close the cap, and centrifuge the tube at maximum speed for 30 sec.

5

Add the filtrate to the cup again and repeat steps 3 and 4.[b] Transfer the filtrate to a 0.5 ml tube and store it at 0-5 °C [c]

Add 200 µl Washing buffer to the cup. *Do not close the cap*. Rotate it with a finger several times to mix the gel.[a]

Close the cap and centrifuge at maximum speed for 30 sec. Discard the filtrate of the reservoir.

8

Repeat steps 6 and 7.

Add 60 µl Catching buffer to a 1.5 ml tube in Protein A pack. Transfer the cup to this tube.[d]

Add 70 µl Elution buffer to the cup. *Do not close the cap*. Rotate the cup several times with a finger to mix the gel.[a]

Close the cap and centrifuge at maximum speed for 30 sec.

12

Repeat steps 10 and 11.

Remove the cup.[e] Close the cap and voltex to mix. Store the solution at 0-5°C .[f]

a) Slant the cartridge tube and rotate the cup with a finger 10-20 times.
b) The IgG recovery increases 25 to 30% more by this process. You may repeat this process to get a maximum IgG recovery.
c) If the recovery of the IgG is low, use this filtrate to recover IgG. Follow the protocol starting at step 2.
d) Do not discard the reservoir. It will be used in step 13.
e) Return the cup to the reservoir kept in step 9. Wash gel with Washing buffer by following steps 6 and 7. Add 200 µl Washing buffer and keep the protein A gel cartridge tube at 0-5 °C. Centrifuge the cartridge tube to remove Washing buffer prior to use.
f) You may add an equal volume of glycerol and store at -20 °C.

Table 1-2 IgG Recovery from 50 µl Serum

Species	Kit-A	Kit-G	Species	Kit-A	Kit-G
Goat	50-100 µg	150-250 µg	Sheep	50-100 µg	150-250 µg
Rabbit	200-300 µg	150-250 µg	Mouse	150-250 µg	100-150 µg
Hamster	150-250 µg	100-150 µg	Rat	50-100 µg	100-200 µg
Human	200-300 µg	200-300 µg	Dog	200-300 µg	100-200 µg
Cat	150-250 µg	100-200 µg	Porcine	200-300 µg	150-250 µg
Calf	200-300 µg	250-350 µg	Horse	150-250 µg	200-300 µg
Guinea pig	150-200 µg	150-250 µg	Donkey	200-300 µg	200-300 µg
Chicken	25-50 µg	10-20 µg			

I-7. Protein Labeling: IgG purification

IgG Purity

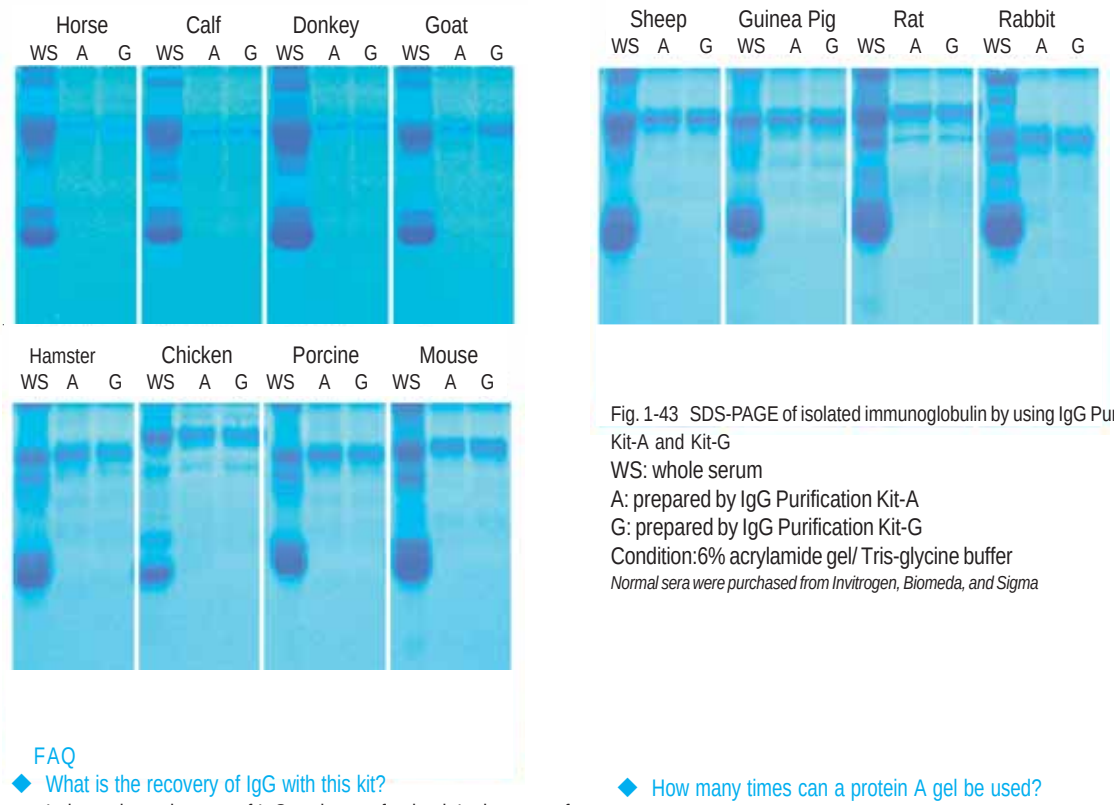


Fig. 1-43 SDS-PAGE of isolated immunoglobulin by using IgG Purification Kit-A and Kit-G
WS: whole serum
A: prepared by IgG Purification Kit-A
G: prepared by IgG Purification Kit-G
Condition:6% acrylamide gel/ Tris-glycine buffer
Normal sera were purchased from Invitrogen, Biomeda, and Sigma

FAQ

- **What is the recovery of IgG with this kit?**
  It depends on the type of IgG and type of animal. In the case of high affinity type IgG for protein A or protein G, about 70-80% IgG is recovered from 100-200 µg IgG or IgG solution containing other proteins or macromolecules.
- **How is the purity of purified IgG using this kit?**
  The purity of the IgG from various serum is indicated in the figure above. Highly purified IgG is available with only a one time purification process.
- **How much IgG can be recovered from serum?**
  About 150-350 µg IgG can be recovered from 50 µl serum.
- **How many times can a protein A gel be used?**
  At least 20 times.
- **Can I use the used protein A gel or protein G gel for the purification of different IgG solutions?**
  Use a new gel for a different sample to avoid contamination.
- **Is a used protein A gel or protein G gel stable?**
  A used protein A gel or protein G gel in Washing buffer is stable at 0-5 ^{o}C for one year.

II. Cell Viability

Introduction

Cell viability and cytotoxicity assays are used for drug screening and cytotoxicity tests of chemicals. They are based on various cell functions such as enzyme activity, cell membrane permeability, cell adherence, ATP production, co-enzyme production, and nucleotide uptake activity. A widely used assay for staining dead cells isTrypan Blue. In this method, cell viability must be determined by counting the unstained cells with a microscope or other instruments. However, Trypan Blue staining cannot be used to distinguish between healthy cells and cells that are alive but losing cell functions. Because of this, enzyme-based methods have been gaining more attention. Fig. 2-1 indicates various reagents used for cell viability detection.

Among the enzyme-based assays, the MTT assay is the best known method for determining mitochondrial dehydrogenase activities in living cells. MTT is reduced to a purple formazan by NADH. MTT formazan, however, has low solubility in water, and it forms purple needle-shaped crystals in cells. Prior to measuring the absorbance, an organic solvent is required to dissolve the crystals. Additionally, the cytotoxicity of MTT formazan makes it difficult to remove cell culture media from the plate wells due to many floating cells with MTT formazan needles, giving significant well-to-well error.

Though the ^{3}[H]thymidine-uptake assay is regarded as a reliable cell viability assay, the use of radioisotope causes various concerns. The ^{51}Cr method is highly sensitive, and is commonly used to determine low levels of cytotoxicity. However, the use of ^{51}Cr also causes problems in handling, storage, and disposal of the material. Cellular enzymes such as lactate dehydrogenase, adenylate kinase, and glucose-6-phosphate dehydrogenase are also used as cell death markers, and there are several such products available on the market. However, adenylate kinase and glucose-6-phosphate are not stable; only lactate dehydrogenase does not lose its activity during cell death assays. Therefore, cell death assays based on lactate dehydrogenase (LDH) activity are more reliable than other enzyme-based cell death assays.

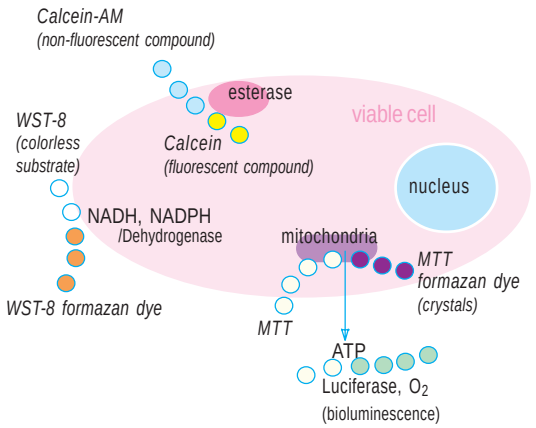


Fig. 2-1 Reagents for cell viability detection

Dehydrogenase Based Assay: Cell Counting Kit-8

Dojindo developed highly water-soluble tetrazolium salts called WSTs. WSTs produce water-soluble formazans and are suitable for cell proliferation and cytotoxicity assays. WSTs receive two electrons from viable cells to generate a yellow, orange, or purple formazan dye. WST-8, a highly stable WST, is utilized in Cell Counting Kit-8 (CCK-8). The electron mediator used in this kit, 1-Methoxy PMS, is also highly stable. Therefore, CCK-8 is stable for at least 6 months at room temperature and one year at 0-5 °C. Since WST-8, WST-8 formazan, and 1-Methoxy PMS have no cytotoxicity in cell culture media, additional experiments may be carried out using the same assay plate. Dehydrogenase-based assays reflect cell conditions with more sensitivity than the other assays because they depend on several elements including dehydrogenases, NAD(H), NADP(H), and mitochondrial activity. The major difference between CCK-8 and the MTT assay, other than MTT's toxicity, is the enzymes involved. The CCK-8 assay involves most of the dehydrogenase in a cell. On the other hand, MTT only involves mitochondrial dehydrogenase. Therefore, the MTT assay depends on mitochondrial activity, not the cell itself. Additionally, CCK-8 is far more sensitive than the MTT assay. Since WST-8 formazan is water soluble, it does not form crystals like MTT. Therefore, after 1-4 hours of incubation with the CCK-8 solution, measurement of O.D. at 450 nm gives the number of viable cells. No extra steps are required.

Esterase Activity Based Assay: Cell Counting Kit-F

Cell Counting Kit-F is another cell viability assay kit based on the esterase activity of viable cells. Fluorescent esterase substrates, such as Calcein-AM, BCECF-AM, CFSE, and FDA, can be passively loaded into viable cells. They are then converted into green fluorescent products by intracellular esterases. These electrically neutral molecules, which have acetyl or acetoxymethyl groups capping the phenolic OH or carboxylic groups of Calcein, freely permeate into the cell. Once converted into fluorescent products by esterase, these compounds are retained by cells. These esterase substrates, therefore, can serve as viability assay probes for cells. Calcein-AM is used for the kit because it is retained inside of cells better than the other fluorescent esterase substrates.

Dead and Viable Cell Staining: Double Staining Kit

To distinguish dead and viable cells simultaneously, propidium iodide and calcein-AM are used. Dead cells can be stained by propidium iodide because it passes through deteriorated cell membranes; viable cells can be stained by calcein-AM because of their esterase activity. Since both calcein and propidium iodide-DNA complex can be excited at 490 nm to emit green and red fluorescence, respectively, dead and viable cells can be observed with a fluorescence microscope. Only dead cells are observed with 545 nm excitation.

Cell Staining Reagents

Fluorescent cell staining reagents are very powerful tools to stain viable cells and dead cells. For more information, see Section III.

Cell Counting Kit-8

Application: Cell viability and cytotoxicity detection

Features: Colorimetric microplate assay
One solution type (ready-to-use solution)
No washing required
No radioisotopes or organic solvents required

Ordering Information

Product code	Unit*
CK04-11	1,000 tests
CK04-13	3,000 tests
CK04-20	10,000 tests

* One test corresponds to one well on a 96-well plate. 500 tests (5 mL) per vial for CK04-11 and CK04-13. 10,000 tests (100 mL) per vial for CK04-20.

Kit Contents:
Cell Counting Kit-8, 1,000 tests ..... 5 ml bottle x 2
Cell Counting Kit-8, 3,000 tests ..... 5 ml bottle x 6
Cell Counting Kit-8, 10,000 tests ..... 100 ml bottle x 1

Storage Condition	Shipping Condition
0-5 °C	ambient temperature

Required Equipment and Materials
plate reader with 450 nm filter, 96-well culture plate, 10 µl, 100-200 µl and multi-channel pipettes, CO_2 incubator

Product Description
Cell Counting Kit-8 (CCK-8) allows convenient assays using Dojindo's tetrazolium salt, WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt), which produces a water-soluble formazan dye upon bioreduction in the presence of an electron carrier, 1-Methoxy PMS (Fig. 2-2). CCK-8 solution is added directly to the cells; no pre-mixing of components is required. CCK-8 is a sensitive nonradioactive colorimetric assay for determining the number of viable cells in cell proliferation and cytotoxicity assays. WST-8 is bioreduced by cellular dehydrogenases to an orange formazan product that is soluble in tissue culture medium (Fig. 2-3). The amount of formazan produced is directly proportional to the number of living cells. Typical cytotoxicity assay and cell proliferation assay curves are shown in Fig. 2-4a and 2-4b. Since adherent cells have a higher metabolism than that of leucocyte cells, the O.D. from adherent cells are much larger than leucocyte cells (Fig. 2-5). The detection sensitivity of cell proliferation assays using WST-8 is higher than assays using other tetrazolium salts such as MTT, XTT, MTS, or WST-1. Since the CCK-8 solution is very stable and has low cytotoxicity, a longer incubation, such as 24 to 48 hours, is possible. Fig. 2-6 shows that the toxicity of CCK-8 is much lower than other cell viabilty assay kits. After 24 hours incubation with CCK-8, most of the HeLa cells have survived. Therefore, after the measurement of cell viability, the cells can be used for further experiments.

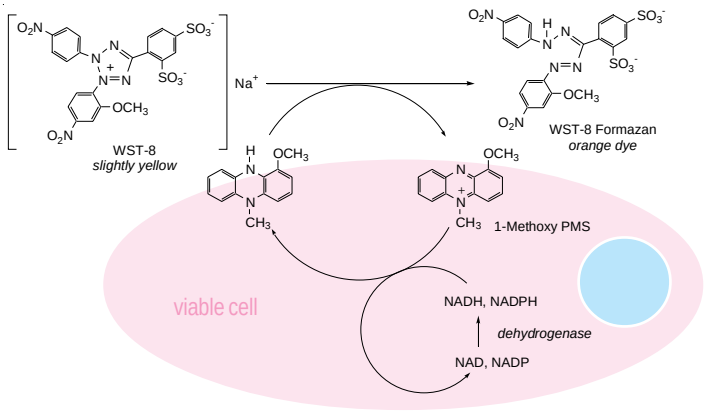


Fig. 2-2 Cell viability detection mechanism with CCK-8

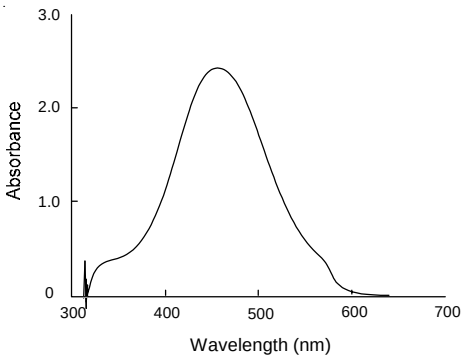
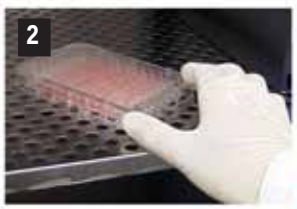


Fig. 2-3 Absorption spectrum of WST-8 formazan dye

Assay Procedure

Add 100 µl of cell suspension[a] to each well.

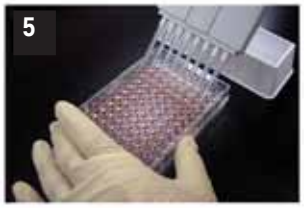


Pre-incubate the plate at 37 °C.[b]

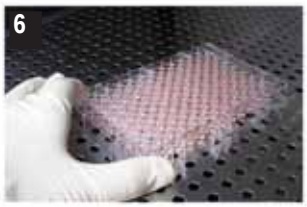
Add 10 µl of various concentration solutions to be tested[c] to each well.

Incubate the plate at 37 °C.

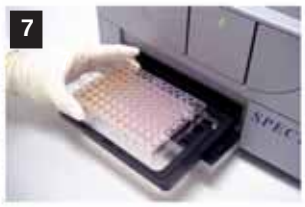
II-1. Cell Viability: Dehydrogenase based



Add 10 µl CCK-8 solution[d] to each well.



Incubate the plate at 37 °C for 1-4 hours.[e]



Read the O.D. at 450 nm and determine the cell viability.

a) Prepare a cell suspension with 50,000-100,000 cells per ml using an appropriate culture medium.
b) Overnight preincubation in a CO_2 incubator is recommended.
c) Use the culture medium or PBS to prepare the solutions.
d) If the solution to be tested has reducing activities, incubate the solution and CCK-8 without cells to determine the background absorbance at 450 nm. If the absorbance is negligibly small, add CCK-8 solution to each well. If the absorbance is high, remove the culture medium and wash cells twice with the medium, then add 100 µl of the culture medium and 10 µl CCK-8 solution.
e) Longer incubation may be necessary for leukocyte cells.

Calculation

$$\text{cell viability* (\%)} = \frac{\text{A(substance) - A(blank)}}{\text{A(0 substance) - A(blank)}} \times 100$$

A(substance): absorbance of a well with cells, CCK-8 soln and substance soln.
A(blank): absorbance of a well with medium and CCK-8 soln, without cells
A(0 substance): absorbance of a well with cells and CCK-8 soln, without substance soln.

*cell viability: proliferation activity or cytotoxicity activity

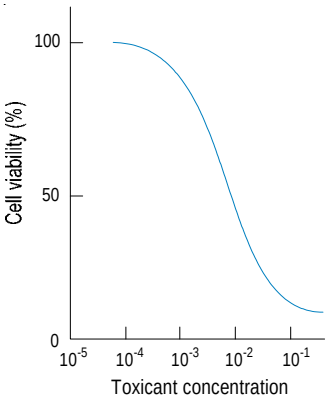


Fig. 2-4a) Typical cytotoxicity assay curve

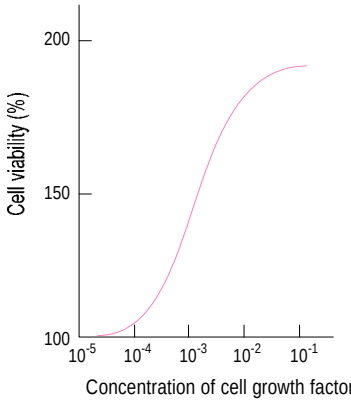


Fig. 2-4b) Typical cell proliferaton assay curve

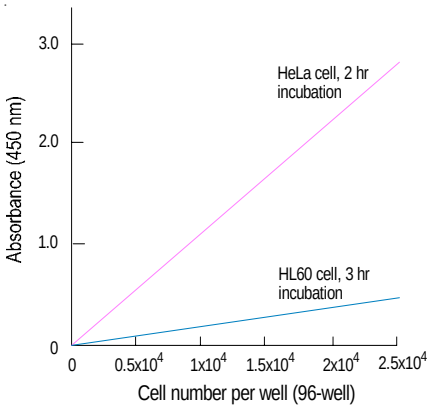


Fig. 2-5 Comparison of signal strength from adherent cells (HeLa) and leucocyte cells (HL69)

Cell Counting Kit-8

Cell viability assay kit solution from another company

Fig. 2-6 Cytotoxicity of CCK-8 and a competitor's cell viability assay kit

Procedures and Conditions: Preincubate HeLa cell/ DMEM culture overnight in a CO_2 incubator and add 10 µl of CCK-8 solution to each well. Incubate cells at 37 °C for various time periods, and observe the cells with a phase-contrast microscope.

As seen in the photos, even after 24-hour incubation with CCK-8, cells continue to proliferate. Other cell viability assay kits are toxic to cells, and most of the cells are killed during the first 3 hours of incubation.

II-1. Cell Viability: Dehydrogenase based

FAQ

◆ How many cells should there be in a well?

For adhesive cells, at least 1,000 cells are necessary per well (100 µl medium) when using a standard 96-well plate. For leukocytes, at least 2,500 cells are necessary per well (100 µl medium) because of low sensitivity. The recommended maximum number of cells per well for the 96-well plate is 25,000. If 24-well or 6-well plates are used for this assay, please calculate the number of cells per well accordingly, and adjust the volume of the CCK-8 solution in each well to 10% of the total volume.

◆ Can this kit be used with a 384-well plate?

Yes, you can use this kit for a 384-well plate. Add CCK-8 solution with 1/10 volume of the culture medium in a well. If the volume of CCK-8 to be added is too small, dilute CCK-8 in half with the medium and add 1/5 of the volume of culture medium in a well.

◆ Can this kit be used with a 24-well plate?

Yes. Add CCK-8 solution with 1/10 volume of the culture medium in a well.

◆ Does CCK-8 stain viable cells?

No, it does not stain viable cells because a water-soluble tetrazolium salt (WST-8) is used for the CCK-8 solution. The electron mediator, 1-Methoxy PMS, receives electrons from viable cells and transfers the electrons to WST-8 in the culture medium. Since its formazan dye is also highly water soluble, CCK-8 cannot be utilized for cell staining.

◆ Does phenol red affect the assay?

No. The absorption value of phenol red in culture medium can be removed by subtracting the absorption value of a blank solution from the absorption value of each well. Therefore, culture medium containing phenol red can be used for the CCK-8 assay.

◆ Is there a correlation between CCK-8 and the Thymidine incorporation assay?

Yes. However, please note that since CCK-8 uses a different assay mechanism from that of the Thymidine assay, so the results may differ. Comparison data are shown in the technical manual, which is available at www.dojindo.com/tm.

◆ Is CCK-8 toxic to cells?

CCK-8 solution itself is cytotoxic because of the high concentration of 1-Methoxy PMS. CCK-8 in the culture medium, however, is not cytotoxic because of the ten times dilution with culture medium. Therefore, long incubation, such as overnight and several days, is possible. The same cell culture can be used for other cell proliferation assays, such as the crystal violet assay, neutral red assay, or DNA fluorometric assays after the CCK-8 assay is completed. Since each cell has a different tolerance to CCK-8, incubate cells with CCK-8 solution and check the cell viability if a longer incubation time is necessary.

◆ Can I use this kit for bacterial cell counting?

You may use this kit for E. coli, but not for yeast. Add 10 µl CCK-8 solution to 100 µl E. coli culture and incubate for 1-4 hours or overnight.

◆ How stable is CCK-8?

CCK-8 is stable over 6 months at room temperature and one year at 0-5 °C with protection from light. For longer storage, we recommend storing the solution at -20 °C ; CCK-8 is stable over 2 years at -20 °C.

◆ I do not have a 450 nm filter. What other filters can I use?

You can use filters with an absorbance between 450 nm and 490 nm.

References

Cell Line	Origin	Reference
293T	human kidney carcinoma	H. Fuda, *et al.*, *J. Lipid Res.*, **48**, 1343 (2007)
3T3-L1	mouse embryonic fibroblast	D. Huang, *et al.*, *FASEB J.*, **19**, 2014 (2005)
A431	human epithelial carcinoma cell	H. Ohori, *et al.*, *Mol. Cancer Ther.*, **5**, 2563 (2006)
A549	human lung cancer cell	C. A. Reilly, et al., *Toxicol. Sci.*, **73**, 170 (2003) T. Kitamuro, et al., *J. Biol. Chem.*, **278**, 9125 (2003) H. Ishibashi, et al., *Cancer Res.*, **65**, 6450 (2005) J. Stankova, et al., *Clin. Cancer Res.*, **11**, 2047 (2005) I. Imoto, et al., *Cancer Res.*, **66**, 4617 (2)
Alexander cell	pancreatic cancer cell	S. Awale, *et al.*, *Cancer Res.*, **66**, 1751 (2006)
AMO1	multiple myeloma	J. Inoue, *et al.*, *Am. J. Pathol.*, **165**, 71 (2004)
AR42J	pancreatic tumor cell	C. Bose, *et al.*, *Am. J. Physiol. Gastrointest. Liver Physiol.*, **289**, G926 (2005)
ARO	human anaplastic thyroid carcinoma	F. Furuya, *et al.*, *Endocrinology*, **145**, 2865 (2004)
AsPC-1	pancreatic cancer cell	T. Mori, *et al.*, *Mol. Cancer Ther.*, **3**, 29 (2004) S. Awale, *et al.*, *Cancer Res.*, **66**, 1751 (2006)
B16F1	murine malignant melanoma	S. Shibata, *et al.*, *J. Immunol.*, **177**, 3564 (2006)
Balb3T3	mouse embryonic cell	H. Tominaga, *et al.*, *Anal. Commun.*, **36**, 47 (1999)
BBMVEC	bovine brain microvascular endothelial	T. Kitamuro, *et al.*, *J. Biol. Chem.*, **278**, 9125 (2003)
BEAS-2B	human bronchial epithelial cell	C. A. Reilly, *et al.*, *Toxicol. Sci.*, **73**, 170 (2003) M. E. Johansen, *et al.*, *Toxicol. Sci.*, **89**, 278 (2006)
BMMSC	bone marrow mesenchymal stem cell	M. Miura, *et al.*, *Stem Cells*, **24**, 1095 (2006)
C33A	human cervical carcinoma	W. Yang, *et al.*, *Mol. Cancer Ther.*, **5**, 1610 (2006)
CHO	chinese hamster ovary cell	A. Kunita, *et al.*, *Am. J. Pathol.*, **170**, 1337 (2007) S. Yokoe, *et al.*, *Cancer Res.*, **67**, 1935 (2007)
cortical neurons, primary	mouse	M. Ikonen, *et al.*, *PNAS*, **100**, 13042 (2003)
COS7	African green monkey kidney cell	T. Kitamuro, *et al.*, *J. Biol. Chem.*, **278**, 9125 (2003) H. Bando, *et al.*, *Clin. Cancer Res.*, **11**, 5784 (2005)

II-1. Cell Viability: Dehydrogenase based

Cell Line	Origin	Reference
CV-1	African green monkey kidney cell	J. Peloponese, *et al.*, *J. Biol. Chem.*, **281**, 8927 (2006)
Daoy	human medulloblastoma	X. Li, *et al.*, *Mol. Cancer Ther.*, **4**, 1912 (2005) S. Kim, *et al.*, *Clin. Cancer Res.*, **12**, 5550 (2006)
DLD-1	human colorectal adenocarcinoma	H. Ohori, *et al.*, *Mol. Cancer Ther.*, **5**, 2563 (2006)
DT40	chicken B-lymphocyte cell	F. Shinozaki, *et al.*, *J. Biol. Chem.*, **281**, 16361 (2006)
DU145	human prostate carcinoma	P. Davis-Searles, *et al.*, *Cancer Res.*, **65**, 4448 (2005) D. J. Son, *et al.*, *Mol. Cancer Ther.*, **6**, 675 (2007)
G361	human melanoma cell	H. Ohori, *et al.*, *Mol. Cancer Ther.*, **5**, 2563 (2006)
H441	human pulmonary adenocarcinoma	H. Shimura, *et al.*, *Cancer res.*, **61**, 3640 (2001)
HCC1937	human breast cancer cell	D. Iliopoulos, *et al.*, *Clin. Cancer Res.*, **13**, 268 (2007)
HEK293	human embryonic kidney cell	S. Semba, *et al.*, *J. Biol. Chem.*, **281**, 28244 (2006)
HeLa	human cervical carcinoma	M. Ishiyama, *et al*., *Talanta*, **44**, 1299 (1997) H. Tominaga, *et al.*, *Anal. Commun.*, **36**, 47 (1999) H. Shimura, *et al.*, *Cancer res.*, **61**, 3640 (2001) C. Shi, *et al.*, *J. Biol. Chem.*, **279**, 17224 (2004)
Hep3B	human hepatocellular carcinoma	J. K. Sicklick, *et al.*, *Carcinogenesis*, **27**, 748 (2006)
HepG2	Human hepatocellular liver carcinoma	C. A. Reilly, *et al.*, *Toxicol. Sci.*, **73**, 170 (2003) T. Ohuchida, *et al.*, *Cancer*, **100**, 2430 (2004) T. Shiokawa, *et al.*, *Clin. Cancer Res.*, **11**, 2018 (2005) H. Ohori, *et al.*, *Mol. Cancer Ther.*, **5**, 2563 (2006)
Hippocampal neuron, primary	from Wistar rat embryos	K. Kurata, *et al.*, *J. Pharmacol. Exp. Ther.*, **311**, 237 (2004)
HL60	human acute promyelonic leukemia	H. Tominaga, *et al.*, *Anal. Commun.*, **36**, 47 (1999)
hMSC	human mesenchymal stem cell	D. Huang, *et al.*, *FASEB J.*, **19**, 2014 (2005) L. Song, *et al.*, *Stem Cells*, **24**, 1707 (2006)
HT22	mouse hippocampal cell	H. Sohn, et al., FASEB J., 20, 1428 (2006).
ILT-Hod	T-cell	T. Kasai, *et al.*, *J. Biol. Chem.*, **277**, 5187 (2002)
Jurkat	human T cell	T. Kasai, *et al.*, *J. Biol. Chem.*, **277**, 5187 (2002) L. Lu, *et al.*, *J. Biochem.*, **141**, 157 (2007)
Kasumi-1	acute myeloid leukemia cell	G. Zhou, *et al.*, *Blood*, **109**, 3441 (2007)
KMS-11	multiple myeloma	J. Inoue, *et al.*, *Am. J. Pathol.*, **165**, 71 (2004)
KYSE	esophageal squamous cell carcinoma	I. Imoto, *et al.*, *Cancer Res.*, **61**, 6629 (2001) K. Nakakuki, *et al.*, *Carcinogenesis*, **23**, 19 (2002)
L929	mouse fibroblast	H. Tominaga, *et al.*, *Anal. Commun.*, **36**, 47 (1999) Y. Morita, *et al.*, *PNAS*, **97**, 5405 (2000)
LK87	human lung adenocarcinoma	H. Ohori, *et al.*, *Mol. Cancer Ther.*, **5**, 2563 (2006)
LNCaP	human prostate carcinoma	D. J. Son, *et al.*, *Mol. Cancer Ther.*, **6**, 675 (2007)
Macrophage	mouse	Y. Miyake, *et al.*, *J. Immunol.*, **178**, 5001 (2007)
MDCK	canine kidney epithelial cell	H. Shimura, *et al.*, *Cancer res.*, **61**, 3640 (2001)
MiaPaCa-2	pancreatic cancer cell	A. Aghdassi, *et al.*, *Cancer Res.*, **67**, 616 (2007)
MT4	T-cell	T. Kasai, *et al.*, *J. Biol. Chem.*, **277**, 5187 (2002) K. V. Kibler, *et al.*, *J. Biol. Chem.*, **279**, 49055 (2004)
NIH3T3	mouse fibroblast	R. Yu, *et al.*, *Toxicol. Sci.*, **93**, 82 (2006)
NT2N	human embryonal carcinoma	J. Tessier, *et al.*, *Infect. Immun.*, **75**, 1895 (2007)
PANC-1	pancreatic cancer cell	T. Mori, *et al.*, *Mol. Cancer Ther.*, **3**, 29 (2004) S. Awale, *et al.*, *Cancer Res.*, **66**, 1751 (2006) A. Aghdassi, *et al.*, *Cancer Res.*, **67**, 616 (2007)
PC6	human lung small-cell carcinoma	M. Spencer, *et al.*, *J. Biol. Chem.*, **277**, 20160 (2002)
PSN-1	pancreatic cancer cell	S. Awale, *et al.*, *Cancer Res.*, **66**, 1751 (2006)
RAW 264	mouse macrophage	M. Shiga, *et al.*, *Anesth. Analg.*, **92**, 128 (2001)
RAW 264.7	mouse macrophage	S. Oyadomari, *et al.*, *PNAS*, **98**, 10845 (2001) D. J. Son, *et al.*, *Mol. Cancer Ther.*, **6**, 675 (2007)
RP9	B lymphoblastoid cell	R. A. Dalloul, *et al.*, *Poult. Sci.*, **85**, 446 (2006)
SH10TC	human gastric cancer cell	H. Ohori, *et al.*, *Mol. Cancer Ther.*, **5**, 2563 (2006)
SH-SY5Y	human neuroblastoma	T. Nakagawa, *et al.*, *Mol. Cell. Biol.*, **22**, 2575 (2002)
SK-N-SH	human neuroblastoma	Y. Wang, *et al.*, *J. Virol.*, **78**, 7916 (2004)

Cell Counting Kit-SK

Application: Cell viability and cytotoxicity detection

Features: Colorimetric microplate assay
One solution type
No washing required
No radioisotopes or organic solvents required
High sensitive

Ordering Information

Product code	Unit*
CK10-11	1,000 tests
CK10-13	3,000 tests

* One test corresponds to one well on a 96-well plate. 500 tests (5 mL) per bottle for CK10-11 and CK10-13

Kit Contents:
Cell Counting Kit-SK, 1,000 tests ..... 5 ml bottle x 2
Cell Counting Kit-SK, 3,000 tests ..... 5 ml bottle x 6

Storage Condition	Shipping Condition
0-5 °C	ambient temperature

Required Equipment and Materials
plate reader with 450 nm filter, 96-well culture plate, 10 µl, 100-200 µl and multi-channel pipettes, CO_2 incubator

Product Description
Cell Counting Kit-SK (CCK-SK) is an alternative kit to CCK-8 for cells or cell lines with low metabolism. The O.D. of the CCK-8 for leukocyte cells is sometimes very low, such as 0.2-0.4 after 2-4 hours incubation because of lower metabolism compared with adherent type cells. CCK-SK enables a shorter incubation time to obtain a higher O.D. increase within 30 min to 1 hour. Assay mechanism and assay protocol are the same as CCK-8 (page 55).
Fig. 2-7shows that the sensitivity of various assays.

Comparison of sensitivities

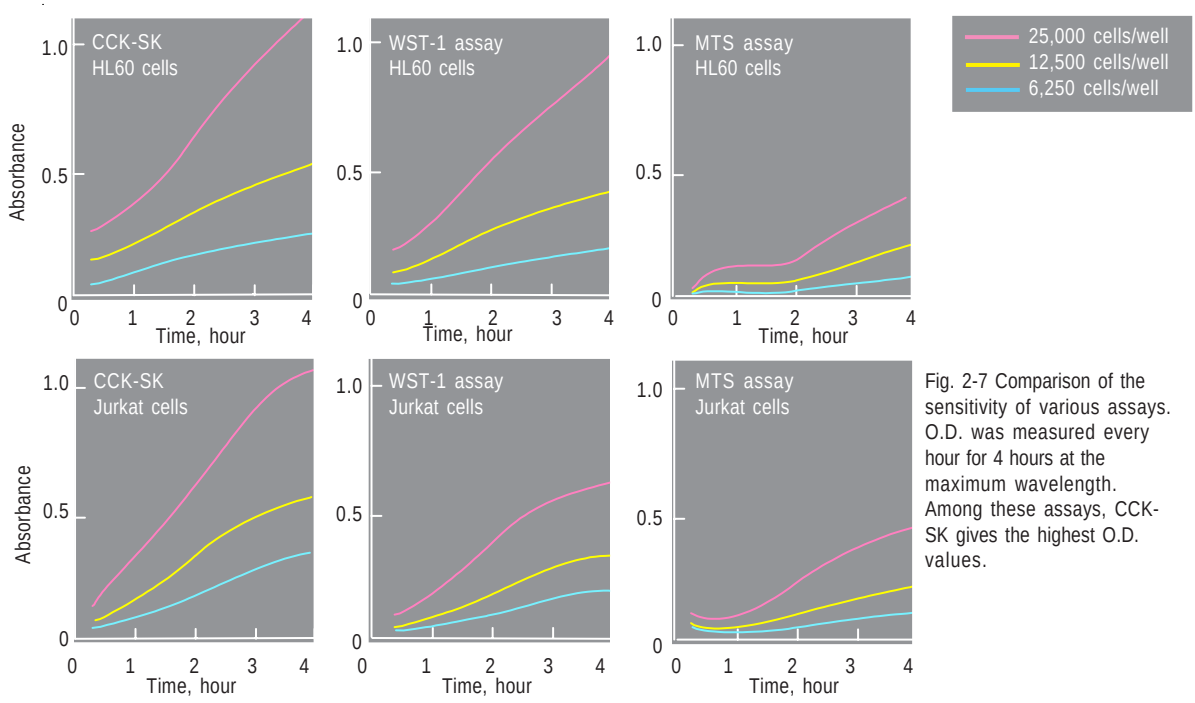


Fig. 2-7 Comparison of the sensitivity of various assays. O.D. was measured every hour for 4 hours at the maximum wavelength. Among these assays, CCK-SK gives the highest O.D. values.

Cell Counting Kit-F

Application: Cell viability and cytotoxicity detection

Features: Fluorometric microplate assay
Highly sensitive: as low as 50 cells per well
Quick detection: 10-30 min incubation
No radioisotopes or organic solvents required

Ordering Information

Product code	Unit
CK06-10	500 tests

* One test corresponds to one well of the 96-well plate.

Kit Components

Calcein-AM Solution .....1 vial

Storage Condition
-20 °C

Shipping Condition
with blue ice or dry ice

Required Equipment and Materials

plate reader with 485 nm excitation and 535 nm emission filters, 96-well culture plate, black or white, 10 µl, 100-200 µl and multi-channel pipettes, CO_2 incubator

Product Description

Cell Counting Kit-F(CCK-F) is utilized for the fluorometric determination of living cell numbers. The amount of the fluorescent dye, calcein, hydrolyzed by esterases in cells is directly proportional to the number of viable cells in culture media (Fig. 2-8). The 96-well microplate CCK-F assay has a detection range of less than 50 cells to more than 25,000 cells per well (Fig. 2-9). Since esterases and phenol red in the culture medium interfere with the fluorescence measurement, replacing the cell culture medium with PBS is necessary prior to adding the Calcein-AM assay solution. The excitation and the emission wavelengths of calcein are 485 nm and 535 nm, respectively (Fig. 2-9). An incubation of 10 to 30 minutes gives sufficient fluorecence intensity for the cell viability determination.

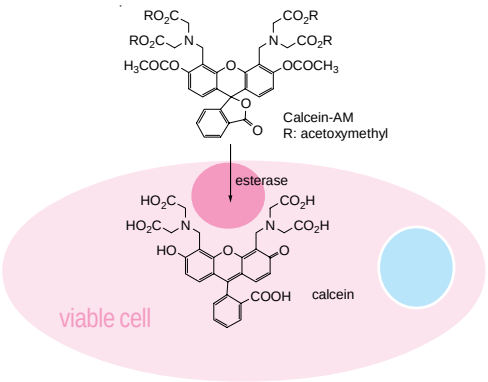


Fig. 2-8 Cell viability detection mechanism with CCK-F

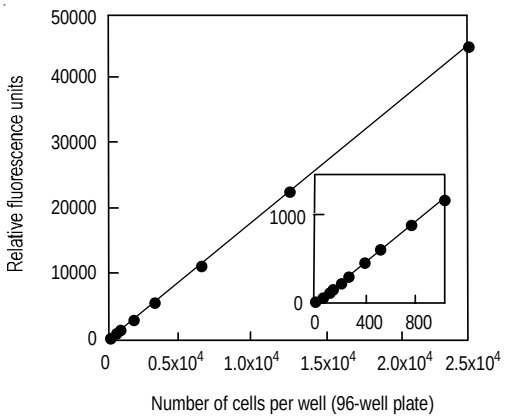


Fig. 2-9 Typical calibration curve using CCK-F
Cell line: HL60
Culture medium: RPMI1640, 10% FCS, L-glutamine
Incubation: 37 °C, 5% CO_2, 30 min.
Detection: 485 nm excitation, 535 nm emission

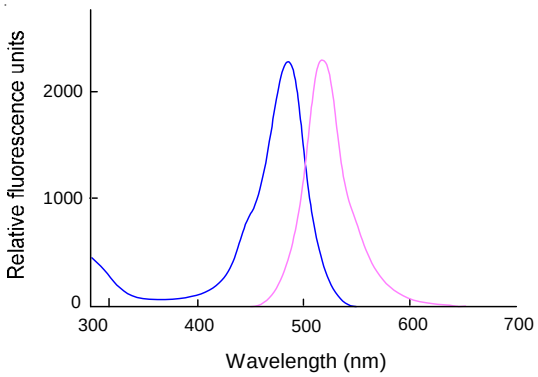


Fig. 2-10 Fluorescence spectrum of Calcein/ PBS solution, pH 7.4

II-2. Cell Viability: Esterase based

Assay Procedure

1. Prepare a cell suspension with 10,000-100,000 cells/ml using an appropriate culture medium.
2. Add 100 µl of the cell suspension to each well.
3. Pre-incubate the plate at 37 °C.[a)]
4. Add 10 µl of various concentration solutions[b)] to be tested to each well.
5. Incubate the plate at 37 °C for a certain time period such as 24, 48, or 72 hours.
6. Remove the medium from each well, and wash cells twice with 100 µl of PBS[c)]. Then, add 100 µl of PBS.
7. Mix 100 µl Calcein-AM solution with 5 ml PBS to prepare an assay solution.
8. Add 10 µl of the assay solution to each well, and incubate the plate at 37 °C for 10-30 min.
9. Measure the fluorescence intensity using a fluorescent microplate reader with 485 nm excitation and 535 nm emission filter.

a) Overnight preincubation in a CO_2 incubator is recommended.
b) Use the culture medium or PBS to prepare the solutions.
c) Warm the PBS solution to 37 °C.

FAQ

- **How many cells should there be in a well?**
  At least 50 cells are necessary per well (100 µl medium) when using a standard 96-well plate. However, we recommend using at least 1,000 cells per well for more reliable and consistent data.
- **Does calcein stain viable cells?**
  Yes, calcein stays inside of the cell. The viable cell can be visualized using a fluorescent microscope. If a longer observation period is necessary, please try CFSE (Product Code: C375-10).
- **Does phenol red affect the assay?**
  Yes. A washing process is required prior to adding assay solution to the plate. Wells with only PBS also have a rather high fluorescent background, so it will be necessary to subtract the background fluorescence from the total fluorescence.
- **Can I use this kit for bacterial cell counting?**
  No. Calcein-AM cannot pass through bacterial cell walls, so they cannot be stained using this kit.
- **Is the CCK-F assay solution stable?**
  No. Calcein-AM in CCK-F is easily hydrolyzed in PBS. Prepare only the required volume of assay solution for the experiment.
- **Is there a correlation between CCK-F and the thymidine incorporation assay?**
  Yes. However, please note that since CCK-F uses a different assay mechanism from that of the thymidine assay, results may differ. Comparison data are shown in the technical manual, which is available at www.dojindo.com/tm.
- **Is CCK-F assay solution toxic to cells?**
  Since the assay solution is prepared with PBS, some cells may be affected by PBS. Also, calcein binds calcium ions in the cell, so the reduction of the free calcium ions causes some damage to cell functions.
- **What other filters can I use?**
  You can use excitation filters between 460 nm and 490 nm and emission filters between 510 nm and 540 nm.
- **Can I use CCK-F for 384-well plates?**
  CCK-F can be used for 384-well plates by adding 5 µl (instead of 10 µl) CCK-F assay solution to 50 µl PBS solution per well.

References

S. A. Weston, *et al.*, *J. Immunol. Methods*, **133**, 87 (1990); X. M. Wang, *et al.*, *Hum. Immunol.*, **37**, 264 (1993); R. Lichtenfels, *et al.*, *J. Immunol. Methods*, **172**, 227 (1994); P. Rat, *et al.*, *Cell. Biol. Toxicol.*, **10**, 329 (1994); F. Braut-Boucher, *et al.*, *J. Immunol. Methods*, **178**, 41 (1995); G. Liminga, *et al.*, *Anticancer Drugs*, **6**, 578 (1995); G. Sunder-Plassmann, *et al.*, *Immunol. Invest.*, **25**, 49 (1996); B. Jonsson, *et al.*, *Eur. J. Cancer*, **32A**, 883 (1996); C. M. De Gendt, *et al.*, *Clin. Chim. Acta*, **249**, 189 (1996); F. Tiberghien, *et al.*, *Anticancer Drugs*, **7**, 568 (1996); P. Decherchi, *et al.*, *J. Neurosci. Methods*, **71**, 205 (1997); D. Imbert, *et al.*, *Cornea*, **16**, 666 (1997); R. Monette, *et al.*, *Brain Res. Brain Res. Protoc.*, **2**, 99 (1998); M. Essodaigui, *et al.*, *Biochemistry*, **37**, 2243 (1998); S. Dhar, *et al.*, *Eur. J. Pharmacol.*, **346**, 315 (1998); R. Gatti, *et al.*, *J. Histochem. Cytochem.*, **46**, 895 (1998); H. B. Oral, *et al.*, *Endothelium.*, **6**, 143 (1998); V. Petronilli, *et al.*, *Biophys. J.*, **76**, 725 (1999); E. Giacomello, *et al.*, *Biotechniques*, **26**, 758 (1999); M. M. Roden, *et al.*, *J. Immunol. Methods*, **226**, 29 (1999); T. K. Petersen, *et al.*, *Vet. Immunol. Immunopathol.*, **68**, 283 (1999); C. R. Jarvis, *et al.*, *Neuroimage*, **10**, 357 (1999); M. Adler, *et al.*, *Neurotoxicology*, **20**, 571 (1999); O. Legrand, *et al.*, *Adv. Exp. Med. Biol.*, **457**, 161 (1999); M. Sandbacka, *et al.*, *Toxicol. In Vitro*, **14**, 61 (2000); S. Neri, *et al.*, *Clin. Diagn. Lab. Immunol.*, **8**, 1131 (2001); X. Wang, *et al.*, *Invest. Ophthalmol. Vis. Sci.*, **44**, 2067 (2003); E. Plantin-Carrenard, *et al.*, *Cell Biol. Toxicol.*, **19**, 121 (2003); A. J. Mathew, *et al*, *Tissue Eng.*, **10**, 1662 (2004); J. B. Kerrison, *et al.*, *Mol. Vis.*, **11**, 208 (2005).

Microbial Viability Assay Kit-WST

Application: Bacterial cell viability detection

Features: Colorimetric microplate assay
Wide variety of microorganism detection
No harvesting or washing required

Ordering Information

Product code	Unit*
M439-10	500 tests

* One test corresponds to one well on a 96-well plate.

Kit Contents:
WST solution .....1 ml x 5 tubes
Electron mediator reagent/ DMSO ..... 0.5 ml x 1 tube

Storage Condition: 0-5 °C
Shipping Condition: ambient temperature

Required Equipment and Materials
plate reader with 450 nm filter, 96-well culture plate, 10 µl, 100-200 µl and multi-channel pipettes, incubator

Product Description

Viable bacterial cell detections are very important for analyzing bacteria contamination in food or evaluating the cleanliness of facilities in order to protect us from food poisonings and infections. Bacterial cell detections are also used for the screening of sanitizing agents and drug resistance detections. Generally, counting the number of colonies on an agar plate is the standard method for determining the number of viable bacterial cells in samples. However, colony formations require one to several days. Dojindo's Microbial Viability Assay Kit-WST can be used to determine the number of viable bacterial cells in a sample by a colorimetric method and can be applied to 96-well microplate assays. The electron mediator in the kit receives electrons from viable bacterial cells and transfers the electrones to WST, one of the water-soluble tetrazolium salts developed by Dojind. Bacterial cell viability then can be determined by monitoring the color intensity of WST formazan dye. Since several types of media used for bacterial cell cultures and components do not interfere with the assay(Fig. 2-15), simply add the assay solution and incubate for 1 to several hours to determine the initial number of viable bacterial cells in the sample (Fig. 2-12).

This assay kit was co-developed with Biotechnology and Food Research Institute, Fukuoka Industrial Technology Center.

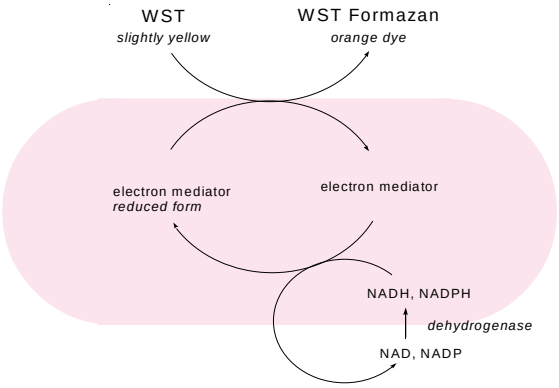


Fig. 2-11 Bacterial cell viability detection mechanism.

General Procedure 1

Determination of the initial number of viable *E. coli* cells
1. Culture *E. coli* with Mueller-Hinton medium.
2. Add Microbial Viability Assay solution with 1/20 volume of the culture medium to the *E. coli* culture
3. Measure the D.D. every 10 to 15 min at 450 nm.
4. Determine the number of *E. coli* from proliferation assay data prepared from a known number of *E. coli* cells (Fig. 2-12[a)]).

a) Fig. 2-12 indicates the correlation between the initial number of E. coli and time-dependent D. D. increase.

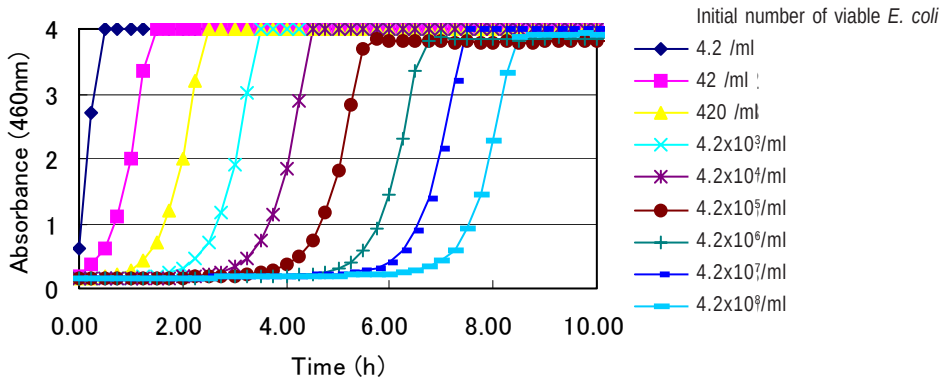


Fig. 2-12 Correlation between initial number of *E. coli* and time-dependent O.D. increase. The initial number of viable *E.coli* were determined by a colony counting method.

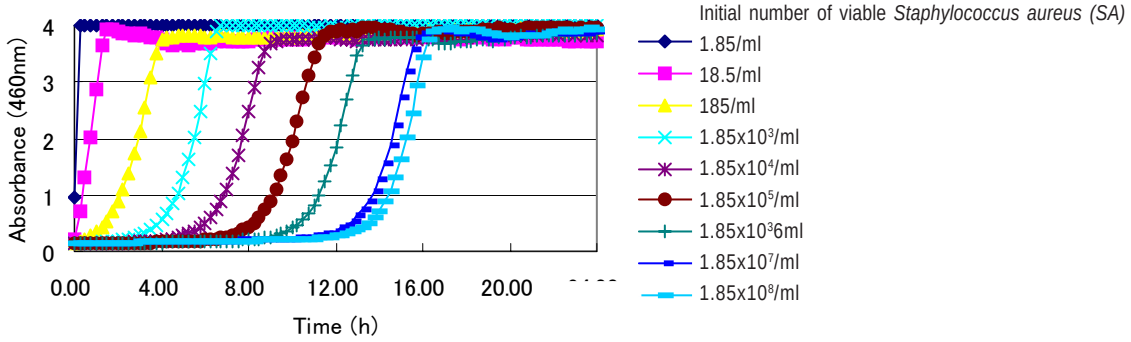


Fig. 2-13 Correlation between the initial number of *SA* and time-dependent O.D. increase. The initial number of viable *SA* were determined by the colony counting method.

General Procedure 2

Determination of the susceptibility of *Staphylococcus aureus* to oxacillin
Oxacillin: antimicrobial agent: 0-64 μg/ml
Microorganism: *Staphylococcus aureus* (*SA*)
Methicillin-resistant *Staphylococcus aureus* (*MRSA*)

1. Culture *SA* or *MRSA* with Mueller-Hintonmedium containing various concentrations of Oxacillin for 6 hours at 35 °C.
2. Add Microbial Viability Assay solution equal to 1/10 the volume of the culture medium.
3. Incubate for 2 hours at 35 °C.
4. Measure the O.D. at 450 nm to determine the MIC (Minimum inhibitory concentration).

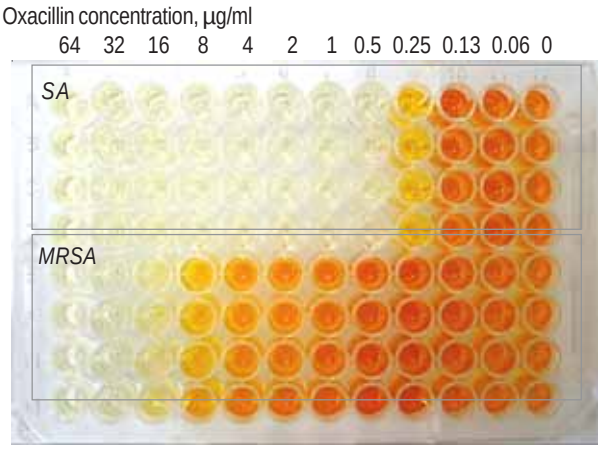


Fig. 2-14 Susceptuvity test of *SA* and *MRSA* against Oxacillin. The data indicated that MRSA has lower susceptivity than SA. The MICs of *MRSA* (32 μg/ml) and *SA* (0.5 μg/ml) are close to the MICs determined by the CLSI (Clinical and Laboratory Standards Institute) method.

II-3. Cell Viability: Microorganism

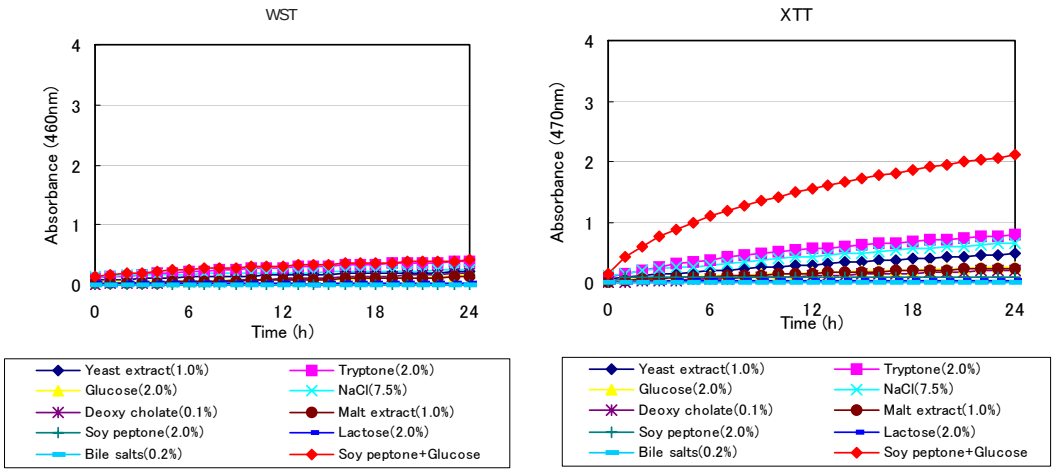


Fig. 2-15 Influence of culture media or substances used for bacterial cell culture.
The data indicated that WST is less sensitive to various culture media or substances which are used for bacterial cell culture. WST is a better tetrazolium salt than XTT for bacterial cell viability assays.

Table 2-1 Initial cell number can reach O.D. 0.5 with 1-hour and 4-hour incubation.

Microorganism		Cell density (CFU/ml)	
		1h	4h
Yeast	*Candida utilis*	5.53×10^{7}	6.18×10^{6}
	Saccharomyces cerevisiae	8.70×10^{5}	2.65×10^{5}
	Zygosaccharomyces rouxii	1.65×10^{5}	2.47×10^{4}
Gram-positive bacteria	*Bacillus cereus*	6.70×10^{5}	6.77×10^{4}
	Bacillus subtilis	2.45×10^{6}	6.71×10^{5}
	Corynebacterium glutamicum	1.69×10^{6}	2.47×10^{5}
	Enterococcus faecalis	5.18×10^{7}	1.76×10^{6}
	Lactobacillus casei	8.40×10^{7}	2.34×10^{6}
	Listeria monocytogenes	5.07×10^{6}	6.46×10^{5}
	Micrococcus luteus	8.29×10^{5}	1.29×10^{5}
	Staphylococcus aureus	2.78×10^{6}	2.71×10^{5}
	Staphylococcus epidermidis	5.53×10^{6}	1.12×10^{6}
Gram-negative bacteria	*Acetobacter sp.*	2.53×10^{7}	7.39×10^{6}
	Escherichia coli	1.31×10^{7}	2.86×10^{5}
	Klebsiella pneumoniae	1.76×10^{7}	5.59×10^{5}
	Proteus mirabilis	7.42×10^{6}	1.35×10^{6}
	Pseudomonas aeruginosa	1.76×10^{8}	1.78×10^{7}
	Salmonella enteritidis	2.55×10^{7}	1.06×10^{6}
	Salmonella typhimurium	1.73×10^{7}	2.60×10^{6}
	Serratia marcescens	7.15×10^{7}	5.08×10^{6}
	Vibrio parahaemolyticus	2.90×10^{7}	1.03×10^{7}
	Yersinia enterocolitica	1.92×10^{7}	5.46×10^{6}

The initial cell number of each microorganism was determined by colony counting. Each microorganism cell culture was diluted with medium and 190 µl of the cell culture was added to each well. Then 10 µl of assay solution was added. The cells were incubated at 30 °C or 37 °C for 1 hour and 4 hours to determine how many cells are required to reach O.D.=0.5 at 460 nm.

Reference

T. Tsukatani, *et al.*, *J. Microbiol. Methods*, **75**, 109 (2008).

III. Staining

Introduction

Visualization of a cell with fluorescent compounds provides a wide variety of information for the analysis of cell functions. Various activities and structures of a cell can be targeted for staining with fluorescent compounds (Fig. 3-1). The most commonly stained cell components are cell membranes, proteins, and nucleotides. Small neutral molecules and positively charged molecules can pass through viable cell membranes and remain inside of cells, depending on their reactivity or hydrophilicity. Negatively charged molecules cannot pass through viable cell membranes. Positively charged molecules are usually cell membrane permeable and accumulate in mitochondria. Ester is a suitable functional group for staining viable cells. Ester can pass through viable cell membranes, where it is hydrolyzed by cellular esterases into a negatively charged molecule under physiological conditions. Several fluorescein analogs with ester groups in their structure are available for viable cell staining. Succinimidyl ester compounds can also be used to improve the retention of the fluorescent derivative within the cell. These compounds are neutral molecules that pass through cell membranes and covalently conjugate with cell proteins. Covalently-conjugated molecules can stay in the cell for several weeks. Nucleotide staining with fluorescent intercalators is mostly applied to dead cell detection.

Cell Cytosol Staining

Fluorogenic esterase substrates that can be passively loaded into viable cells, such as Calcein-AM, BCECF-AM, Carboxyfluorescein succinimidyl ester (CFSE), and Fluorescein diacetate (FDA), are converted by intracellular esterases into fluorescein analogs with green fluorescence. Calcein and BCECF are converted into electrically neutral molecules by the addition of acetyl or acetoxymethyl groups to their phenolic OH or carboxylic groups, which allows them to freely permeate into the cell. Once converted into fluorescent products by esterase, these compounds are retained by cells because of their negative charges. These esterase substrates, therefore, can serve as cell viability assay probes.

Fluorescent esterase substrates may also be used in cell viability assays in place of tetrazolium analogs such as MTT or WST. The mechanism of the determination of cell viability is different: Though both assays determine cell metabolism, esterase substrates detect esterase activity, and tetrazolium salts detect dehydrogenase activity of viable cells. CFSE is also an ester compound that passes through viable cell membranes. Since it has an amine-reactive succinimidyl group, fluorescein derived from CFSE can covalently bind to proteins or other amino groups in the cell or on the cell membrane. This covalently-attached fluorescein is stable enough to trace the cell over several weeks.

Mitochondria Staining

Mitochondria exist in most eukaryotic cells and play a very important role in oxidative metabolism by generating ATP as an energy soruce. The average number of mitochondria per cell is from 100 to 2,000. Though the typical size is about 0.5-2mm, the shape, abundance, and location of mitochondria vary by cell type, cell cycle, and cell viability. Therefore, visualization of mitochondra is important. Since mitochondria have electron transport systems, they can be stained with various redox dyes. MitoRed and Rh123 readily pass through cell membranes and accumulate in mitochondria. The fluorescence intensity of Rh123 reflects the amount of ATP generated in mitochondria.

Nucleus Staining

Fluorescent dyes with aromatic amino or guanidine groups, such as propidium iodide (PI), ethidium bromide (EB), diaminophenylindole (DAPI), acridine orange (AO), and Hoechst dyes, interact with nucleotides to emit fluorescence. EB and PI molecules intercalate inside the DNA double helix. DAPI and Hoechst dye molecules attach at the minor groove of the DNA double helix. On the other hand, AO can form complexes with either double-stranded DNA or single-stranded DNA and RNA. One molecule of AO can intercalate with three base pairs of double-stranded DNA to emit green fluorescence with the maximum wavelength at 526 nm. One molecule of AO can also interact with one phosphate group of single-stranded DNA or RNA to form an aggregated, or stacked, structure that emits red fluorescence with the maximum wavelength at 650 nm. These fluorescent dyes, except for the Hoechst dyes, are impermeable through the cell membranes of viable cells, and can be used as fluorescent indicators of dead cells. Hoechst dyes are positively charged under physiological conditions and can pass through viable cell membranes.

Bacterial Cell Staining

There are several ways to detect bacteria such as from agar plate cultivation to bacteria specific DNA amplification. Fluorescent staining using CTC is one of the methods used to detect viable bacterial cells. The advantage of this method is very quick detection and the possibility of VNC (viable but culturable) bacterial cell detection. CTC is a tetrazolium salt that is converted to formazan dye by bacterial cell activity. The solid state of the formazan dye emits red fluorescence. Therefore, viable bacterial cells can be stained by CTC and are easily detected by fluorescent microscopy.

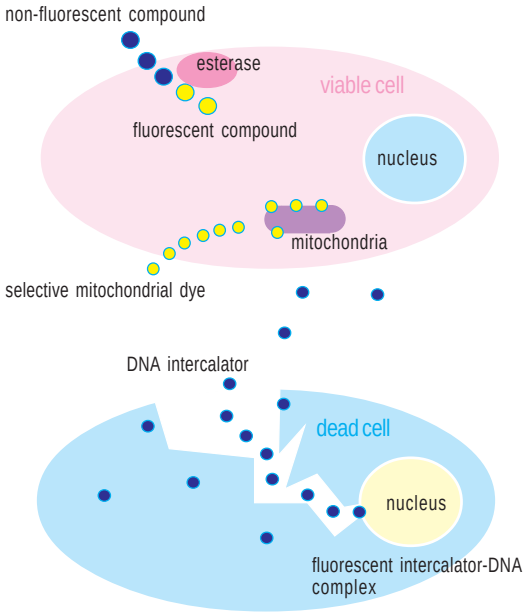


Fig. 3-1 Cell staining method

III-1. Staining: Microorganism

-*Bacstain*-CTC Rapid Staining Kit
for Flow cytometry (BS01-10)
for Microscopy (BS02-10)

Application: Aerobic bacterial cell staining
Hard to culture bacteria detection

Features: Highly sensitive fluorescence detection
No washing required

Ordering Information

Product code	Unit*
BS01-10	100 assays
BS02-10	100 assays

* One kit can stain 100 samples when using the standard protocol.

Contents of the Kits:

CTC Rapid Staining Kit - for Flow cytometry (BS01-10)
CTC ..... 10 mg x 3 vials Enhancing reagent A ..... 100 µl x 1 vial
CTC Rapid Staining Kit- for Microscopy (BS02-10)
CTC ..... 10 mg x 3 vials Enhancing reagent B ..... 500 µl x 1 vial

Storage Condition	Shipping Condition
0-5 °C	ambient temperature

Required Equipment and Materials

10 µl, 1000 µl pipettes, incubator, Microscope (blue excitation filter and red emission filter) or flow cytometer (488 nm blue laser)

Product Description

In order to count bacterial cells, colony formation using an agar plate is a very common and reliable method. However, it takes quite a long time to incubate. Therefore, alternative detection methods have been developed. Bacteria-specific gene amplification methods such as PCR, LAMP, and nucleus staining are quite rapid, but these methods count dead bacteria as well. Therefore, detection of live cell functions is essential to determining the actual number of living bacteria in a sample. Tetrazolium salts can be used to detect respiratory activity of bacterial cells or mitochondria. CTC is one of the tetrazolium salts and is reduced by this respiratory activity to form fluorescent CTC formazan on the cell surface. Therefore, CTC is used for specific staining of an aerobic live bacteria and can be applied to hard to culture bacteria (VBNC: viable but non-culturable). CTC forms a fluorescent formazan by an electron transfer system. However, CTC alone is not sensitive enough to stain single cells. Therefore, CTC-Rapid Staining Kit contains an enhancing reagent which improves the CTC staining efficiency. Compared with CTC only staining, this staining kit enables rapid and sensitive staining of microorganism (Fig. 3-2). Maximum wavelengths of the CTC formazan dye are 450 nm amd 480 nm for excitation and 630 nm for emission.

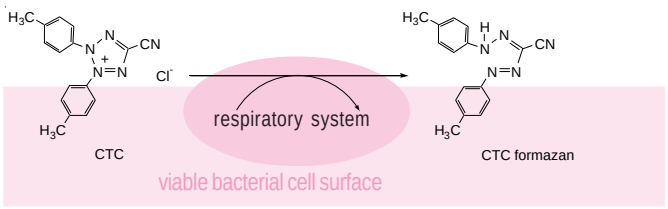


Fig. 3-2 Bacterial cell viability detection mechanism with CTC

General Staining Protocol

Microscopy detection

1. Centrifuge bacteria culture and remove the supernatant, and then resuspend the bacteria pellet with PBS(-).
2. Add CTC + Enhancing reagent-B. Incubate at 37 °C for 1 hour.
3. Prepare a slide and detect fluorescence by B-excitation filter set.

Flow cytometry detection

1. Centrifuge bacteria culture and remove the supernatant, and then resuspend the bacteria pellet with PBS(-).
2. Add CTC + Enhancing reagent-B. Incubate at 37 °C for 1 hour.
3. Analyze the cells with a flow cytometry: 488 nm excitation, 630 nm emission.

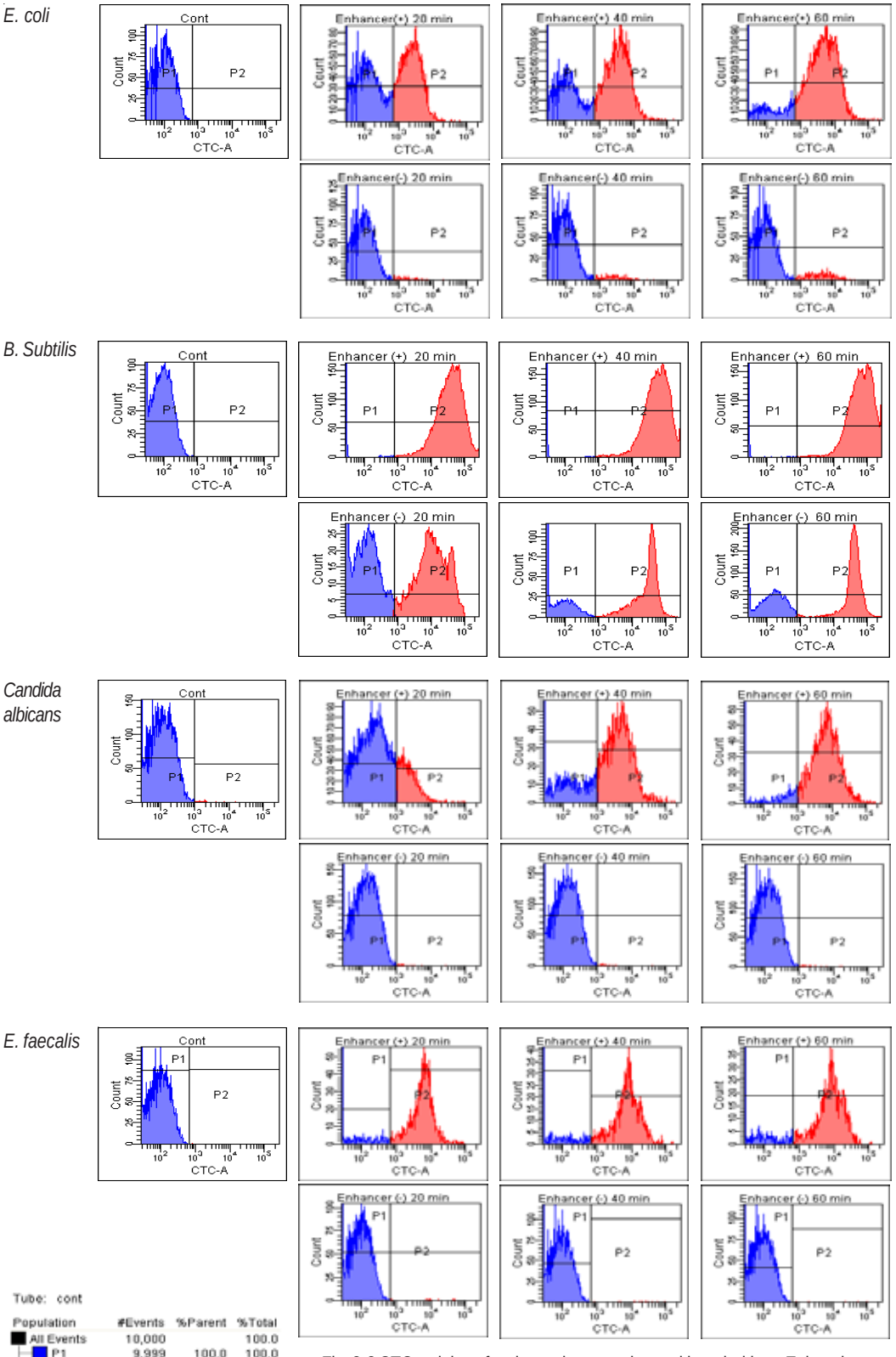


Fig. 3-3 CTC staining of various microorganisms with and without Enhancing reagent.

III-1. Staining: Microorganism

Experimental Example

Double staining with CTC and DAPI

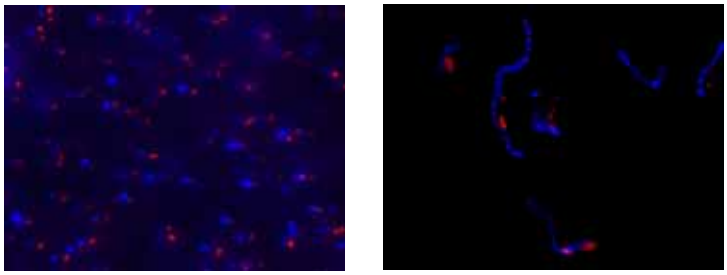


Fig. 3-4 *E. coli* staining (left) and *L. casei* staining (right) with CTC and DAPI.
Bacterial cells were stained with CTC first, and then 1 µl of DAPI solution was added. The cells were incubated at room temperature for 5 min.
Formaldehyde fixation with 1-4% formaldehyde can be performed before DAPI staining.

References

A. Hiraishi, *et al.*, *Microbes Environ.*, **19**, 61 (2004), A. Kitaguchi, *et al.*, *Appl. Environ. Microbiol.*, **71**, 2748 (2005).

CTC

5-Cyano-2,3-ditolyl-2H-tetrazolium chloride [CAS: 90217-02-0]

Application: Bacterial Counting

Features: Selectively staining possible
Fast results
Fluorescent microscopy and flow cytometry detection

Ordering Information

Product code	Unit
C440-10	100 mg

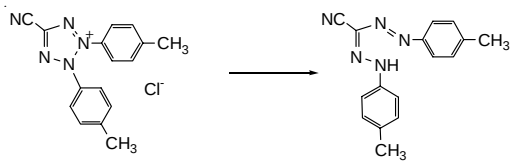
Storage Condition
0-5 °C, protect from light

Shipping Condition
ambient temperature

Product Description

As a way to detect bacteria, CTC can be used in various ways depending on the objective. Detection methods include the agar plate cultivation method which involves counting colonies that arise from bacteria cultivation, staining bacteria using the fluorescent stain method, testing for bacteria that has potential for growth using the DVC method, and detecting particular bacteria using the FISH method-DNA amplification method. CTC is reduced to CTC formazan (CTF) by electron transfer by way of respiratory activity, and builds up as fluorescent sedimentation inside a cell that has become insoluble in water. CTC itself is water-soluble and non-fluorescent in aqueous solution. On one hand, CTF is not fluorescent in fluids with low viscosity. However, in fluids with high viscosity and in a solid state, it gives off a red fluorescence. It is possible to search for cells with respiratory activity after incubating CTC with reagent, by counting under a fluorescent microscope or analyzing by flow cytometry. By using with nucleic acid staining reagent to count the total cell population and the number of living cells, or using with the FISH method to count selectively a particular viable cell type, it is possible to collect a higher level of data. Since the existence of bacteria that's living but has difficulty in culture (VNC: viable but non-culturable) has become evident, the demand for a rapid detection method of microorganisms is on the rise and is expected to become a technique in sanitation testing.

Chemical Structure

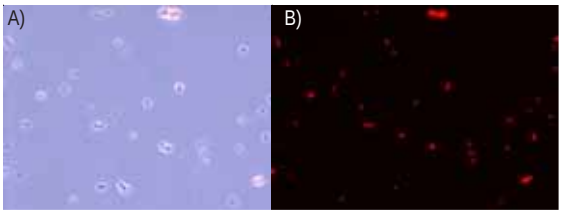


CTC CTC formazan

References

A. W. Coleman, *Limnol. Oceanogr.*, **25**, 948 - 951 (1980); E. Severin, *et al.*, *Anal. Chim. Acta*, **170**, 341 - 346 (1985); G. G. Rodriguez, *et al.*, *Appl. Environ. Microbiol.*, **58**(6), 1801 - 1808 (1992); G. Schaule, et al., *Appl. Environ. Microbiol.*, **59**(11), 3850 - 3857 (1993); R. A. Bovill, *et al.*, *J. Appl. Bacteriol.*, **77**(4), 353 - 358 (1994); M. T. E. Suller, *et al.*, *Antimicrob. Agents Chemother.*, **42**(5), 1195 - 1199 (1998); M. Kawai, *et al.*, *J. Appl. Microbiol.*, **86**, 496 - 504 (1999); N. Yamaguchi, *et al.*, *Cytometry*, **54A**, 27 - 35 (2003); A. Kitaguchi, *et al.*, *Appl. Environ. Microbiol.*, **71**(5), 2748 -2752 (2005)

E. coli DH5a



B. subtilies JCM1465

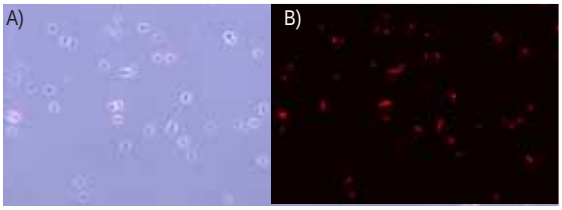


Fig. 3-5 Microorganism staining with CTC

E. coli staining Condition
E. coli culture was stained with 5 mg/ml CTC for 4 hours at 37 °C.
A) Phase-contrast microscope
B) fluorescent microscope (485 nm, 510 nm filters)

B. subtiles staining Condition
B. subtilis culture was stained with 5 mg/ml CTC for 4 hours at 37 °C.
A) Phase-contrast microscope
B) fluorescent microscope (485 nm, 510 nm filters)

-Bacstain- Series

Products Description

The *Bacstain* series can be used for microorganism staining as indicated in Fig. 3-6. CFDA is used for staining of viable microorganisms. CFDA is bacterial cell wall and cell membrane permeable, and hydrolized by esterase of the cell to stay inside of the cell. Other *Bacstain* reagents are used for nucleotide staining and are cell wall permeable except for PI. Therefore, using one of these nucleotide staining reagents and CFDA or *Bacstain*-CTC Rapind Staining Kit, it is possible to stain both membrane intact cells and membrane damaged cells simultaneously (Fig. 3-4 and 3-5). Since PI can stain only membrane damaged cells, membrane intact cells are not stained by this compound. PI is also used for double staining coupled with CFDA and CTC.

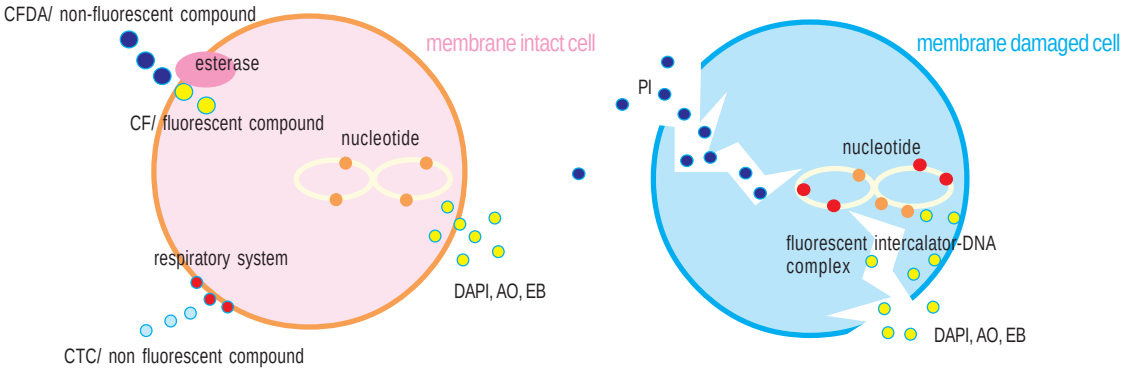


Fig. 3-6 Microorganism staining mechanism with *Bacstain* reagents

*-Bacstain-*CFDA solution

5(6)-Carboxyfluorescein diacetate, DMSO solution [CAS: 79955-27-4]

Application: Viable microorganim staining

Features: Fluorescence detection
Ready-to-use solution

Ordering Information

Product code	Unit
BS03-10	100 assays

Contents of the Kits:
CFDA solution ..... 375 µl x 4 vials

Storage Condition
0-5 °C, protect from light

Shipping Condition
ambient temperature

III-1. Staining: Microorganism

Mechanism of Viable Cell Staining

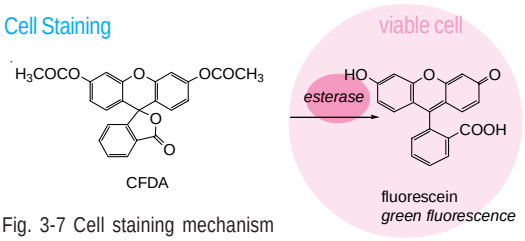


Fig. 3-7 Cell staining mechanism

Required Equipment and Materials

10 µl, 1000 µl pipettes, incubator, Microscope (blue excitation filter and red emission filter) or flow cytometer (488 nm blue laser)

Staining procedure

1. Allow CFDA solution to stand at room temperature for 30 min in order to thaw. Solution should be protected from light.
2. Resuspend the organism with an appropriate buffer (phosphate buffer, saline,etc) [a] and adjust the number of cells to 10^6cells/mL(flow cytometry) or 10^8-10^9cells/mL(microscopy).
3. Add CFDA solution into the microbial cell suspension and vortex gently to mix. Use 5 µl for flow cytometry and 15 µl for microscopy analysis. The maximum wavelengths of the dye are 493 nm for excitation and 515 nm for emission.
4. Incubate the microbial cell at 37 ºC for 5 min [b].
5. Fix the microbial cell by addition of formaldehyde (1-4% final concentration).
6. Remove the buffer by filtration or centrifugation, and resuspend with buffer.
7. Analyze the stained-cells by a flow cytometer or a microscope.

a) Gram-negative bacteria tend to exhibit lower fluorescence intensity than Gram-positive bacteria because of their cell structure (outer membrane impedes penetration of CFDA). For the staining of Gram-negative bacteria, use 0.1 M pphosphate buffer, 0.9 M NaCl, 0.5 mM EDTA, pH 8.5.
b) If CFDA staining is not sufficient with 5 min incubation, increase the incubation time.

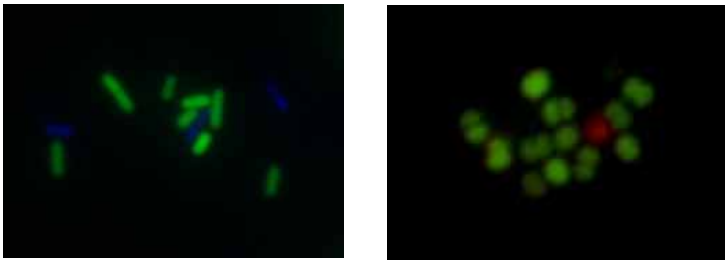


Fig. 3-8 *B. cereus* stained with CFDA and DAPI (left). *S. aureus* stained with CFDA and PI (right).

References

N. Yamaguchi, *et al*., *J. Appl. Microbiol*., **83**, 43 (1997), M. Kawai, *et al*., *J. Appl. Microbiol*., **86**, 496 (1999), T. Someya, *et al., J of the Science of Soil and Manure*, **76**, 401 (2005).

-Bacstain-DAPI solution

4',6-Diamidino-2-phenylindole, dihydrochloride, solution [CAS: 27718-90-3]

Application: Microorganism staining

Features: Fluorescence detection
Ready-to-use solution

Ordering Information

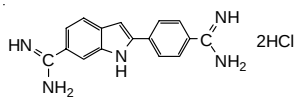
Product code	Unit
BS04-10	100 assays

Kit Contents:
DAPI solution ..... 25 µl x 4 vials

Storage Condition
0-5 °C

Shipping Condition
ambient temperature

Chemical Structure



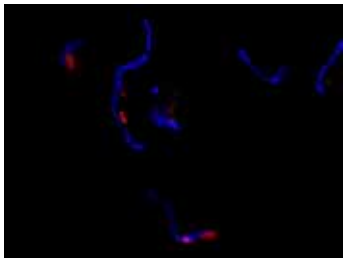
Required Equipment and Materials

10 µl, 1000 µl pipettes, incubator, Microscope (blue excitation filter and red emission filter) or flow cytometer (488 nm blue laser)

Staining Procedure

1. Allow DAPI solution[a)] to stand at room temperature for 30 min. Solution should be protected from light.
2. Resuspend the organisms with PBS(-) or saline and adjust the number of cells to 10^6cells/mL(flow cytometry) or 10^8-10^9cells/mL(microscopy).
3. Add 1 µL of DAPI solution into the microbial cell suspension and vortex gently to mix. Formaldehyde-fixation can be recommended if necessary.
4. Incubate the microbial cells at room temperature for 5 min.
5. Analyze the stained-cells by a flow cytometer or a microscope. The maximum wavelength of the dye are 360 nm for excitation and 460 nm for emission.

a) Since DAPI may be carcinogenic, be careful when handling and disposing.



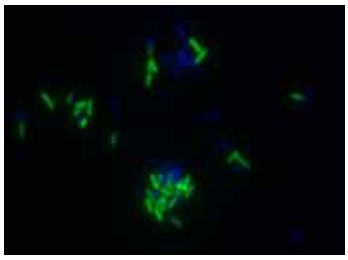


Fig. 3-9 *L. casei* stained with CTC and DAPI (left). *B. cereus* stained with CFDA and DAPI (right).

-*Bacstain*-AO solution

3,6-Bis(dimethylamino)acridine hydrochloride, solution [CAS: 65-61-2]

Application: Microorganism staining

Features: Fluorescence detection
Ready-to-use solution

Ordering Information	
Product code	Unit*
BS05-10	100 assays

Contents of the Kits:
AO solution ..... 75 µl x 4 vials

Storage Condition	Shipping Condition
-20 °C	ambient temperature

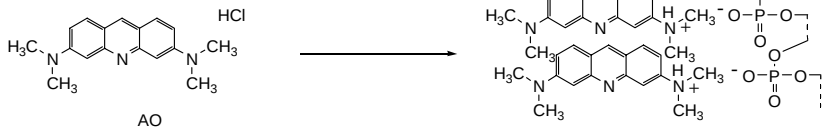


Fig. 3-10 Cell staining mechanism

Required Equipment and Materials

10 µl, 1000 µl pipettes, incubator, Microscope (blue excitation filter and red emission filter) or flow cytometer (488 nm blue laser)

Staining Protocol

1. Allow AO solution[a)] to stand at room temperature for 30 min in order to thaw. Solution should be protected from light.
2. Resuspend the organisms with PBS(-) or saline and adjust the number of cells to 10^6cells/mL(flow cytometry) or 10^8-10^9cells/mL(microscopy).

III-1. Staining: Microorganism

3. Add 3 µl of AO solution into the 1 mL of microbial cell suspension and vortex gently to mix.
   Formaldehyde-fixation can be carried out if necessary.
4. Incubate the microbial cells at room temperature for 5 min.
5. Analyze the stained-cells with a flow cytometer or a microscope. The maximum wavelengths of the dye with ssDNA are 420-460 nm for excitation and 630-650 nm for emission. The maximum wavelengths of the dye with dsDNA are 500 nm for excitation and 520 nm for emission.

a) Since AO may be carcinogenic, be careful when handling and disposing.

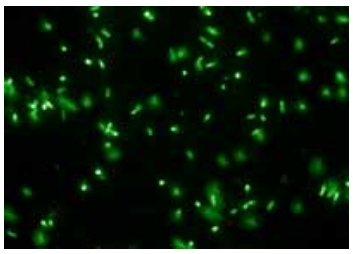


Fig. 3-11 *B. subtils*stained with AO.

References
J. E. Hobbie, *et al*., *Appl. Environ. Microbiol*., **May 1997**, 1225 (1997), S. F. Nishino, *et al*., *Appl. Environ. Microbiol*., **Sept. 1986**, 602 (1986).

-Bacstain-EB solution

3,8-Diamino-5-ethyl-6-phenylphenanthridinium bromide, solution

Application: Microorganism staining

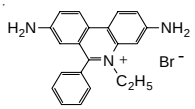
Features: Fluorescence detection
Ready-to-use solution

Ordering Information	
Product code	Unit*
BS06-10	100 assays

Contents of the Kit:
EB solution ..... 250 µl x 4 vials

Storage Condition	Shipping Condition
-20 °C	ambient temperature

Chemical Structure



Required Equipment and Materials
10 µl, 1000 µl pipettes, incubator, Microscope (blue excitation filter and red emission filter) or flow cytometer (488 nm blue laser)

Staining procedure
1. Allow EB solution[a)] to stand at room temperature for 30 min in order to thaw.
   Solution should be protected from light.
2. Resuspend the organisms with PBS(-) or saline and adjust the number of cells to 10^6cells/mL(flow cytometry) or 10^8-10^9cells/mL(microscopy).
3. Add 10 µl of EB solution into the 1 mL of microbial cell suspension and vortex gently to mix.
   Formaldehyde-fixation can be carried out if necessary.
4. Incubate the microbial cells at room temperature for 5 min.
5. Analyze the stained-cells with a flow cytometer or a microscope. The maximum wavelengths of the dye are 520-525 nm for excitation and 615 nm for emission.

a) Since EB may be carcinogenic, be careful when handling and disposing.

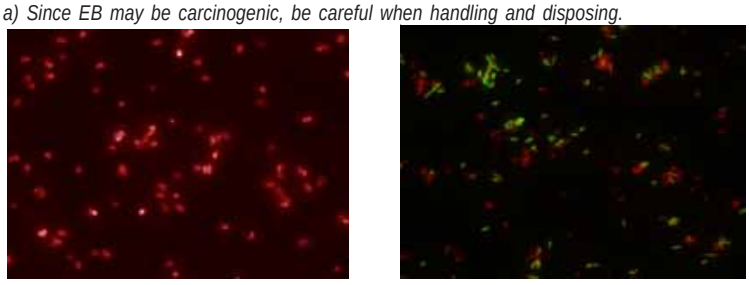


Fig. 3-12 *S. epidermidis* stained with EB (left).
B. cereus stained with CFDA and EB (right)

-*Bacstain*-PI solution

3,8-Diamino-5-[3-(diethylmethylammonio)propyl]-6-phenylphenanthridinium diiodide, solution

Application: Membrane damaged microorganism staining

Features: Fluorescence detection
Ready-to-use solution

Ordering Information

Product code	Unit*
BS07-10	100 aasays

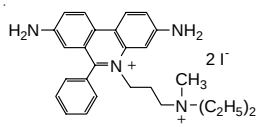
Contents of the Kit:

PI solution ..... 25 µl x 4 vials

Storage Condition
0-5 °C

Shipping Condition
ambient temperature

Chemical Structure



Required Equipment and Materials

10 µl, 1000 µl pipettes, incubator, Microscope (blue excitation filter and red emission filter) or flow cytometer (488 nm blue laser)

Staining Procedure

1. Allowe PI solution[a)] to stand at room temperature for 30 min in order to thaw.
   Solution should be protected from light.
2. Resuspend the organisms with PBS(-) or saline and adjust the number of cells to 10^6cells/mL(flow cytometry) or 10^8-10^9cells/mL(microscopy).
3. Add 10 µl of PI solution into the 1 mL of microbial cell suspension and vortex gently to mix.
   Formaldehyde-fixation may be carried out, if necessary.
4. Incubate the microbial cells at room temperature for 5 min.
5. Analyze the stained-cells with a flow cytometer or a microscope. The maximum wavelength of the dye are 530 nm for excitation and 620 nm for emission.

a) Since PI may be carcinogenic, be careful for handling and disposing.

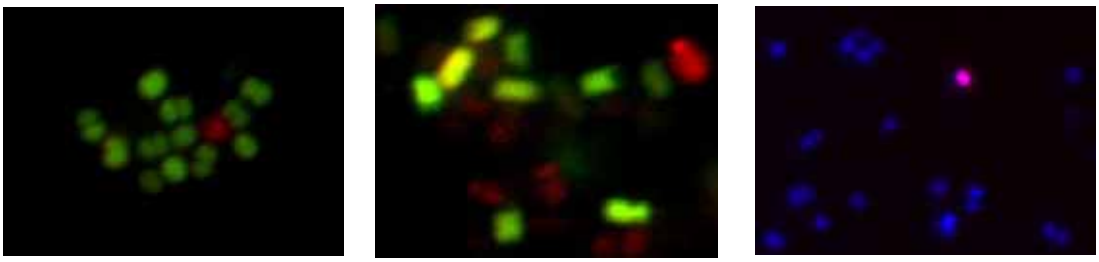


Fig. 3-13 *S. aureus* stained with CFDA and PI (left). *E. coli* stained with CFDA and PI (middle). *S. aureus* stained with DAPI and PI (right).

Reference

N. Yamaguchi, *et al*., *J. Appl. Microbiol*., **83**, 43 (1997).

III-2. Staining: Cytosol

BCECF-AM

3'-*O*-Acetyl-2',7'-bis(carboxyethyl)-4 or 5-carboxyfluorescein, diacetoxymethyl ester [CAS:117464-70-7]

Application: Viable cell staining

Appearance: orange or orange-brown crystals
Purity: >90.0% (HPLC)
MW: 688.59, $C_{35}H_{28}O_{15}$

Ordering Information	
Product code	Unit
B262-10	1 mg

Storage Condition
-20 °C, protect from light

Shipping Condition
ambient temperature

Product Description

BCECF-AM is cell membrane permeable and is easily hydrolyzed by esterases to BCECF. BCECF is not cell membrane permeable, and it accumulates inside viable cells (Fig. 3-14). BCECF emits a strong green fluorescence; therefore, it is easy to visualize viable cells. BCECF-AM is also utilized as an intracellular pH indicator. The excitation and emission wavelengths of BCECF are 500 nm and 530 nm, respectively. Usually, over 80% of loaded BCECF will remain inside the cell for at least 2 hours.

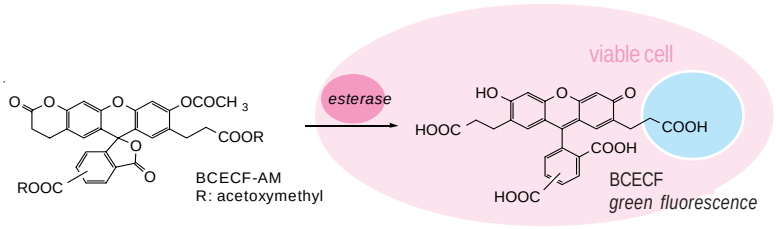


Fig. 3-14 Cell staining mechanism

General Protocol (for Human Neutrophil)*

Reagents:
1 mM BCECF-AM/DMSO solution (1 mg BCECF in 1.45 ml DMSO)
HEPES buffer saline (20 mM HEPES, 153 mM NaCl, 5 mM KCl, 5 mM glucose, pH 7.4)

Protocol:
1. Suspend cells in HEPES buffer solution to prepare $4x10^7$ cells per ml.
2. Add 1 mM BCECF-AM/DMSO solution to the cell suspension to prepare 3 µM BCECF-AM (1/300 vol of cell suspension) as the final concentration.
3. Incubate the cell suspension at 37 °C for 30 min.
4. Wash the cells 3 times with HEPES buffer saline and then prepare $3x10^6$ cells per ml of the cell suspension.
5. Determine the fluorescence intensity using a fluorescence microscope or a confocal laser microscope coupled with an image analyzer.

* Cell staining conditions differ by cell type, so it is necessary to optimize the conditions for each experiment.

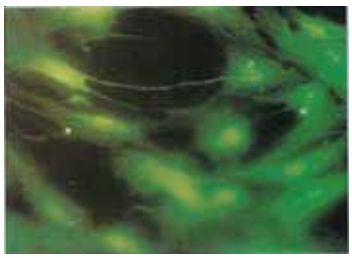


Fig. 3-15[a)] Cell staining with BCECF-AM
Cell type: HeLa

Fig. 3-15[b)] Cell staining with BCECF-AM
Cell type: HeLa/ trypsin treated

References
S. A. Weston, *et al., J. Immunol. Methods*, **133**, 87 (1990); S. A. Weston, *et al., Cytometry*, **13**, 739 (1992); L. S. De Clerck, *et al., J. Immunol. Methods*, **172**, 115 (1994); V. Radosevic, *et al., Cytometry*, **20**, 281 (1995); P. Franck, *et al., J. Biotechnol.*, **46**, 187 (1996). M. Ikuma, *et al., J. Gerontol. A Biol. Sci. Med. Sci.*, **51**, B346 (1996); A. Sachinidis, *et al., Br. J. Pharmacol.*, **119**, 787 (1996); T. Hirohashi, *et al., J. Pharmacol. Exp. Ther.*, **280**, 813 (1997); D. W. Johnson, *et al., Am. J. Physiol.*, **272**, F484 (1997); B. M. Wolska, *et al., J. Mol. Cell. Cardiol.*, **29**, 2653 (1997); N. A. Ritucci, *et al., Am. J. Physiol.*, **275**, R1152 (1998); M. Ikuma, *et al., J. Membr. Biol.*, **166**, 205 (1998); S. F. Yew, *et al., Cardiovasc. Res.*, **40**, 538 (1998). T. Atsumi, *et al., Oral Dis.*, **4**, 248 (1998). P. Pickkers, *et al., Hypertension*, **33**, 1043 (1999); Y. Aoyama, *et al., Jpn. J. Physiol.*, **49**, 55 (1999); U. Bonnet, *et al., Brain Res.*, **840**, 16 (1999).

-*Cellstain*-Calcein-AM

3',6'-Di(*O*-acetyl)-4',5'-bis[*N*,*N*-bis(carboxymethyl)aminomethyl]fluorescein tetraacetoxymethyl ester [CAS: 148504-34-1]

Application: Viable cell staining

Appearance: white or slightly yellow crystals
Purity: > 90.0% (HPLC)
MW: 994.86, $C_{48}H_{46}N_2O_{23}$

Storage Condition	Shipping Condition
-20 °C	ambient temperature or with blue ice

Ordering Information	
Product code	Unit
C326-10	1 mg

-*Cellstain*-Calcein-AM solution

3',6'-Di(*O*-acetyl)-4',5'-bis[*N*,*N*-bis(carboxymethyl)aminomethyl]fluorescein tetraacetoxymethyl ester, DMSO solution [CAS: 148504-34-1]

Application: Viable cell staining

Product: 1 mM Calcein-AM DMSO solution
(1.0 mg Calcein-AM per 1ml DMSO)
Appearance: colorless solution
MW: 994.86, $C_{48}H_{46}N_2O_{23}$

Storage Condition	Shipping Condition
-20 °C	with blue ice or dry ice

Ordering Information	
Product code	Unit
C396-10	1 ml

Product Description

Calcein-AM readily passes through the cell membrane of viable cells because of its enhanced hydrophobicity as compared to Calcein. After Calcein-AM permeates into the cytoplasm, it is hydrolyzed by esterases to Calcein, which remains inside the cell (Fig. 3-16). Among other reagents, including BCECF-AM and Carboxy-fluorescein diacetate, Calcein-AM is the most suitable fluorescent probe for staining viable cells because of its low cytotoxicity. Calcein does not inhibit any cellular functions such as proliferation or chemotaxis of lymphocyte. In addition, viability assays using Calcein are reliable and correlate well with the standard ^{51}Cr-release assay. The excitation and emission wavelengths of calcein are 490 nm and 515 nm, respectively.

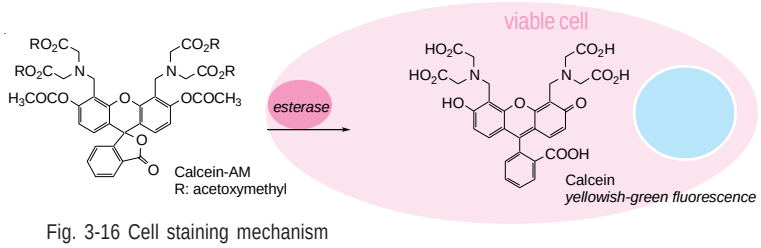


Fig. 3-16 Cell staining mechanism

Staining Procedure

1. Prepare 1 mM Calcein-AM solution with DMSO and dilute to prepare 1-50 µM Calcein-AM solution with PBS.[a)]
2. Add Calcein-AM solution with 1/10 of the volume of cell culture medium to the cell culture.[b)]
3. Incubate the cell at 37 °C for 15-30 min.
4. Wash cells twice with PBS or an appropriate buffer.
5. Observe the cells using a fluorescence microscope with 490 nm excitation and 515 nm emission filters.

a) If the Calcein-AM has difficulty loading into cells, use a detergent such as Pluronic F127.
b) Or you may replace the culture medium with 1/10 concentration of Calcein-AM buffer solution.

III-2. Staining: Cytosol

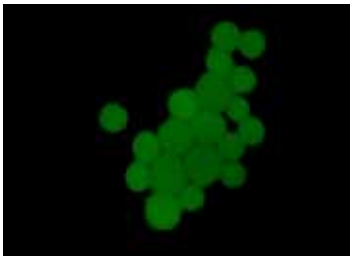


Fig. 3-17 Cell staining with Calcein-AM
Cell type: HeLa

References

S. A. Weston, *et al.*, *J. Immunol. Methods*, **133**, 87 (1990); X. M. Wang, *et al.*, *Hum. Immunol.*, **37**, 264 (1993); R. Lichtenfels, *et al.*, *J. Immunol. Methods*, **172**, 227 (1994); P. Rat, *et al.*, *Cell. Biol. Toxicol.*, **10**, 329 (1994); F. Braut-Boucher, *et al.*, *J. Immunol. Methods*, **178**, 41 (1995); G. Liminga, *et al.*, *Anticancer Drugs*, **6**, 578 (1995); G. Sunder-Plassmann, *et al.*, *Immunol. Invest.*, **25**, 49 (1996); B. Jonsson, *et al.*, *Eur. J. Cancer*, **32A**, 883 (1996); C. M. De Gendt, *et al.*, *Clin. Chim. Acta*, **249**, 189 (1996); F. Tiberghien, *et al.*, *Anticancer Drugs*, **7**, 568 (1996); P. Decherchi, *et al.*, *J. Neurosci. Methods*, **71**, 205 (1997); D. Imbert, *et al.*, *Cornea*, **16**, 666 (1997); R. Monette, *et al.*, *Brain Res. Brain Res. Protoc.*, **2**, 99 (1998); M. Essodaigui, *et al.*, *Biochemistry*, **37**, 2243 (1998); S. Dhar, *et al.*, *Eur. J. Pharmacol.*, **346**, 315 (1998); R. Gatti, *et al.*, *J. Histochem. Cytochem.*, **46**, 895 (1998); H. B. Oral, *et al.*, *Endothelium.*, **6**, 143 (1998); V. Petronilli, *et al.*, *Biophys. J.*, **76**, 725 (1999); E. Giacomello, *et al.*, *Biotechniques*, **26**, 758 (1999); M. M. Roden, *et al.*, *J. Immunol. Methods*, **226**, 29 (1999); T. K. Petersen, *et al.*, *Vet. Immunol. Immunopathol.*, **68**, 283 (1999); C. R. Jarvis, *et al.*, *Neuroimage*, **10**, 357 (1999); M. Adler, *et al.*, *Neurotoxicology*, **20**, 571 (1999); O. Legrand, *et al.*, *Adv. Exp. Med. Biol.*, **457**, 161 (1999).

*-Cellstain-*CFSE 5- or 6-(*N*-Succinimidyloxycarbonyl)-fluorescein 3',6' diacetate [CAS: 150347-59-4]

Application: Viable cell staining

Appearance: white or slightly yellow powder
Purity: >95.0% (HPLC)
MW: 557.46, $C_{29}H_{19}NO_{11}$

Ordering Information	
Product code	Unit
C375-10	1 mg

Storage Condition	Shipping Condition
-20 °C	ambient temperature

Product Description

CFSE is cell membrane permeable and readily accumulates inside of viable cells where it covalently attaches to intracellular proteins (Fig. 3-18). Hydrolyzed CFSE emits fluorescence, and covalently-attached fluorescein molecules do not leak from cells. CFSE-labeled cells can be monitored over several weeks *in vivo*. Therefore, CFSE is utilized for viable cell detection as well as for the long-term observation of cell activities by fluorescent microscopy. The excitation and emission wavelengths of CFSE-labeled cells are 500 nm and 520 nm, respectively. A photo of the CFSE-stained cells are shown in Fig. 3-19.

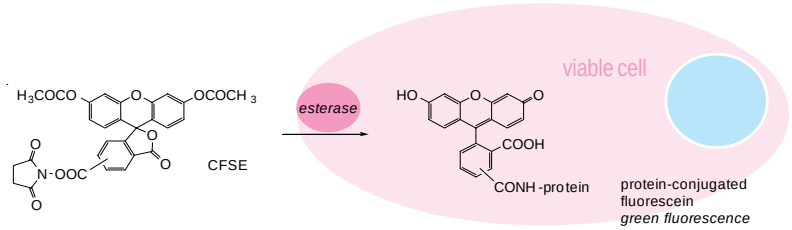


Fig. 3-18 Cell staining mechanism

Staining Procedure

1. Prepare 1 mM CFSE solution with DMSO. Dilute it to prepare 10-50 μM CFSE solution with PBS or an appropriate buffer.
2. Add CFSE solution with 1/10 of the volume of cell culture medium to the cell culture.
3. Incubate the cell at 37 °C for 15 to 30 min.
4. Wash cells twice with PBS or an appropriate buffer.
5. Observe the cells using a fluorescence microscope with 490 nm excitation and 530 nm emission filters.

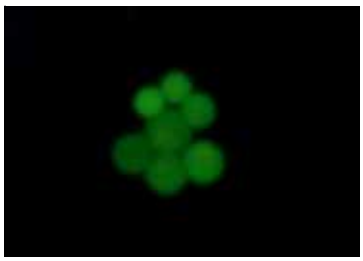


Fig. 3-19 Cell staining with CFSE
Cell type: HeLa

References

S. A. Weston, *et al*., *J. Immunol. Methods*, **133**, 87 (1990); L. S. De Clerck, *et al*., *J. Immunol. Methods*, **172**, 115 (1994); H. Fujioka, *et al*., *Cell Transplant*, **3**, 397 (1994); K. Tokita, *et al*., *Invasion Metastasis*, **15**, 46 (1995); D. A. Fulcher, *et al*., *J. Exp. Med*., **183**, 2313 (1996); S. Oehen, *et al*., *J. Immunol. Methods*, **207**, 33 (1997); L. Y. Khil, *et al*., *Mol. Cells*, **7**, 742 (1997); M. Graziano, *et al*., *Exp. Cell Res*., **240**, 75 (1998); L. P. Logan, *et al*., *J. Immunol. Methods*, **213**, 19 (1998); R. Hokari, *et al*., *Gastroenterology*, 115, 618 (1998). W. T. Lee, *et al*., *Cell Immunol*., 188, 1 (1998); R. A. Oostendorp, *et al*., *Leukemia*, **13**, 499 (1999); H. K. Song, *et al*., *Transplantation*, **68**, 297 (1999); A. Tsujikawa, *et al*., *Invest. Ophthalmol. Vis. Sci*., **40**, 2918 (1999); B. F. Groth, *et al*., *Immunol. Cell. Biol*., **77**, 530 (1999); J. Mintern, *et al*., *Immunol. Cell. Biol*., 77, 539 (1999); H. S. Warren, *Immunol. Cell. Biol*., 77, 544 (1999); D. Fulcher, *et al*., *Immunol. Cell. Biol*., 77, 559 (1999).

-Cellstain-CytoRed Solution

7-Isobutyloxycarbonyloxy-*3H*-phenoxazin-3-one

Application: Viable cell staining

Product: 1 mM CytoRed DMSO solution
(0.31 mg CytoRed per 1 ml DMSO)

Appearance: yellowish-orange solution

MW: 313.31, $C_{17}H_{15}NO_5$

Ordering Information	
Product code	Unit
C410-10	1 ml

Storage Condition	Shipping Condition
-20 °C	with blue ice or dry ice

Product Description

CytoRed is cell membrane permeable and accumulates inside of viable cells as resorufin (Fig. 3-20). CytoRed has a much wider spectrum than BCECF or Calcein, so filters for fluorescein and rhodamine can also be used. The excitation and emission wavelengths of resorufin are 560 nm and 590 nm, respectively. A photo of cells stained with CytoRed is shown in Fig. 3-21.

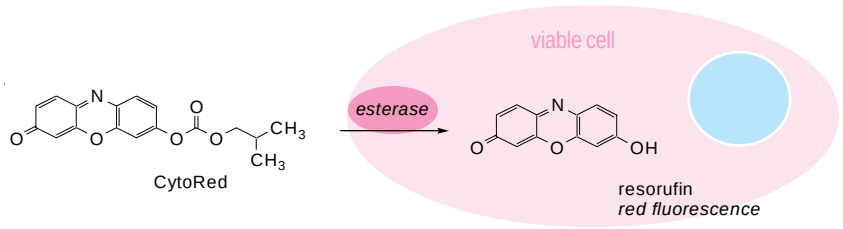


Fig. 3-20 Cell staining mechanism

Staining Procedure

1. Prepare 1 mM CytoRed solution with DMSO. Dilute it to prepare 10 µM CytoRed solution with culture medium or an appropriate buffer.[a)]
2. Prepare a $1x10^5$-$1x10^6$ cells/ml cell suspension and culture the cells in a chamber slide.
3. Remove culture medium and wash cells with culture medium (PBS-Hanks medium, etc).
4. Add CytoRed solution to the cells, and incubate the chamber at 37 °C for 30 min to 1 hour.
5. Remove the culture medium from cells and add new medium.[b)]
6. Wash cells twice with PBS or an appropriate buffer.
7. Observe the cells using a fluorescence microscope with 560 nm excitation and 590 nm emission filters.

a) Incubate the MitoRed buffer solution at 37 °C prior to adding to cells.
b) For fixing after washing cells, add 10% formarin buffer and incubate for 15-20 min, and then wash with PBS.

III-2. Staining: Cytosol

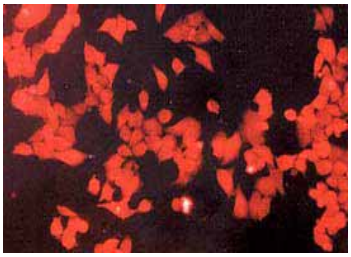


Fig. 3-21 Cell staining with CytoRed
Cell type: HeLa

Reference

K. Sasamoto, *et al.*, *Anal. Sci.*, **15**, 1025 (1999).

-*Cellstain*-FDA

Fluorescein diacetate [CAS: 596-09-8]

Application: Viable cell staining

Appearance: white crystalline powder
Purity: >99.0% (HPLC)
MW: 416.38, $C_{24}H_{16}O_7$

Ordering Information

Product code	Unit
F209-10	1 mg

Storage Condition
-20 °C, protect from light

Shipping Condition
ambient temperature

Product Description

FDA is cell membrane permeable and accumulates inside of viable cells as fluorescein (Fig. 3-22). Since fluorescein is less hydrophilic than BCECF or Calcein, the leakage of fluorescein from cells is rather high. FDA is also utilized for flow cytometry. The excitation and emission wavelengths of fluorescein are 488 nm and 530 nm, respectively. A photo of FDA-stained cells is shown in Fig. 3-23.

Mechanism of Viable Cell Staining

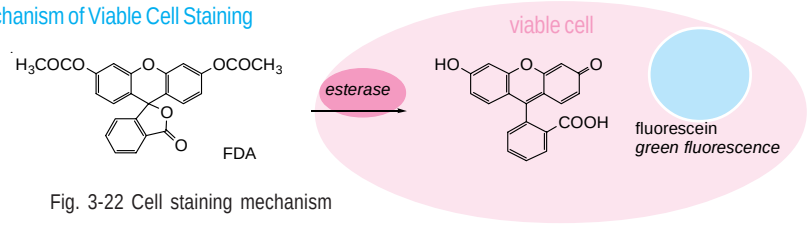


Fig. 3-22 Cell staining mechanism

Staining Procedure

1. Prepare 0.5 mg/ml FDA stock solution with DMSO. Dilute 10 ul of the stock solution with 5 ml PBS(-).
2. Prepare a cell suspension and wash cells with PBS(-). Prepare $1x10^5$-$1x10^6$ cells/ml cell suspension
3. Add 15 ul FDA solution to 30 ul cell suspension, and incubate at 37 °C for 15-30 min.
4. Put 10 ul stained cell suspension on a glass slide and cover with a cover glass.
5. Observe the cells using a fluorescence microscope with 488 nm excitation and 530 nm emission filters.

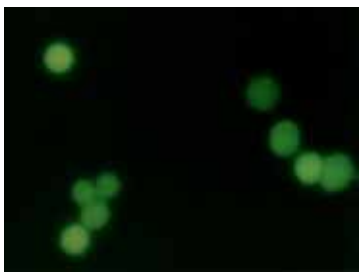


Fig. 3-23 a) Cell staining with FDA, Cell type: HeLa

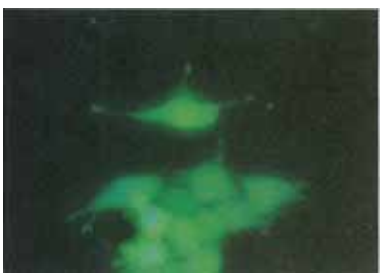


Fig. 3-23 b) Cell staining with FDA, Cell type: HeLa

References

K. H. Jones, *et al.*, *J. Histochem. Cytochem.*, **33**, 77 (1985); D. W. Gray, *et al.*, *Stain Technol.*, **62**, 373 (1987); D. D. Ross, *et al.*, *Cancer Res.*, **49**, 3776 (1989); E. Prosperi, *Histochem. J.*, **22**, 227 (1990); V. Jayapal, *et al.*, *Lepr. Rev.*, **62**, 310 (1991); M. Nijs, *et al.*, *Biotech. Histochem.*, **67**, 351 (1992); S. L. Nyberg, *et al.*, *Biotech. Histochem.*, **68**, 56 (1993); S. Patel, *et al.*, *Int. Endod. J.*, **27**, 1 (1994); P. Breeuwer, *et al.*, *Appl. Environ. Microbiol.*, **61**, 1614 (1995); M. L. Skoog, *et al.*, *Photodermatol. Photoimmunol. Photomed.*, **13**, 37 (1997); A. Kovarik, *et al.*, *Biotechniques*, **27**, 685 (1999).

-*Cellstain*-MitoRed

9-[2-(4'-Methylcoumarin-7'-oxycarbonyl)phenyl]-3,6-bis(diethylamino)xanthylium chloride

Application: Mitochondrial staining

Appearance: purplish-brown powder
Purity: >80.0% (HPLC)
MW: 637.17, $C_{38}H_{37}ClN_2O_5$

Ordering Information

Product code	Unit
R237-10	50 µg x 8 vials

Storage Condition
0-5 °C, protect from light and moisture

Shipping Condition
ambient temperature

Product Description
MitoRed is a cell membrane permeable rhodamine-based dye. It localizes in mitochondria and emits red fluorescence (Fig. 3-24). The interaction of MitoRed with mitochondria depends on the membrane potential of the mitochondria. Mitochondria can be stained with 20 to 200 nM MitoRed. The excitation and emission wavelengths of MitoRed are 560 nm and 580 nm, respectively.

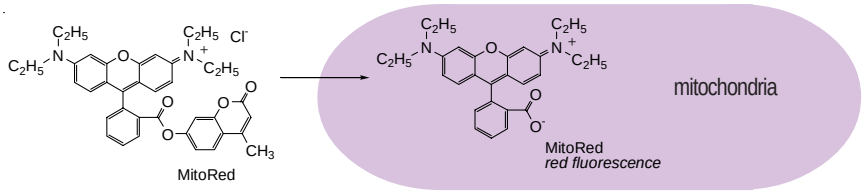


Fig. 3-24 Cell staining mechanism

Staining Procedure
1. Dissolve 50µg MitoRed (1 tube) in 78 µl of DMSO to prepare 1 mM MitoRed-DMSO solution.
2. Prepare cells with a glass slide. The cell number should be $5x10^4$ to $5x10^5$ cells per ml.
3. Incubate the slide and wash cells with PBS or Hank's medium.
4. Dilute the 1 mM MitoRed solution with culture medium to prepare 20-200 nM MitoRed buffer solution.
5. Add the MitoRed buffer solution[a)] to the glass slide and incubate at 37 °C for 30 min to 1 hour.
6. Remove the MitoRed buffer solution and wash cells with culture medium.[b)]
7. Observe the cells using a fluorescence microscope with a rhodamine filter.

a) Incubate the MitoRed buffer solution at 37 °C prior to adding to cells.
b) For fixing after washing cells, add 10% formarin buffer and incubate for 15-20 min, and then wash with PBS.

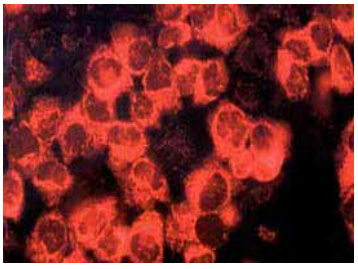


Fig. 3-25 Cell staining with MitoRed
Cell type: HeLa

III-3. Staining: Mitochondria

-Cellstain-Rh123

Rhodamine 123 [CAS: 62669-70-9]

Application: Mitochondrial staining

Appearance: reddish-brown powder
Purity: > 90.0% (HPLC)
MW: 380.82, $C_{21}H_{17}ClN_2O_3$

Ordering Information	
Product code	Unit
R233-10	1 mg

Storage Condition	Shipping Condition
ambient temperature	ambient temperature

Product Description

Rhodamine 123 (Rh123) is cell membrane permeable and it localizes in mitochondria of viable cells to emit yellowish-green fluorescence (Fig. 3-26). Rh123 is utilized for staining a wide variety of cells, including plant cells and bacteria. Since there is a correlation between the amount of ATP in a cell and the fluorescence intensity of Rh123, this compound is used for the detection of intracellular ATP. Rh123 is also used in cancer research.

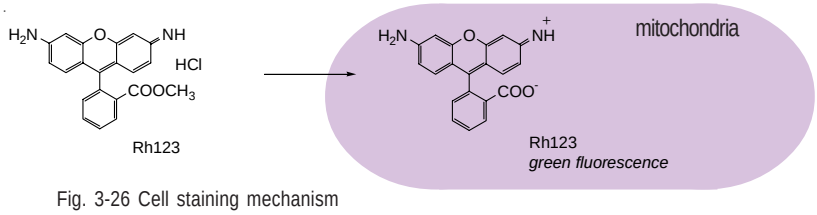


Fig. 3-26 Cell staining mechanism

Staining Procedure

1. Dissolve 0.4 mg Rh123 in 1 ml DMSO to prepare 1 mM Rh123-DMSO solution.
2. Prepare cells with a glass slide. The cell number will be $5x10^4$ to $5x10^5$ cells per ml.
3. Incubate the slide and wash cells with PBS or Hank's medium.
4. Dilute the 1 mM Rh123 solution with culture medium to prepare 1-20 μM Rh123 buffer solution.
5. Add the Rh123 buffer solution[a)] to the glass slide and incubate at 37 °C for 30 min to 1 hour.
6. Remove the Rh123 buffer solution and wash cells with culture medium.[b)]
7. Observe the cells using a fluorescence microscope with a fluorescein filter.

a) Incubate the Rh123 buffer solution at 37 °C prior to adding to cells.
b) For fixing after washing cells, add 10% formarin buffer and incubate for 15-20 min, and then wash with PBS.

References

L. V. Johnson, *et al.*, *Proc. Natl. Acad. Sci. USA*, **77**, 990 (1980); C. S. Downes, *et al.*, *Carcinogenesis*, **6**, 1343 (1985); G. Varbiro, *et al.*, *Free Radic. Biol. Med.*, **31**, 548 (2001).

-Cellstain-AO

3,6-Bis(dimethylamino)acridine, hydrochloride [CAS: 65-61-2]

Application: DNA, RNA staining

Appearance: reddish-brown powder
Purity: pass test (TLC)
MW: 301.81, $C_{17}H_{20}ClN_3$

Ordering Information	
Product code	Unit
A386-10	1 mg

Storage Condition	Shipping Condition
0-5 °C, protect from light	ambient temperature

-*Cellstain*-AO Solution

3,6-Bis(dimethylamino)acridine, hydrochloride, aqueous solution [CAS: 65-61-2]

Application: DNA, RNA staining

Product: 3.3 mM AO aqueous solution
(1 mg AO per 1 ml water)
Appearance: orange or yellow solution
MW: 301.81, $C_{17}H_{20}ClN_3$

Ordering Information	
Product code	Unit
A430-10	1 ml

Storage Condition
-20 °C, protect from light

Shipping Condition
ambient temperature

Product Description

Acridine orange (AO) forms a complex with double-stranded DNA to emit green fluorescence (Fig. 3-27). AO also forms a complex with single-stranded DNA or RNA to emit red fluorescence. One molecule of AO intercalates with three base pairs of double-stranded DNA and emits green fluorescence with the maximum wavelength at 526 nm (excitation 502 nm). One molecule of AO can also interact with one phosphate group of single-stranded DNA or RNA to form an aggregated, or stacked, structure, that emits red fluorescence with the maximum wavelength at 650 nm (excitation 460 nm). Therefore, AO is utilized for the detection of both double-stranded DNA and single-stranded DNA or RNA. It enables simultaneous determination of DNA and RNA with argon laser excitation or flow cytometry.

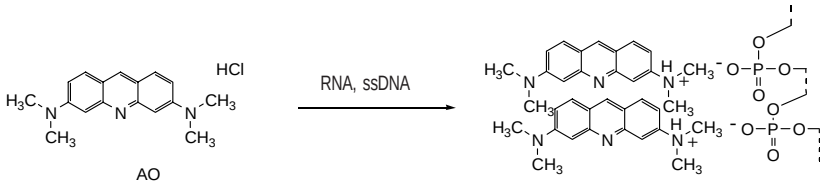


Fig. 3-27 Cell staining mechanism

Staining Procedure

1. Prepare 10-50 µM AO solution with PBS or an appropriate buffer.[a)]
2. Add AO solution with 1/10 of the volume of cell culture medium to the cell culture.[b)]
3. Incubate the cell at 37 °C for 10-20 min.
4. Wash cells twice with PBS or an appropriate buffer.
5. Observe the cells using a fluorescence microscope with 500 nm excitation and 530 nm emission filters.

a) Since AO may be carcinogenic, extreme care is necessary during handling.
b) You may replace the culture medium with 1/10 concentration of AO buffer solution.

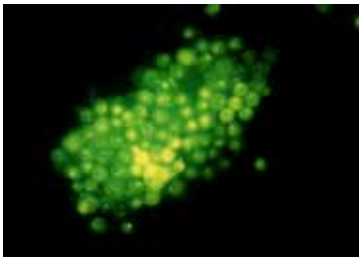


Fig. 3-28 Cell staining with AO
Cell type: HeLa

References

I. W. Taylor, *et al., J. Histochem. Cytochem*., **28**, 1224 (1980); N. Miyoshi, *et al., Photochem. Photobiol*., **47**, 685 (1988); Z. Darzynkiewicz, *Methods Cell Biol*., **33**, 285 (1990); Z. Darzynkiewicz, *Methods Cell Biol*., **41**, 401 (1990); J. Delic, *et al., Exp. Cell Res*., **194**, 147 (1991); A. K. El-Naggar, *et al*., *Cytometry*, **12**, 330 (1991); A. Myc, *et al., Cytometry*, **13**, 103 (1991).

III-4. Staining: Nucleus

-*Cellstain*-DAPI

4',6-Diamidino-2-phenylindole, dihydrochloride [CAS: 28718-90-3]

Application: DNA staining

Appearance: yellow powder
Purity: pass test (TLC)
MW: 350.25, $C_{16}H_{17}Cl_2N_5$

Ordering Information	
Product code	Unit
D212-10	1 mg

Storage Condition	Shipping Condition
-20 °C, protect from light	ambient temperature

-*Cellstain*-DAPI Solution

4',6-Diamidino-2-phenylindole, dihydrochloride, aqueous solution [CAS: 28718-90-3]

Application: DNA staining

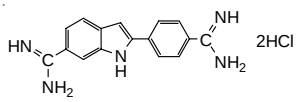
Product: 2.9 mM DAPI buffer solution
(1 mg DAPI per 1 ml buffer)
Appearance: yellow solution
MW: 350.25, $C_{16}H_{17}Cl_2N_5$

Ordering Information	
Product code	Unit
D523-10	1 ml

Storage Condition	Shipping Condition
0-5 °C, protect from light	ambient temperature

Product Description

DAPI is an AT-sequence specific DNA intercalator that attaches to DNA at the minor groove of the double helix like Hoechst dyes. Though DAPI is not permeable through viable cell membranes, it passes through disturbed cell membranes to stain the nucleus. DAPI has a high photo-bleaching tolerance level. DAPI is utilized for the detection of mitochondrial DNA in yeast, chloroplast DNA, virus DNA, micoplasm DNA and chromosomal DNA. The excitation and emission wavelengths of DAPI-DNA complex are 360 nm and 460 nm, respectively.



Staining Procedure

1. Prepare 10-50 μM DAPI solution with PBS or an appropriate buffer.[a)]
2. Add DAPI solution with 1/10 of the volume of cell culture medium to the cell culture.[b)]
3. Incubate the cell at 37 °C for 10-20 min.
4. Wash cells twice with PBS or an appropriate buffer.
5. Observe the cells using a fluorescence microscope with 360 nm excitation and 460 nm emission filters.

a) Since DAPI may be carcinogenic, extreme care is necessary during handling.
b) Or you may replace the culture medium with 1/10 concentration of DAPI buffer solution.

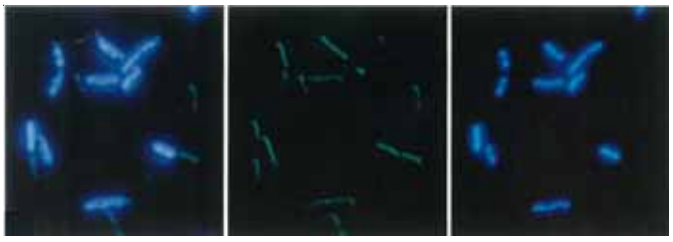


Fig. 3-29 Cell staining with DAPI

References
W. Schnedl, *et al.*, *Hum. Genet.*, **36**, 167 (1977); I. W. Taylor, *et al.*, *J. Histochem. Cytochem.*, **28**, 1224 (1980); F. Otto, *et al.*, *Stains Technol.*, **60**, 7 (1985); I. Vollenweider, *et al.*, *J. Immunol. Methods*, **149**, 133 (1992); M. A. Hotz, *et al.*, *Cytometry*, **15**, 234 (1994); N. Poulin, *et al.*, *Cytometry*, **16**, 227 (1994); C. Souchier, *et al.*, *Cytometry*, **20**, 203 (1995); J. Kapuscinski, *et al.*, *Biotech. Histochem.*, **70**, 220 (1995); S. M. Bilinski, *et al.*, *Histochem. J.*, **28**, 651 (1996); S. Burde, *et al.*, *Cytometry*, **25**, 295 (1996); E. A. Moscone, *et al.*, *Chromosoma*, **105**, 231 (1996); S. Saby, *et al.*, *Appl. Environ. Microbiol.*, **63**, 1564 (1997); K. Kameyama, *et al.*, *Oncol. Rep.*, **6**, 1345 (1999).

-*Cellstain*-EB

3,8-Diamino-5-ethyl-6-phenylphenanthridium bromide [CAS: 1239-45-8]

Application: DNA staining

Appearance: reddish-brown powder
Purity: pass test (TLC)
MW: 394.31, $C_{21}H_{20}BrN_3$

Ordering Information

Product code	Unit
E262-10	1 mg

Storage Condition
0-5 °C, protect from light

Shipping Condition
ambient temperature

-*Cellstain*-EB Solution

3,8-Diamino-5-ethyl-6-phenylphenanthridium bromide, aqueous solution [CAS: 1239-45-8]

Application: DNA staining

Product: 2.5 mM EB aqueous solution
(1 mg EB per 1 ml water)
Appearance: orange or red solution
MW: 394.31, $C_{21}H_{20}BrN_3$

Ordering Information

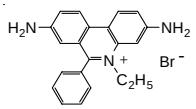
Product code	Unit
E272-10	1 ml

Storage Condition
-20 °C, protect from light

Shipping Condition
ambient temperature

Product Description

Ethidium bromide (EB) is commonly used for staining DNA in agarose gel. EB does not permeate viable cell membranes. However, it passes through the disrupted membranes of dead cells to stain nucleic DNA. The excitation and emission wavelengths of EB-DNA complex are 518 nm and 605 nm, respectively.



Staining Procedure

1. Prepare 10-50 μM EB solution with PBS or an appropriate buffer.[a)]
2. Add EB solution with 1/10 of the volume of cell culture medium to the cell culture.[b)]
3. Incubate the cell at 37 °C for 10-20 min.
4. Wash cells twice with PBS or an appropriate buffer.
5. Observe the cells using a fluorescence microscope with 515 nm excitation and 600 nm emission filters.

a) Since EB is carcinogenic, extreme care is necessary during handling.
b) Or you may replace the culture medium with 1/10 concentration of EB buffer solution.

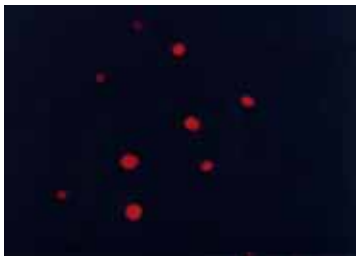


Fig. 3-30 Cell staining with EB
Cell type: HeLa

III-4. Staining: Nucleus

References
I. W. Taylor, *et al.*, *J. Histochem. Cytochem.*, **28**, 1224 (1980); D. Roser, *et al.*, *J. Appl. Bacteriol.*, **56**, 343 (1984); I. P. Beletsky, *et al.*, *J. Immunol. Methods*, **134**, 201 (1990); C. M. Davies, *Lett. Appl. Microbiol.*, **13**, 58 (1991); C. Lazaro, *et al.*, *Mol. Cell. Probes*, **6**, 357 (1992); W. G. Ballhausen, *et al.*, *Appl. Theor. Electrophor.*, **3**, 129 (1993); S. S. Chong, *et al.*, *Am. J. Med. Genet.*, **51**, 522 (1994); M. W. Jernaes, *et al.*, *Cytometry*, **17**, 302 (1994); M. S. Liu, *et al.*, *Biotechniques*, **18**, 316 (1995); W. D. Harriman, *et al.*, *Anal. Biochem.*, **228**, 336 (1995); D. Gilligan, *et al.*, *Int. J. Radiat. Biol.*, **69**, 251 (1996); J. G. Bruno, *et al.*, *Biotech. Histochem.*, **71**, 130 (1996); M. F. Shubsda, *et al.*, *J. Biochem. Biophys. Methods*, **34**, 73 (1997); M. J. Lucey, *et al.*, *Biotechniques*, **23**, 780 (1997); T. Xie, *et al.*, *Mol. Pathol.*, **50**, 276 (1997); M. Shono, *et al.*, *Biochem. Mol. Biol. Int.*, **46**, 1055 (1998); T. N. Ferraro, *et al.*, *Psychiatr. Genet.*, **8**, 227 (1998); J. Frohlich, *et al.*, *J. Microbiol. Methods*, **35**, 121 (1999).

*-Cellstain-*Hoechst 33258 Solution

Bisbenzimide, 2'-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5'-bi-*1H*-benzimidazole trihydrochloride, aqueous solution [CAS: 23491-44-3]

Application: DNA staining

Product: 1.9 mM Hoechst 33258 aqueous solution
(1 mg Hoechst 33258 per 1 ml water)
Appearance: yellow solution
MW: 533.88, $C_{25}H_{27}Cl_3N_6O$

Ordering Information

Product code	Unit
H341-10	1 ml

Storage Condition
0-5 °C, protect from light

Shipping Condition
ambient temperature

*-Cellstain-*Hoechst 33342 Solution

Bisbenzimide, 2'-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5'-bi-*1H*-benzimidazole trihydrochloride, aqueous solution [CAS: 23491-52-3]

Application: DNA staining

Product: 1.8 mM Hoechst 33342 aqueous solution
(1 mg Hoechst 33342 per 1 ml water)
Appearance: yellow solution
MW: 561.93, $C_{27}H_{31}Cl_3N_6O$

Ordering Information

Product code	Unit
H342-10	1 ml

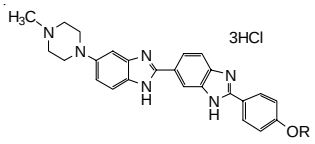
Storage Condition
0-5 °C, protect from light

Shipping Condition
ambient temperature

Product Description

Hoechst dyes are cell membrane permeable and stain DNA to emit intense blue fluorescence. They bind to DNA in the minor groove of poly-AT sequence rich areas. Both Hoechst 33342 and Hoechst 33258 are water-soluble and stable in aqueous solutions. The excitation and emission wavelengths of Hoechst-DNA complex are 350 nm and 460 nm, respectively.

Chemical Structure



Hoechst 33258: R=OH
Hoechst 33342: R=C_2H_5

Staining Procedure

1. Prepare 10-50 μM Hoechst dye solution with PBS or an appropriate buffer.[a)]
2. Add Hoechst dye solution with 1/10 of the volume of cell culture medium to the cell culture.[b)]
3. Incubate the cell at 37 °C for 10-20 min.
4. Wash cells twice with PBS or an appropriate buffer.
5. Observe the cells using a fluorescence microscope with 350 nm excitation and 460 nm emission filters.

a) Since Hoechst dyes may be carcinogenic, extreme care is necessary during handling.
b) Or you may replace the culture medium with 1/10 concentration of Hoechst dye buffer solution.

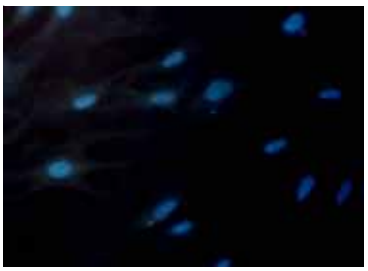


Fig. 3-31 Cell staining with Hoechst 33258
Cell type: human fetal cell

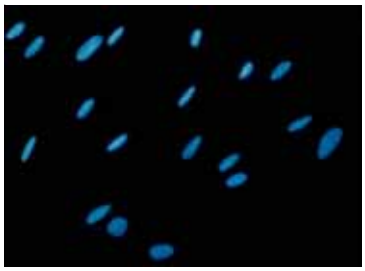


Fig. 3-32 Cell staining with Hoechst 33342
Cell type: human fetal cell

References

M. E. Lalande, *et al.*, *J. Histochem. Cytochem.*, **27**, 394 (1979); M. J. Lydon, *et al.*, *J. Cell Physiol.*, **102**, 175 (1980); H. M. Shapiro, *et al.*, *Cytometry*, **2**, 143 (1981); T. Hoshino, *et al.*, *Cancer*, **50**, 997 (1982); G. C, Rice, *et al.*, *Cancer Res.*, **44**, 2368 (1984); D. Evenson, *et al.*, *Cytometry*, **7**, 45 (1986); A. Krishan, *et al.*, *Cytometry*, **8**, 642 (1987); H. A. Crissman, *et al.*, *Cancer Res.*, **48**, 5742 (1988). D. Loeffler, *et al.*, *J. Immunol. Methods*, **119**, 95 (1989); J. M. Morrell, *et al.*, *Mutat. Res.*, **224**, 177 (1989); J. W. Ellwart, *et al.*, *Cytometry*, **11**, 239 (1990); L. Karawajew, *et al.*, *J. Immunol. Methods*, **129**, 277 (1990); M. Montag, *et al.*, *J. Microsc.*, **163**, 201 (1991); J. A. Hardin, *et al.*, *J. Immunol. Methods*, **154**, 99 (1992); D. P. Green, *et al.*, *J. Reprod. Fertil.*, **96**, 581 (1992); R. C. Boltz, *et al.*, *Cytometry*, **15**, 28 (1994); F. Belloc, *et al.*, *Cytometry*, **17**, 59 (1994); S. Reid, *et al.*, *J. Immunol. Methods*, **192**, 43 (1996); M. A. DeMaria, *et al.*, *Cytometry*, **25**, 37 (1996); J. E. Reynolds, *et al.*, *Cytometry*, **25**, 349 (1996); X. Zhang, *et al.*, *Ann. Clin. Lab. Sci.*, **27**, 260 (1997); S. L. Catt, *et al.*, *Mol. Hum. Reprod.*, **3**, 821 (1997); G. Whiteside, *et al.*, *Brain Res. Brain Res. Protoc.*, **2**, 160 (1998); I. Tatischeff, *et al.*, *Cell Mol. Life Sci.*, **54**, 476 (1998); X. Zhang, *et al.*, *Arch. Pathol. Lab. Med.*, **123**, 921 (1999).

-*Cellstain*-PI

3,8-Diamino-5-[3-(diethylmethylammonio)propyl]-6-phenylphenanthridinium diiodide [CAS: 25535-16-4]

Application: DNA staining

Appearance: reddish-brown powder
Purity: pass test (TLC)
MW: 668.39, $C_{27}H_{34}I_2N_4$

Ordering Information	
Product code	Unit
P346-10	1 mg

Storage Condition
0-5 °C, protect from light

Shipping Condition
ambient temperature

-*Cellstain*-PI Solution

3,8-Diamino-5-[3-(diethylmethylammonio)propyl]-6-phenylphenanthridinium diiodide, aqueous solution [CAS: 25535-16-4]
Application: DNA staining

Product: 1.5 mM PI aqueous solution
(1 mg PI per 1 ml water)
Appearance: orange or red solution
MW: 668.39, $C_{27}H_{34}I_2N_4$

Ordering Information	
Product code	Unit
P378-10	1 ml

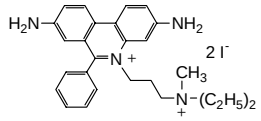
Storage Condition
-20 °C, protect from light

Shipping Condition
ambient temperature

Product Description

Propidium iodide (PI) is an ethidium bromide analog that emits red fluorescence upon intercalation with double-stranded DNA. Though PI does not permeate viable cell membranes, it passes through disturbed cell membranes and stains the nuclei. PI is often used in combination with a fluorescein compound, such as Calcein-AM or FDA, for simultaneous staining of viable and dead cells. The excitation and emission wavelengths of PI-DNA complex are 535 nm and 615 nm, respectively.

Chemical Structure



III-4. Staining: Nucleus

Staining Procedure

1. Prepare 10-50 μM PI solution with PBS or an appropriate buffer.[a)]
2. Add PI solution with 1/10 of the volume of cell culture medium to the cell culture.[b)]
3. Incubate the cell at 37 °C for 10-20 min.
4. Wash cells twice with PBS or an appropriate buffer.
5. Observe the cells using a fluorescence microscope with 535 nm excitation and 615 nm emission filters.

a) Since PI may be carcinogenic, extreme care is necessary during handling.
b) Or you may replace the culture medium with 1/10 concentration of PI buffer solution.

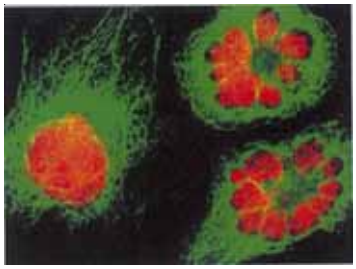


Fig. 3-33 Cell staining with PI

References

I. W. Taylor, *et al.*, *J. Histochem. Cytochem.*, **28**, 1224 (1980); W. M. J. Vuist, *et al.*, *Cancer Res.*, **49**, 3783 (1989); A. Pollack, *et al.*, *Methods Cell. Biol.*, **33**, 315 (1990); A. Bertuzzi, *et al.*, *Cell. Biophys.*, **17**, 257 (1990); A. K. El-Naggar, *et al.*, *Cytometry*, **12**, 330 (1991); I. Nicoletti, *et al.*, *J. Immunol. Methods*, **139**, 271 (1991); I. Vollenweider, *et al.*, *J. Immunol. Methods*, **149**, 133 (1992); M. G. Ormerod, *et al.*, *Cytometry*, **13**, 678 (1992); T. Crompton, *et al.*, *Biochem. Biophys. Res. Commun.*, **183**, 532 (1992); M. Vitale, *et al.*, *Histochemistry*, **100**, 223 (1993); P. L. Olive, *et al.*, *Cytometry*, **16**, 305 (1994); N. M. Poulin, *et al.*, *J. Histochem. Cytochem.*, **42**, 1149 (1994); F. Belloc, *et al.*, *Cytometry*, **17**, 59 (1994); C. A. Van Hooijdonk, *et al.*, *Cytometry*, **17**, 185 (1994); D. L. Garner, *et al.*, *J. Androl.*, **15**, 620 (1994); C. Souchier, *et al.*, *Cytometry*, **20**, 203 (1995); D. L. Garner, *et al.*, *Biol. Reprod.*, **53**, 276 (1995); M. Wulf, *et al.*, *Biotechniques*, **19**, 368 (1995); K. Wrobel, *et al.*, *J. Immunol. Methods*, **189**, 243 (1996); W. E. Corver, *et al.*, *Cytometry*, **28**, 329 (1997).

-*Cellstain*-Double Staining Kit

Features: Fluorometric microscope detection
Simultaneous determination of viable and dead cells

Ordering Information

Product code	Unit
CS01-10	1 set

Kit Components:

Solution A (Calcein-AM) ..... 4 vials Solution B (PI) ..... 1 vial

Storage Condition	Shipping Condition
-20 °C	with blue ice or dry ice

Required Equipment and Materials

microscope with 490 nm excitation filter and 530 nm emission filter, glass slide or glass bottom plate, CO_2 incubator, 10 μl and 200 μl adjustable pipettes, PBS

Product Description

-*Cellstain*-Double Staining Kit is utilized for simultaneous fluorescence staining of viable and dead cells. This kit contains Calcein-AM and Propidium Iodide (PI) solutions, which stains viable and dead cells, respectively (Fig. 3-34). Calcein-AM, acetoxymethyl ester of calcein, is highly lipophilic and cell membrane permeable. Though Calcein-AM itself is not a fluorescent molecule, the calcein generated from Calcein-AM by esterase in a viable cell emits a strong green fluorescence (excitation: 490 nm, emission: 515 nm). Therefore, Calcein-AM only stains viable cells. On the other hand, PI, a nuclei staining dye, cannot pass through a viable cell membrane. It reaches the nucleus by passing through disordered areas of dead cell membrane, and intercalates with the DNA double helix of the cell to emit red fluorescence (excitation: 535 nm, emmision: 617 nm). Since both calcein and PI-DNA can be excited with 490 nm, simultaneous monitoring of viable and dead cells is possible with a fluorescence microscope. With 545 nm excitation, only dead cells can be observed (Fig. 3-35). Since optimal staining conditions differ from cell line to cell line, we recommend that a suitable concentration of PI and Calcein-AM be individually determined. Please note that PI is suspected to be highly carcinogenic; careful handling is required.

III-4. Staining: Nucleus

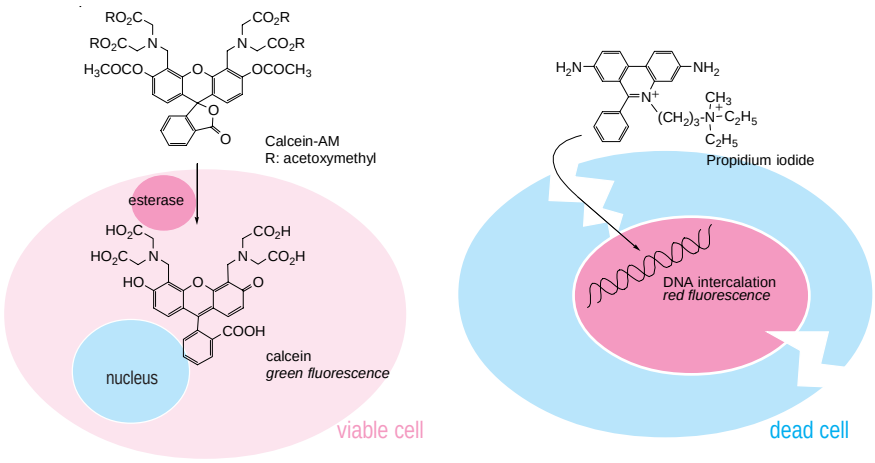


Fig. 3-34 Assay system to determine viable cells and dead cells

Assay Procedure

1. Add 10 µl Solution A and 5 µl Solution B to 5 ml PBS to prepare assay solution.*
2. Wash the cell with PBS several times to remove residual esterase activity.
3. Add 100 µl of assay solution to the cell and incubate the mixture at 37 °C for 15 min.
4. Detect fluorescence using a fluorescence mircoscope with 490 nm excitation for simultaneous monitoring of viable and dead cells. With 545 nm excitation, only dead cells can be observed.

* The concentration of each reagent should be optimized. The following steps may be necessary to determine the suitable concentration of each reagent:
  1. Prepare dead cells by 10 min incubation in 0.1% saponin or 0.1-0.5% digitonin or by 30 min incubation in 70% ethanol.
  2. Stain dead cells with 0.1-10 µM PI solution to find a PI concentration that stains the nucleus only, not the cytosol.
  3. Stain dead cells with 0.1-10 µM Calcein-AM solution to find a Calcein-AM concentration that does not stain the cytosol. Then stain viable cells with that Calcein-AM solution to check whether the viable cell can be stained.

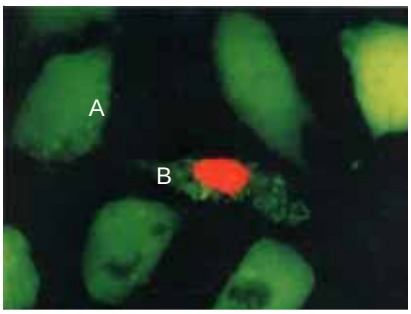


Fig. 3-35 Cell staining with Double Staining Kit
MHD-1 cell, incubated with assay solution for 15 min.
A) viable cell
B) dead cell

References

E. S. Kaneshiro, *et al., J. Microbiol. Methods*, **17**, 1 (1993); L. S. De Clerck, *et al., J. Immunol. Methods*, **172**, 115 (1994); N. G. Papadopoulus, *et al., J. Immunol. Methods*, **177**, 101 (1994); M. Adler, *et al., Neurotoxicology*, **20**, 571 (1999); P. G. Bush, *et al., Osteoarthritis Cartilage*, **13**, 54 (2005).

III-5. Staining: Membrane

-*Cellstain*-Trypan Blue

3,3'-{[3,3'-Dimethyl-(1,1'-biphenyl)-4,4'-diyl]bis(azo)}-bis(5-amino-4-hydroxy-2,7-naphthalenedisulfonic acid), tetrasodium salt [CAS: 72-57-1]

Application: Dead cell staining

Appearance: dark brown powder
Molar absorptivity: >6.9x10^4 at 609 nm
MW: 960.81, $C_{34}H_{24}N_6Na_4O_{14}S_4$

Ordering Information

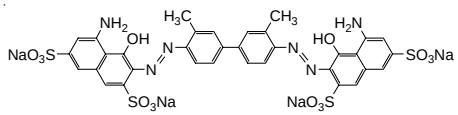
Product code	Unit
T375-10	5 g

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Product Description

Trypan Blue is commonly utilized for dead cell staining, in what is called the dye exclusion test. Viable cells are not stained by Trypan Blue. Therefore, dead Trypan Blue-stained cells are easily recognized by microscopy and can be counted using a hematocytometer. Erythrosin B, negrosine, eosin Y, AO and EB are also utilized for this purpose. Though it is hard to detect cells in early to middle stages of apoptosis, Trypan Blue staining is a very simple and widely used method to visualize dead cells.

Chemical Structure



References

E. Hegazy, *et al.*, *Eur. J. Cell Biol.*, **30**, 132 (1983); K. H. Jones, *et al.*, *J. Histochem. Cytochem.*, **33**, 77 (1985); E. Walum, *et al.*, *Xenobiotica*, **15**, 701 (1985); M. A. Linshaw, *et al.*, *Am. J. Physiol.*, **251**, F214 (1986); G. J. Lees, *et al.*, *Histochemistry*, **91**, 357 (1989); A. Lewin, *et al.*, *J. Reprod. Med.*, **35**, 136 (1990); M. Nijs, *et al.*, *Biotech. Histochem.*, **67**, 351 (1992); S. A. Altman, *et al.*, *Biotechnol. Prog.*, **9**, 671 (1993); J. Hu, *et al.*, *Life Sci.*, **55**, 1009 (1994); S. W. Perry, *et al.*, *Biotechniques*, **22**, 1102 (1997); D. S. Reynolds, *et al.*, *J. Neurosci. Methods*, **79**, 115 (1998); N. Georgiadis, *et al.*, *Ophthalmologica*, **213**, 8 (1999); G. R. Melles, *et al.*, *J. Cataract Refract. Surg.*, **25**, 7 (1999).

FSB Solution

1-Fluoro-2,5-bis(3-carboxy-4-hydroxystyryl)benzene, 1% w/v DMSO solution

Application: Amyloid staining

Properties: High affinity with β-sheet structure
High detection sensitivity

Ordering Information

Product code	Unit
F308-10	100 µl

Appearance: pale yellow to tannish soluton
Absorbance: 0.6-0.85 (around 370nm)
MW: 420.39, $C_{24}H_{17}FO_6$

Storage Condition: 0-5 °C, protect from light
Shipping Condition: ambient temperature

Product Description

Amyloidosis, a disease which has been identified as a particular disorder by the Ministry of Health, is an illness which involves an abnormal protein called amyloid that has a β sheet structure, aggregates in fibers, and is deposited on the outside of internal organs and systems, inhibiting the function of those organs and systems. Disorders among many Japanese include immunocytic amyloidosis (AL amylodosis), responsive AA amyloidsis, familial amyloid polyneuropathy (FAP), dialysis amyloidsis (DRA), and it is estimated that there are hundreds of patients throughout Japan. Amyloidosis can be largely divided into two groups: amylids that are deposited in various organs throughout the body [systemic amyloidsis] such as the disorders listed above, and [localized amyloidosis] which amyloids are deposited in a particular organ such as the brain in the case of Alzheimer's disease.

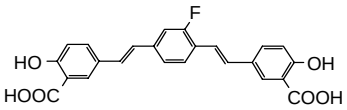
1-Bromo-2,5-bis(3-carboxy-4-hydroxystyryl)benzene (BSB) has been used for detecting amyloids because of its high affinity with amyloid β

III-6. Staining: β-Amyloid

peptide (A β), the amyloid associated with Alzheimer's disease. Skovronsky confirmed that the dye accumulates in senile plaque of brain tissue of transgenic mice Tg2576 which express the amyloid precursor protein of A β (APP) 18 hours after the intravenous injection of BSB.[1)] Not limited to A β, Ando and others have announced that amyloid deposits in various systemic amyloidosis (AA, AL, ATTR, Ascr, A β 2M) are stained more sensitively with BSB than Congo red, which is a common dye used for β sheet staining. BSB has twice the fluoresence strength as Congo red. In addition, BSB is not only a staining dye, it is also able to block the amyloid formation FAP's amyloid precursor TTR.

Newly developed FSB is also utilized for highly sensitive amyloid staining. This is made possible by the bromine in BSB changing to fluorine, and recovered from the fluorescence quenching caused by the heavy electron effect of bromine. From the results of the stains of Alzheimer patients' brain tissue (Fig. 3-36) and the heart tissue of AL amyloidosis patients (Fig 3-37), FSB detects amyloid deposits more

Chemical Structure



Protocol

1. Add 50% EtOH to the product and dilute to concentration of 0.01~0.0001% FSB solution.
2. Soak a slice in FSB stain for 30 minutes. After soaking the slice in saturated lithium carbonate, wash with 50% EtOH
3. Detect stained area under UV light (V excitation)

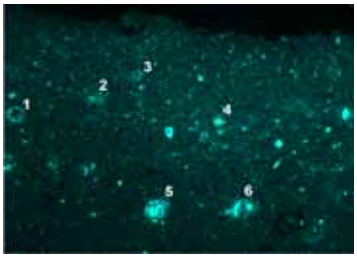


Fig. 3-36 Tissue staining with FSB
A stained image of a segment of the frontal cortex of a patient with Alzheimer's disease. The tissue was fixed with ethanol. The illuminating portions are amyloids. The numbers in the sub-adjacent slice figures correspond to each senile plaque. (Image was courteously provided by Dr. Higuchi, Dr. Saido,Laboratory for proteolytic Neuroscience, RIKEN Brain Science Institute.)

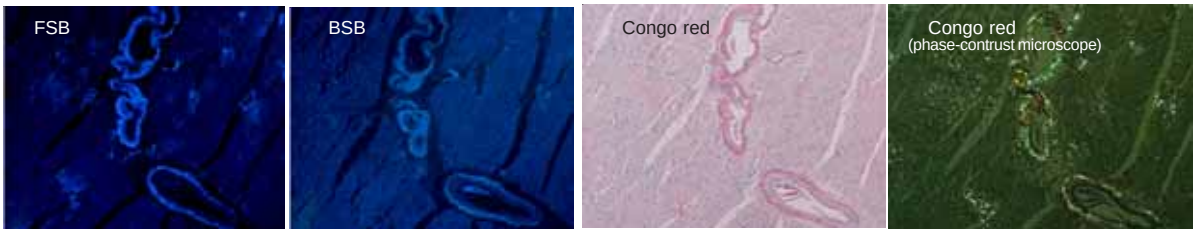


Fig. 3-37 Tissue staining with FSB, BSA, and Congo red
A Slice of heart tissue of a patient with AL amyloidosis (Congo red is auburn colored, the luminated portions of BSB and FSB are amyloids). These are sub-adjacent slices. It is possible to examine finer portions by FSB, and the contrast with the amyloid deposit portions are clear. (Image was courteously provided by Dr. Andoh: Department of Laboratory Medicine, Kumamoto University School of Medicine.)

References

D.M.Skovronsky, B. Zhawng, M.-P.Kung, J.Q. Trojanowski, V.M.-Y.Lee, *Proc. Natl. Acad. Soc.*, **97**, 7609 (2000); Y. Ando, *Dojindo News*, **104**, 1 (2002); Y. Ando, Y. Tanoue, K. Haraoka, K. Ishikawa, S. Katsuragi, M. Nakamura, X. Sun, K. Nakagawa, K. Sasamoto, K. Takesako, T. Ishizaki, K. Doh-ura, *Lab. Invest.*, **83**, 1751 (2003); Higuchi, M., et al, Nat. Neurosci., **8**, 527 (2005); Sato, K., et al., Eur. J. Med. Chem., **39**, 573 (2004).

IV. ACE Assay

ACE Kit-WST

Application: ACE activity detection. Screening of ACE inhibitors

Features: Colorimetric microplate assay
Simple protocol
No organic solvent required
High reproducibility

Ordering Information

Product code	Unit*
A502-10	100 tests

* One test corresponds to one well on a 96-well plate. 100 tests kit contains 2 sets of 50 tests kits.

Contents of the Kit:

Enzyme A ..... 2 tubes	Enzyme B .....2 tubes
Enzyme C ..... 2 tubes	Coenzyme .....2 tubes
Substrate buffer ..... 1 ml x 2	Indicator solution ..... 5 ml x 2

Storage Condition	Shipping Condition
0-5 °C	ambient temperature

Required Equipment and Materials

plate reader with 450 nm filter, 96-well culture plate, 10 µl, 100-200 µl and multi-channel pipettes, 37 °C incubator

Product Description

This kit is used for the determination of ACE (angiotensin-converting enzyme) inhibition activity. ACE works in the Renin-Angiotensin system, which is one of the mechanisms of blood pressure control, to convert Angiotensin I to the vasopressor Angiotensin II. This enzyme also contributes to elevated blood pressure due to the activity of breaking down the antihypertensive peptide Bradykinin. In recent years, food and supplements containing ingredients that have ACE blocking activity have received attention for their use in preventing high blood pressure.The conventional method of measuring ACE inhibition employs the synthetic substrate Hippuryl-His-Leu. Hippuric acid from the synthetic substrate is extracted with ethyl acetate, condensed, redissolved, and then read at an absorbance of 228 nm. This method is cumbersome and measurement is subjected to error due to residual ethyl acetate. ACE inhibition Assay Kit enzymatically detects 3-Hydroxybutyric acid (3HB) which is made from 3-Hydryoxybutyryl-Gly-Gly-Gly (3HB-GGG). Using a 96-well format, it is possible to test multiple samples at one time. In addition, there is no need to use harmful organic solvents, resulting in a safe, simple, and highly reproducible assay.

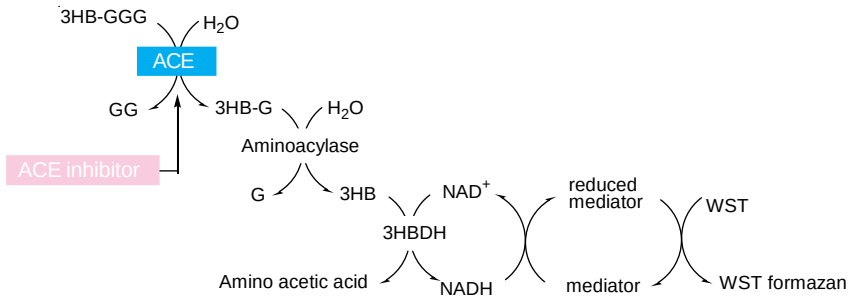


Fig. 4-1 Principle of the assay system to determine ACE activity or inhibition activity.

Preparation of solutions

Enzyme Working Solution

Add 2 ml of purified water to Enzyme B bottle to prepare an Enzyme B solution.[a)] Then, add 1.2 ml of Enzyme B solution to the Enzyme A bottle to prepare a working solution.[b)]

a) *Enzyme A and B are freeze-dried and closed with a rubber cap under vacuum pressure. The contents may fly out of the container when the rubber cap is removed. Add purified water or Enzyme B solutions using a syringe, and then open the bottle after dissolving the contents.*

b) Use with in several hours after making a solution. 1.2 ml of Enzyme working solution is sufficient for 50 tests.

Indicator working solution

Add 3 ml of purified water to each Enzyme C and Coenzyme bottle respectively and dissolve.[c)] Then, add 2.8 ml each of Enzyme C and Coenzyme to the Indicator solution to prepare an Indicator working solution.[d)]

c) Enzyme C and Coenzyme are freeze-dried and closed with a rubber cap under vacuum pressure. The contents may fly out of the container when the rubber cap is removed. Add pure water or Enzyme B solutions using a syringe, and then open the bottle after dissolving the contents.

IV. ACE Assay

d) Use with in several hours after making a solution. 11.2 ml of Indicator working solution is sufficient for 50 tests.

Sample solution

Dilute sample solution with purified water.

example: dilution ratio: 1(no dilution), 1/5, 1/25, 1/625, 1/3125, 1/15625, 1/78125

Table 4-1 Solution and buffer volumes in each well

	sample	blank 1	blank 2
Sample solution	20 µl	-	-
Purified water	-	20 µl	40 µl
Substrate buffer	20 µl	20 µl	20 µl
Enzyme working solution	20 µl	20 µl	-
Indicator working solution	200 µl	200 µl	200 µl

Assay Protocol

1. Add 20 µl of the sample solution (sample) or purified water (blank 1, blank 2) to each well.
2. Add 20 µl of the Substrate buffer to each well.
3. Add 20 µl of purified water to the blank 2 well.
4) Add 20 µl of the Enzyme working solution to the wells containing Sample solution and blank 1.

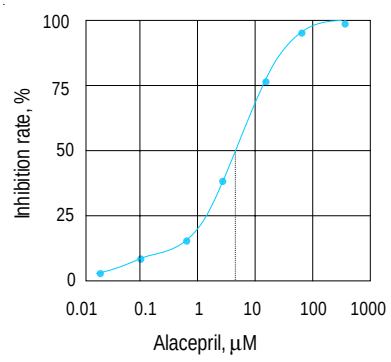
**Hydroxybutyric acid (3HB) is produced immediately upon addition of Enzyme working solution. In order to reduce time lag from well to well, please use a multichannel pippette.*

5. Incubate the plate at 37 °C for 60 min.
6. Add 200 µl Indicator working solution to each well.

**3-Hydroxyburyric acid (3HB) is produced immediately upon addition of Indicator working solution. In order to reduce time lag from well to well, please use a multichannel pippette.*

7. Incubate for 10 min at room temperature.
8. Measure the absorbance of each well at 450 nm.
9. Use the following equation to calculate the ACE inhibition activity (percent inhibition).

ACE inhibition (percent inhibition) = $[(A_{blank\,1}-A_{sample}) / (A_{blank\,1}-A_{blank\,2})] \times 100$



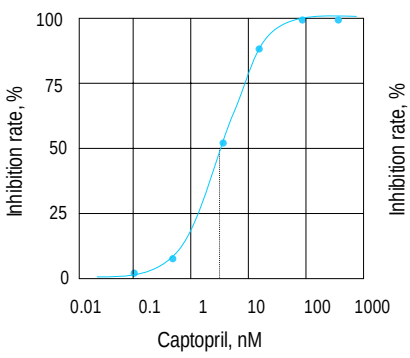


Fig. 4-2. Inhibition curves prepared by Alacepril and Captopril. IC_{50} of Alacepril and Captopril are 3.62 µM and 2.14 nM, respectively. Both compounds are ACE inhibitors.

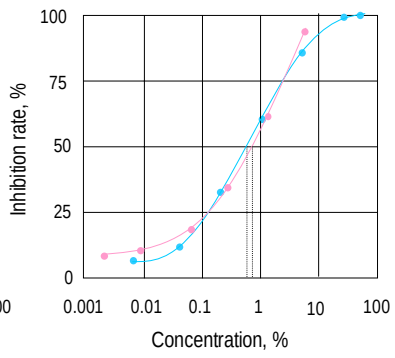


Fig. 4-3. Inhibition curves prepared by two beverages containing a valyl-tyrosine (●) or lacto tripeptide (●). IC_{50} of these beverages are 0.56% and 0.69%,* respectively. It is known that these substances have antihypertensive effects.
** Concentration of the beverage in the sample solution.*

V. Oxidative Stress

Introduction

Oxygen is a very important molecule for the synthesis of biologically active materials such as hormones and ATP. The acquisition of the ability to utilize oxygen was a significant driving force for the evolution of life. Oxygen activates various enzymes in cells, and activated oxygen species are involved in the operation of cell functions. Though oxygen itself is an essential element of life, molecules in cells, such as DNA and proteins, are sometimes damaged by reactive oxygen species (ROS) in so-called oxidative stress. Oxidative stress in cells is caused by ROS created by metabolism, ionizing radiation, and carcinogenic compounds that directly interact with DNA. During metabolism, a small portion of oxygen is converted to superoxide anion by one electron reduction; superoxide anion is then converted to oxygen and hydrogen peroxide by superoxide dismutase (SOD). Hydrogen peroxide is reduced to water by catalase or glutathione peroxidase. However, if hydrogen peroxide is not completely reduced by these enzymes, it can generate an extremely reactive hydroxy radical when oxidized by iron (Fenton reaction). Hydroxy radical is also generated by UV irradiation or directly from water by ionizing radiation. Hydroxy radical reacts with lipid to generate lipid peroxide. However, not all ROS are unwanted. Hypochlorite ion, an ROS derived from hydrogen peroxide by myeloperoxidase in neutrophils, has germicidal activity. Nitric oxide, also known as endothelial-derived relaxation factor, is generated by NO synthetase. However, NO and superoxide anion may react to generate peroxynitrite, which is cytotoxic.

The ROS and reactive nitrogen compounds have many different activities in biological systems. In response, aerobic organisms created defense mechanisms to avoid oxidative stress. Oxidative stress has recently become the focus of many studies conducted for the understanding of defense mechanisms and relationships between oxidative damage and disease or aging processes. To this end, many assay methods have been developed for the detection of ROS-related or ROS-derived substances such as superoxide anion, superoxide dismutase, glutathione, glutathione reductase, glutathione peroxidase, DNA lesions, 8-oxoguanine, 8-nitroguanosine, and protein carbonyl.

DNA Damage by Oxidative Stress

Oxidative damage to DNA is a result of the interaction of DNA with ROS, in particular, hydroxy radicals. Hydroxy radicals, which are produced from superoxide anion and hydrogen peroxide by the Fenton reaction, cause multiple modifications in DNA. Oxidative attacks by hydroxy radicals on the deoxyribose moiety lead to the release of free bases from DNA, generating strand breaks with various sugar modifications and simple abasic sites. In fact, these are one of the major types of DNA damage generated by ROS. Measurement of these modifications is important for understanding the mechanisms of oxidative DNA damage and its biological repercussions. Most of the time, the DNA repair system eliminates such damage by severing the disease process and maintain the integrity of the form of life. However, clinical data clearly indicates that oxidative DNA damage is related to a number of disease processes such as carcinogenesis and neurodegenerative diseases.

DNA Damage Quantification Kit is utilized for the determination of the number of simple abasic sites (AP sites) in genomic DNA isolated from cell and tissue samples. The DNA damage level can be monitored in the range of 1 to 40 AP sites per 10^5 base pairs. Therefore, DNA Damage Quantification Kit is useful for the detection of carcinogen toxicity and for the detection of DNA repair activities.

8-Nitroguanosine, another oxidatively modified molecule, is a nitrated base of DNA or RNA. It is known that a large amount of nitric oxide and superoxide molecules, generated by inflammation, can cause nitration of guanosine. Since chemically-modified nucleotides cause mutation during DNA replication, 8-nitroguanosine is thought to be a marker of DNA damage related to mutation and cancer. 8-Nitroguanosine is involved in controlling cell functions and is related to oxidizing and reducing reactions. 8-Nitroguanosine antibodies and assay kits are available from Dojindo.

Oxidative Stress Related Components

Superoxide dismutase (SOD) is one of the most important antioxidative enzymes for scavenging toxic superoxides *in vivo*. SOD catalyzes the dismutation of the superoxide anion into molecular oxygen and hydrogen peroxide, which are much less oxidatively reactive. SOD is a potential therapeutic agent in diseases related to oxidative stress, and as a material for moderating the aging process, because of its high antioxidant efficiency (much higher than that of glutathione or other reducing agents) and early action in the initial stages of ROS metabolism. Since glutathione also has superoxide-scavenging activity, it contributes to a reductive atmosphere in cells and tissues to avoid oxidative damage.

SOD Assay Kit-WST is for the determination of superoxide quenching ability, based on an inhibition assay, and is suitable for evaluating SOD or SOD-like activities in samples. Cytochrome C is a commonly utilized agent for SOD activity detection. However, its reactivity with superoxide anion is too high to determine low SOD levels. Since the reactivity of WST-1 with superoxide anion is much lower than that of cytochrome C, low levels of SOD activity can be determined.

Total Glutathione Quantification Kit is for measuring the amount of glutathione in samples. The combination of DTNB (Ellman's Reagent) and glutathione reductase in this kit enables highly sensitive detection of total glutathione. DTNB generates an intense yellow dye upon reaction with the reduced form of glutathione. The assay range of this kit is from 1 μM to 100 μM glutathione.

Nitric Oxides

Nitric oxide (NO) has been identified as an endothelial-derived relaxation factor and antiplatelet substance. It serves as a neurotransmitter when derived from a neutrophil, and as a cytotoxic substance when derived from an activated macrophage. NO reacts with superoxide anion to generate highly toxic peroxynitrite. The reaction rate of NO with superoxide is three times that of SOD. In some cases, NO also activates cyclooxygenase. The most important role of NO is thought to be the activation of guanylate cyclase. Recently, there have been many contradictory reports published in NO research. These contradicting results are due to NO's unique chemical properties. Since NO is a free radical, it is very reactive and unstable. NO changes its form in a complex manner immediately after appearing in a biological environment. Each of NO's metabolites might have different bioactivities from NO itself. For this reason, it is vital to separately investigate each function of the NO-related metabolites.

V. Oxidative Stress

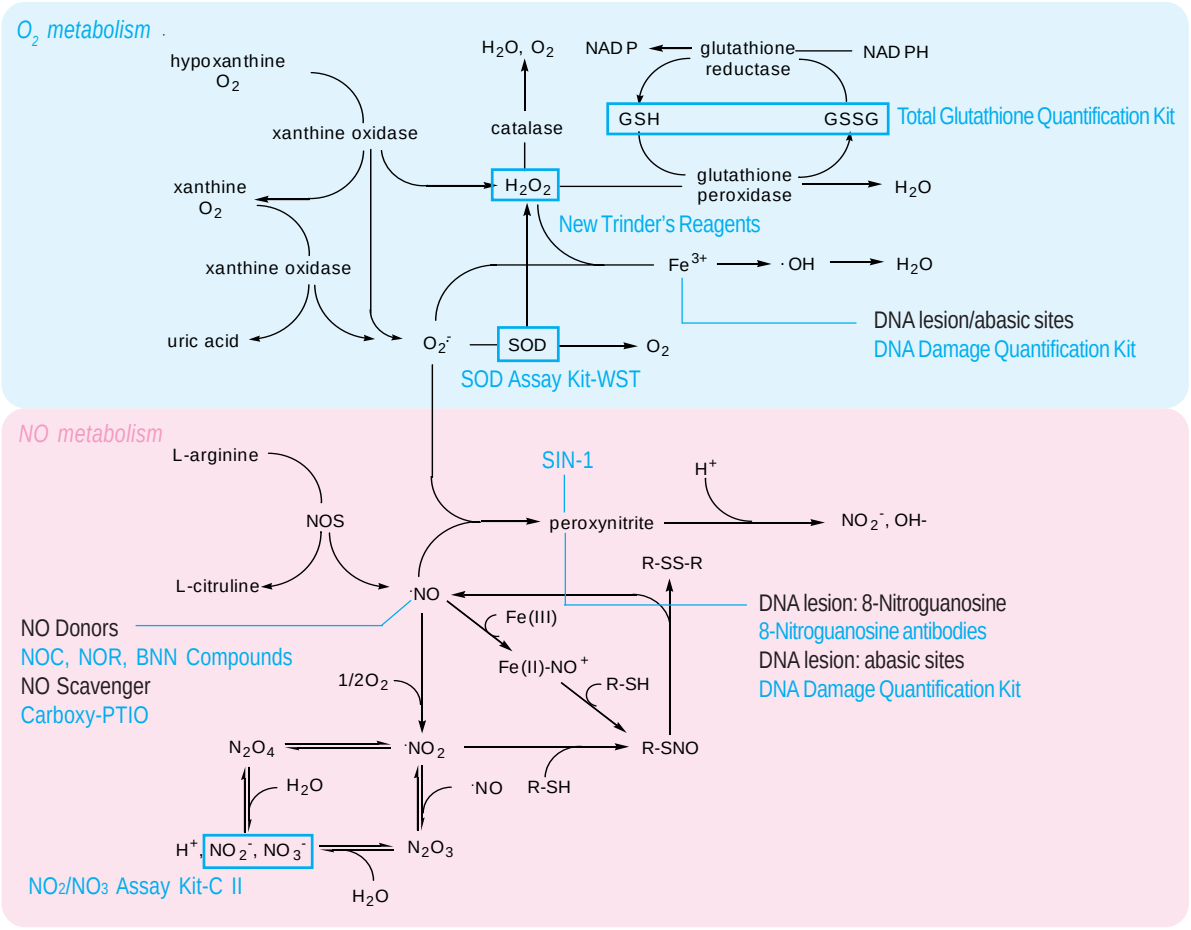


Fig. 5-1 O_2 and NO Metabolism Chart

V-1. Oxidative Stress: DNA Damage

DNA Damage Quantification Kit-AP Site Counting-

Application: Abasic site quantification in genomic DNA

Features: Determine the number of abasic sites in genomic DNA samples.
Colorimetric microplate assay.
Detection range: 1-40 abasic sites per 1x10^5 base pairs DNA.

Ordering Information

Product code	Unit
DK02-10	5 samples
DK02-12	20 samples

Contents of the Kit: 5 samples

ARP solution	100 µl x 1 tube	DNA binding solution	10 ml x 1 bottle
ARP-DNA standard soln.	250 µl each	Substrate solution	10 ml x 1 bottle
Filtration tube	5 tubes	TE buffer	15 ml x 1 bottle
Washing buffer	1 packet	HRP-streptavidin	25 µl x 1 tube
96-well Microplate	1 plate		

Contents of the Kit: 20 samples

ARP solution	250 µl x 1 tube	DNA binding solution	10 ml x 1 bottle
ARP-DNA standard soln.	250 µl each	Substrate solution	10 ml x 1 bottle
Filtration tube	20 tubes	TE buffer	15 ml x 2 bottles
Washing buffer	1 packet	HRP-streptavidin	25 µl x 1 tubes
96-well Microplate	1 plate		

Storage Condition
0-5 °C, protect from light

Shipping Condition
ambient temperature or with blue ice

Required Equipment and Materials
microplate reader with 650 nm filter, incubator, microcentrifuge, 10 µl and 200 µl adjustable pipettes, multi-channel pipette

Product Description
Oxidative damage to DNA is a result of its interaction with reactive oxygen species (ROS), in particular, the hydroxy radical. Hydroxy radicals, which are produced from superoxide anion and hydrogen peroxide by the Fenton reaction, produce multiple modifications in DNA. Oxidative attacks by hydroxy radicals on the deoxyribose moiety will lead to the release of free bases from DNA, generating strand breaks with various sugar modifications and simple abasic sites (AP sites). In fact, AP sites are one of the major types of damage generated by ROS. Aldehyde Reactive Probe (ARP; *N'*-aminooxymethylcarbonylhydrazin-D-biotin) reacts specifically with an aldehyde group present on the open ring form of the AP sites (Fig. 5-2). This reaction makes it possible to detect DNA modifications that result in the formation of an aldehyde group. After treatment with excess ARP reagent, all of the AP sites on DNA are tagged with a biotin residue. These biotin-tagged AP sites can be quantified using the avidin-biotin assay, followed by colorimetric detection with either peroxidase or alkaline phosphatase conjugated to the avidin. DNA Damage Quantification Kit contains all the necessary solutions for detecting between 1 to 40 AP sites per 1 x 10^5 base pairs.

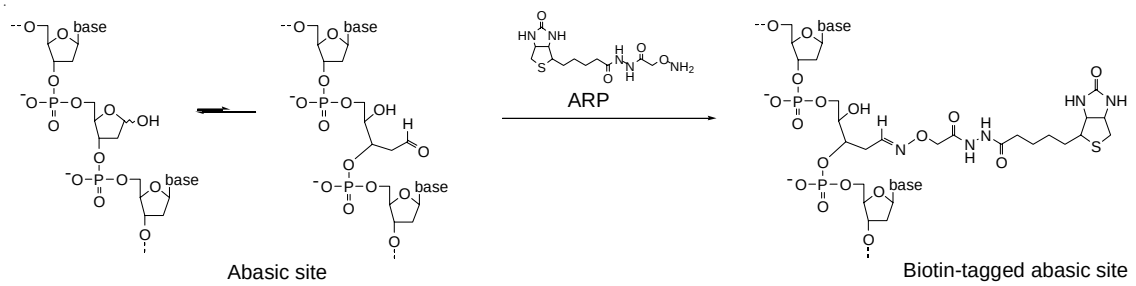
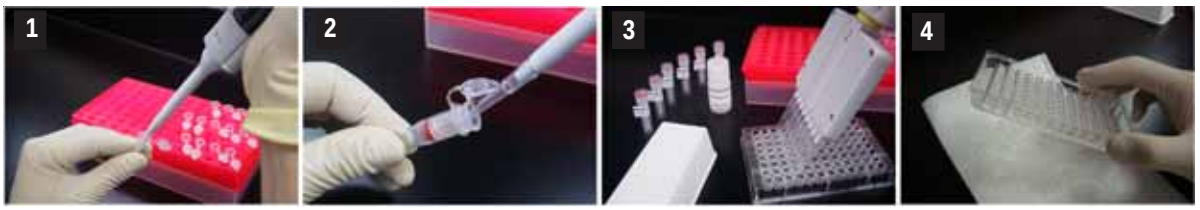


Fig. 5-2 Mechanism of ARP Tagging at an Abasic Site

V-1. Oxidative Stress: DNA Damage

Assay Procedure



Add ARP solution to a sample DNA solution. Incubate at 37 °C for 1 hour.

Transfer the ARP reaction mixture to a Filtration tube, and spin the tube to purify ARP-labeled DNA.

Add the ARP-DNA standard solution or ARP-labeled sample DNA solution to each well. Add DNA binding solution, and leave the plate at room temperature overnight.

Discard the solutions and wash wells with Washing buffer. Tap the plate on a paper towel several times to remove as much of buffer as possible.

Add HRP-streptavidin solution to each well and incubate at 37 °C for 1 hour.

Discard the solutions and wash well with Washing buffer. Tap the plate on a paper towel to remove as much of buffer as possible.

Add Substrate solution to each well and incubate at 37 °C for 1 hour.

Read the O.D. at 650 nm.

ARP Reaction (Preparation of ARP-labeled DNA)

1. Mix 10 µl of purified genomic DNA solution (100 µg per ml) and 10 µl ARP solution in a 0.5 ml tube, and incubate at 37 °C for 1 hour.
2. Wash the inside of Filtration tube cup with 100 µl TE.
3. Add 380 µl TE to the ARP-labeled DNA solution, and transfer the solution to Filtration tube.[a)]
4. Centrifuge Filtration tube at 5,000 g for 15 min, and discard the filtrate solution.
5. Add 400 µl TE to Filtration tube, and re-suspend the DNA on the filter with pipetting.
6. Centrifuge Filtration tube at 5,000 g for 15 min.[b)]
7. Add 200 µl TE to Filtration tube to re-suspend the DNA on the filter with pipetting.
8. Transfer the ARP-labeled DNA solution to a 1.5 ml tube, and again add 200 µl of TE to Filtration tube to completely transfer the ARP-labeled DNA from the filter to the 1.5 ml tube.[c)]
9. Store the ARP-labeled DNA solution at 0-5 °C.

a) Ethanol precipitation can be used in place of a Filtration tube to purify the ARP-labeled DNA. After ethanol precipitation, dissolve the DNA pellet in 100 µl TE, and determine the DNA concentration.
b) If the DNA solution still remains on the filter after centrifuging, centrifuge for another 5 min, and then proceed to step 7.
c) The recovery rate of DNA using a Filtration tube is 90%, so the approximate concentration of ARP-labeled DNA is 2.25 µg per ml. For a more accurate determination of the number of abasic sites in sample DNA, we recommend measuring the actual DNA concentration.

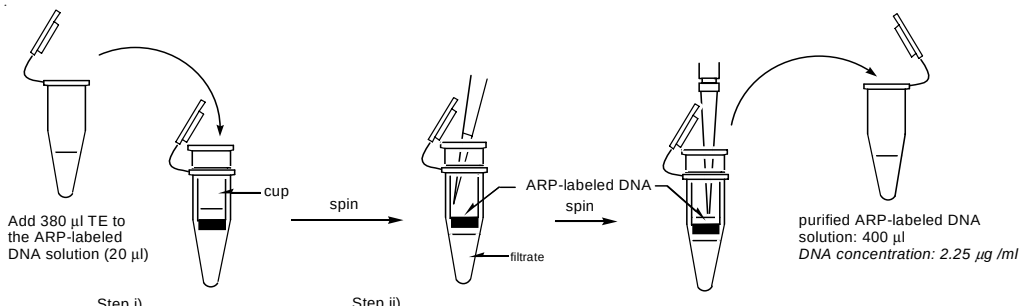


Fig. 5-3 Isolation Process of ARP-labeled genomic DNA

V-1. Oxidative Stress: DNA Damage

Determination of the Number of Abasic Sites in DNA

Day 1:

1. Dilute 90 µl of the ARP-labeled genomic DNA with 310 µl TE.
2. Add 60 µl of ARP-DNA standard solution per well. Use 3 wells per each concentration of the ARP-DNA standard solution.
3. Add 60 µl of the diluted ARP-labeled genomic DNA solution per well. Use 3 wells per sample.
4. Add 100 µl DNA Binding solution to each well and mix. Leave the plate at room temperature overnight.

Day 2:

5. Preparation of Solutions

   Washing buffer solution: Dissolve the contents of the Washing buffer packet in 1 L of deionized or distilled water. Store this Washing Buffer solution at room temperature.

   HRP-Streptavidin solution: Dilute HRP-streptavidin with Washing buffer solution to prepare HRP-streptavidin working solution.
6. Discard the DNA binding solution from all the wells and wash the wells five times with 250 µl of Washing buffer solution. After discarding theWashing buffer solution, invert the plate and tap it on a paper towel several times to completely remove the solution.
7. Add 150 µl of diluted HRP-Streptavidin working solution to each well and incubate the plate at 37 °C for 1 hour.
8. Discard the solution in all wells, and wash the well five times with 250 µl of Washing buffer solution.
9. Add 100 µl Substrate solution to each well and incubate at 37 °C for 1 hour.
10. Measure the O.D. at 650 nm, and prepare a calibration curve using the data obtained from the ARP-DNA standard solution wells.
11. Determine the number of abasic sites in the genomic DNA using the calibration curve.

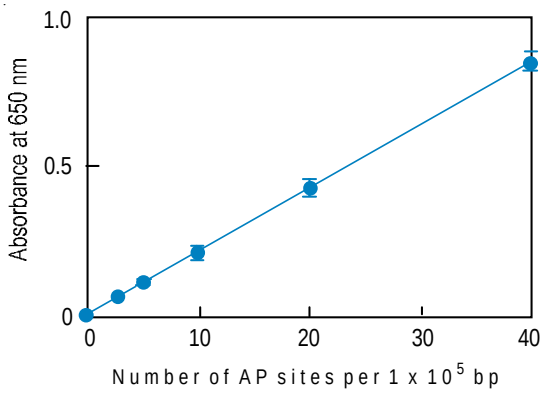


Fig. 5-4 Typical calibration curve of DNA Damage Quantification Kit

How to Prepare a Calibration Curve

1. Calculate the average O.D. of each ARP-DNA standard solution.
2. Subtract the blank O.D. from the average O.D.[a)]
3. Plot the O.D. corresponding to the number of AP sites of the Standard Solution. X-axis is the number of AP sites and Y-axis is the O.D.
4. Determine the number of AP sites in the sample using this calibration curve.

a) The blank O.D. is about 0.04-0.06 and the O.D. of the 40 ARP-DNA Standard Solution is about 0.8-1.0. The O.D. value depends on HRP-Streptavidin activity.

FAQ

◆ **Can I use single-stranded DNA or RNA?**
No, you can not use this kit to determine the number of abasic sites in single-stranded DNA or RNA. The O.D. reading of single-stranded DNA will be nearly twice that of double-stranded DNA because of the binding efficiency on the microplate.

◆ **How should genomic DNA be stored?**
Prepare a DNA pellet and store at -20 °C or -80 °C if the DNA cannot be labeled with ARP immediately after isolation. After ARP labeling, the sample can be stored at 4 °C in TE Buffer for several months.

◆ **How should I prepare the DNA?**
You can use general protocols or commercially available DNA isolation kits. Between 2 to 4 abasic sites per 1 x 10^5 base pairs will be created during DNA isolation process. Therefore, use the same isolation method to prepare each DNA sample.

◆ **How can I determine the number of abasic sites if there are more than 40 per 1 x 10^5 base pairs?**
Simply dilute the ARP-labeled sample DNA with 0.5 µg per ml double-stranded genomic DNA, such as calf thymus or salmon sperm DNA, using TE Buffer.

◆ **What should I do if the sample DNA concentration is less than 100 µg per ml?**
You can either use a Filtration tube to concentrate your sample DNA or ethanol precipitation to recover DNA as a pellet and then re-dissolve it to prepare a 100 µg per ml solution.

◆ **What should I do if the sample DNA is less than 1 µg?**
Add the same volume of ARP solution and follow the manual. The recovery of the ARP-labeled DNA may be lower than the usual reactions, so measure the ARP-labeled DNA solution. The average recovery rate of the 0.5 µg DNA and 0.25 µg DNA is 70% and 50%, respectively.

References

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SOD Assay Kit-WST

Application: SOD or SOD-like activity detection

Features: WST-1 based SOD inhibition assay
Colorimetric microplate measurement
Measures 100% inhibition by SOD
pH-independent IC_{50} determination
Low background noise measurement

Ordering Information

Product code	Unit*
S311-08	100 tests
S311-10	500 tests

* One test corresponds to one well of the 96-well plate.

Contents of the Kit: 100 tests

WST solution ..... 1 ml, 1 tube
Buffer solution..... 11 ml, 2 bottles
Enzyme solution .....20 µl, 1 tube
Dilution buffer.....10 ml,1 bottle

Contents of the Kit: 500 tests

WST solution ..... 5 ml, 1 bottle
Buffer solution..... 100 ml, 1 bottle
Enzyme solution .....100 µl, 1 vial
Dilution buffer.....50 ml,1 bottle

Storage Condition
0-5 °C, protect from light

Shipping Condition
ambient temperature or with blue ice

Required Equipment and Materials
microplate reader with 450 nm filter, 37 °C incubator, 96-well clear plate, 10 µl and 200 µl adjustable pipettes, multi-channel pipette

Product Description
Superoxide dismutase (SOD), which catalyzes the dismutation of the superoxide anion ($O_2^{\cdot -}$) into hydrogen peroxide and molecular oxygen, is one of the most important antioxidative enzymes. In mammals, cytosolic SOD has a greenish color and consists of two subunits: One subunit contains copper and the other zinc (Cu/Zn-SOD). Mitochondrial and bacterial SOD has a reddish-purple color and contains manganese (Mn-SOD). *E. coli* has Mn-SOD and Fe-SOD. Several direct and indirect methods have been developed to determine SOD activity. An indirect method using nitrotetrazolium blue is often used because of its convenience. However, there are several disadvantages to this method, such as poor water solubility of the formazan dye and its reaction with the reduced form of xanthine oxidase. Though cytochrome C is also commonly used for SOD activity detection, its reactivity with superoxide is too high to determine low levels of SOD activity. SOD Assay Kit-WST allows a very convenient and highly sensitive SOD assay by utilizing Dojindo's highly water-soluble tetrazolium salt, WST-1 (2-(4-iodophenyl)- 3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-2*H*-tetrazolium, monosodium salt), which produces a water-soluble formazan dye upon reduction with a superoxide anion (Fig. 5-5). The absorption spectrum is shown in Fig. 4-6. WST-1 is 70 times less reactive with superoxide anion than cytochrome C; therefore, highly sensitive SOD detection is possible and samples can be diluted with buffer to minimize background problems. WST-1 does not react with the reduced form of xanthine oxidase; therefore, even 100% inhibition with SOD is detectable. The rate of WST-1 reduction by superoxide anion is linearly related to the xanthine oxidase activity, and is inhibited by SOD (see figure below). Therefore, the IC_{50} (50% inhibition concentration) of SOD or SOD-like materials can be determined using colorimetric methods (patent filing).

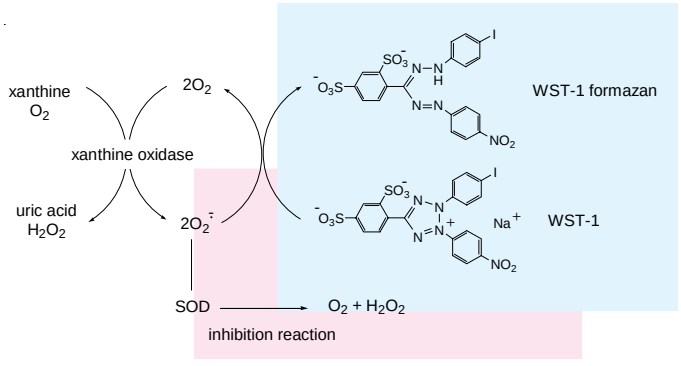


Fig. 5-5 SOD inhibition assay mechanism

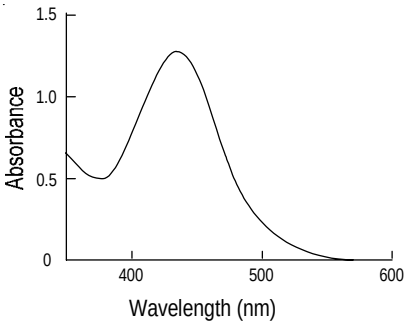


Fig. 5-6 Absorption spectrum of WST-1 formazan

V-2. Oxidative Stress: SOD

Preparation of Working Solutions

WST working solution:
Dilute 1 ml WST soln with 19 ml Buffer soln.

Enzyme working solution:
Dilute 15 µl Enzyme soln with 2.5 ml Dilution soln.

SOD solution (if necessary):
Dilute SOD with Dilution buffer to prepare the following SOD standard solutions:
200 U/ml, 100 U/ml, 50 U/ml, 20 U/ml, 10 U/ml, 5 U/ml, 1 U/ml, 0.1 U/ml, 0.05 U/ml, 0.01 U/ml, 0.001 U/ml

Table 5-1. Solution and Buffer Volumes in Each Well

Solution	Sample	Blank 1	Blank 2	Blank 3
sample solution	20 µl	0 µl	20 µl	0 µl
ddH_2O	0 µl	20 µl	0 µl	20 µl
WST working soln	200 µl	200 µl	200 µl	200 µl
Enzyme working soln	20 µl	20 µl	0 µl	0 µl
Dilution buffer	0 µl	0 µl	20 µl	20 µl

Assay Procedure

1. Add 20 µl sample solution or H_2O to each well
2. Add WST working soln, Dilution buffer, and Enzyme working soln to each well as indicated in Table 1.[a)]
3. Incubate the plate at 37 °C for 20 min.[b)]
4. Read O.D. at 450 nm.

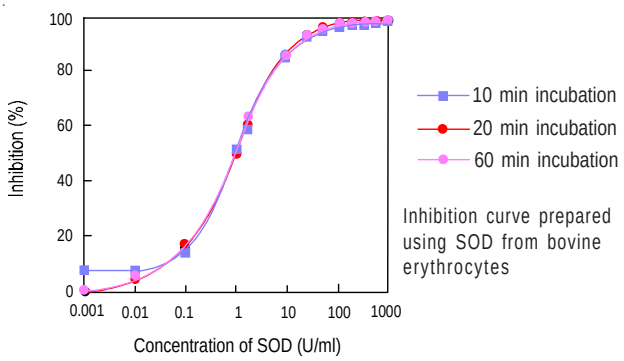
a) After the addition of Enzyme working solution, the mixed solution generates superoxide. Use a multi-channel pipette to add the Enzyme working solution to minimize the reaction time lag.

b) If the microplate reader has a temperature control function, incubate the plate on the microplate holder at 37 °C.

Inhibition Rate Calculation

$$\text{Inhibition rate (\%)} = \frac{(A_{blank\ 1} - A_{blank\ 3}) - (A_{sample} - A_{blank\ 2})}{A_{blank\ 1} - A_{blank\ 3}} \times 100$$

$A_{blank\ 1}$: absorbance of blank 1
$A_{blank\ 2}$: absorbance of blank 2
$A_{blank\ 3}$: absorbance of blank 3
A_{sample}: absorbance of sample well



Inhibition curve prepared using SOD from bovine erythrocytes

Fig. 5-7 Inhibition curve prepared by different data acquisition times

Preparation of Sample Solution

Cells (Adherent cells: $9x10^6$ cells, Leukocytes: $1.2 x10^7$ cells)

1. Harvest cells with a scraper, centrifuge at 2,000 g for 10 min at 4 °C, and discard the supernatant.
2. Wash the cells with 1 ml PBS and centrifuge at 2,000 g for 10 min at 4 °C. Discard the supernatant. Repeat this step.
3. Break cells using the freeze-thaw method (-20 °C for 20 min, then 37 °C bath 10 min, repeat twice).
4. Add 1 ml PBS. If necessary, sonicate the cell lysate on an ice bath (60 W with 0.5 sec interval for 15 min).
5. Centrifuge at 10,000 g for 15 min at 4 °C.
6. Remove the supernatant and dilute it with PBS to prepare sample solution.

Plant or Vegetable (200 mg)

1. Add 1 ml distilled water, and homogenize the sample using a homogenizer with beads.
2. Filter the homogenate with paper filter, and lyophilize the filtrate.
3. Measure the weight of the lyophilized sample, and dissolve with 0.1 M phosphate buffer (pH 7.4) to prepare sample solution.

Tissue (100 mg)

1. Wash the tissue with saline to remove as much blood as possible. Blot the tissue with paper towels and then measure its weight.
2. Add 400-900 µl sucrose buffer (0.25 M sucrose, 10 mM Tris, 1 mM EDTA, pH 7.4) and homogenize the sample using Teflon homogenizer. If necessary, sonicate the homogenized sample on an ice bath (60W with 0.5 second intervals for 15 min).
3. Centrifuge the homogenized sample at 10,000 g for 15 min at 4 °C, and transfer the supernatant to a new tube.
4. Dilute the supernatant with distilled water to prepare sample solution.

Tea (antioxidant activity detection)

1. Add 60 ml boiled water to 10 g of tea, and leave it for 2.5 min.
2. Filter the extract with paper filter and then filter again with a 0.45 µm membrane filter.
3. Dilute the filtrate with distilled water to prepare sample solution.

V-2. Oxidative Stress: SOD

Erythrocytes or Plasma

1. Centrifuge 2-3 ml of anticoagulant-treated blood (such as heparin 10 U/ml final concentration) at 600 g for 10 min at 4 °C.
2. Remove the supernatant and dilute it with saline to use as a plasma sample. Add saline to the pellet to prepare the same volume, and suspend the pellet.
3. Centrifuge the pellet suspension at 600 g for 10 min at 4 °C, and discard the supernatant.
4. Add the same volume of saline, and repeat Step 3 twice.
5. Suspend the pellet with 4 ml distilled water, then add 1 ml ethanol and 0.6 ml chloroform.
6. Shake the mixture vigorously with a shaker for 15 min at 4 °C.
7. Centrifuge the mixture at 600 g for 10 min at 4 °C and transfer the upper water-ethanol phase to a new tube.
8. Mix 0.1 ml of the upper phase with 0.7 ml distilled water, and dilute with 0.25% ethanol to prepare sample solution.

Extracellular SOD (EC-SOD)

1. Prepare a 0.5 ml volume of Con A-sepharose column equilibrated with PBS.
2. Apply supernatant of a tissue homogenate on the column, and leave the column for 5 min at room temperature.
3. Add total 10 ml PBS to wash the column.
4. Add 1 ml of 0.5 M α-methylmannoside/PBS, and collect the eluate. Repeat 5 times.
5. Use the eluate for the SOD assay without dilution. If the SOD activity is high enough, dilute the eluate with PBS.

Wine (antioxidant activity detection)

1. Filter wine with a 0.45 µm membrane filter.
2. Dilute the filtrate with distilled water to prepare sample solution.

FAQ

- **What is the definition of "1 Unit"?**
  One unit is defined as a point where a sample gives 50% inhibition of a colorimetric reaction between reactive dye (such as cytochrome C, WST-1, nitro-tetrazolium blue or XTT) and superoxide anion. For example, if the O.D. of a sample that does not contain any SOD is 1.0, another sample that gives 0.5 O.D. is defined as having 1 unit of SOD activity. You can use this unit to determine the SOD activity of your sample. Therefore, SOD activities determined using different dyes or methods are not comparable with each other.
- **Can I use standard SOD to determine SOD activity in sample solutions?**
  Yes, you can. Prepare a inhibition curve (typical inhibition curve, and determine SOD activity in the sample solution. SOD bovine erythrocytes (CAS# 9054-89-1, EC 1.15.1.1) can be purchased from Sigma (catalog# S7571).
- **Can I use a kinetic method to determine SOD activity?**
  Yes, you can use a kinetic method for SOD assay. Since the rate of the color development remains the same for up to 20 minutes, measure the slope for 5 minutes during this linear phase.
- **The sample has color. Can I still use this sample?**
  Yes, you can still use it for SOD assay. Diluting the sample will minimize the interference. Subtract the O.D. of blank 2 from the O.D. of the sample to cancel out the background color. However, if the SOD activity in the sample is low, it may not be measurable.
- **How do I prepare more Dilution buffer?**
  Dilution buffer is PBS. Please prepare the Dilution buffer with following concentrations; 137 mM NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4.
- **Can I determine Mn-SOD and Cu/Zn-SOD independently using this kit?**
  Yes. In order to measure Mn-SOD activity, blocking the Cu/Zn-SOD activity using potassium cyanide is necessary (KCN). Adding 1 mM KCN to samples can block Cu/Zn-SOD activity completely. To measure Cu/Zn-SOD activity, measure the total SOD activity with and without KCN, and then subtract the Mn-SOD activity from total SOD activity.
- **How long can I store the sample?**
  A sample stored in a freezer at -80 °C is stable for 1 month.
- **Can I measure the levels of superoxide anion using this kit?**
  You could simply use WST-1, instead of this kit, to measure superoxide. However, you would need a standard to determine the amount of superoxide in sample solution. Since superoxide is not stable and reacts with various materials, it might be difficult to determine the total amount of superoxide generated in the system. The xanthine-xanthine oxidase system in this kit can be used as a standard for measuring the relative amount of superoxide production in each sample.

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Total Glutathione Quantification Kit

Application: Total glutathione detection

Features: Colorimetric microplate measurement
Highly sensitive DTNB-based recycling system
Wide detection range of 1 µg to 100 µg

Ordering Information

Product code	Unit
T419-10	100 tests

Contents of the Kit:

Enzyme solution	50 µl, 1 vial	Co-enzyme (lyophilized) .....2 vials	
Substrate.....	2 vials	Standard GSH (lyophilized) ..... 1 vial	
Buffer solution	50 ml, 1 bottle		

Storage Condition
0-5 °C, protect from light

Shipping Condition
ambient temperature or with blue ice

Required Equipment and Materials
microplate reader with 405 or 415 nm filter, 37 °C incubator, 96-well clear plate, 20 µl and 200 µl adjustable pipettes, multi-channel pipette

Product Description

Glutathione (GSH) is the most abundant thiol compound in animal, plant tissues, bacteria, and yeast. GSH has many different roles, including protection against reactive oxygen species and the maintenance of protein thiol groups. During these processes, GSH is converted into its oxidized form, glutathione disulfide (GSSG). Since GSSG is then enzymatically reduced by glutathione reductase, GSH is the dominant form in organisms. DTNB (5,5'-Dithiobis(2-nitrobenzoic acid)), known as Ellman's Reagent, was developed to detect thiol compounds. In 1985, Dr. M. E. Anderson suggested that the glutathione recycling system involving DTNB and glutathione reductase could be used as a highly sensitive glutathione detection method. DTNB and GSH react to generate 2-nitro-5-thiobenzoic acid and GSSG (Fig. 5-8). Since 2-nitro-5-thiobenzoic acid is yellow, the GSH concentration in a sample solution can be determined by O.D. measurement at 412 nm absorbance (Fig. 5-9). GSH is regenerated from GSSG by glutathione reductase, and will again react with DTNB to produce 2-nitro-5-thiobenzoic acid. This recycling reaction improves the sensitivity of total glutathione detection. Total Glutathione Quantification Kit contains all of the necessary reagents for total glutathione measurement, except for those used in sample preparation. 5-Sulfosalicylic acid is recommended for the removal of proteins from sample solutions and for the prevention of GSH oxidation and γ-glutamyl transpeptidase reactions. However, the optimum method for sample preparation differs from sample to sample, so please review the references. This kit can be used to quantify total glutathione concentrations from 1 µM to 100 µM using the standard method. For lower glutathione concentrations, such as in blood samples, longer incubation times are required.

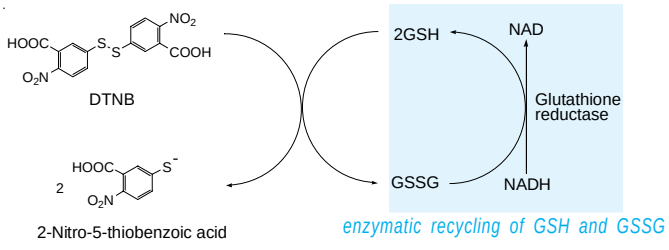


Fig. 5-8 Mechanism of total glutathione quantification

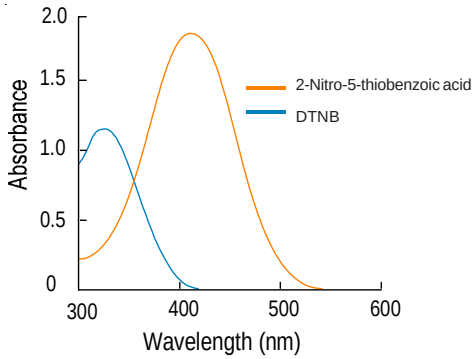
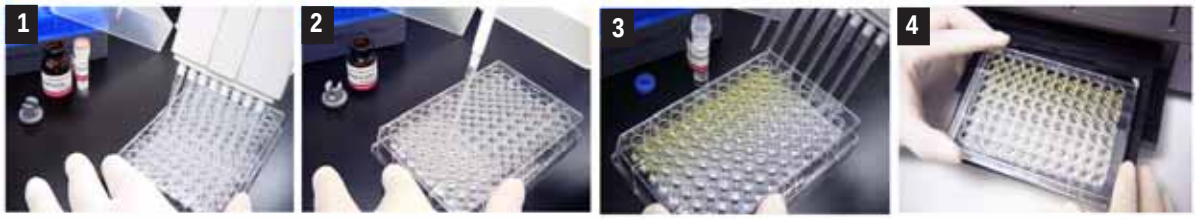


Fig. 5-9 Absorption spectrum of 2-Nitro-5-thiobenzoic acid

Assay Procedure



1 Add enzyme and co-enzyme solns to each well.

2 Add GSH standard soln or sample soln to each well and incubate at 37°C for 10 min.

3 Add substrate soln to each well and incubate at 37°C for 5-15 min.

4 Read the O.D. at 405 or 415 nm.

V-3. Oxidative Stress: GSH

Preparation of 5% 5-Sulfosalicylic Acid (SSA) Solution

Note: SSA is not included in this kit.

1. Dissolve 1 g SSA in 19 ml water.
2. Store the solution at 4°C (stable for 6 months at 4 °C).

Preparation of Sample Solution

Cells (Adhesive cells: $5x10^5$ cells; Leukocyte cells: $1x10^6$ cells)

1. Collect cells by centrifugation at 200 g for 10 min at 4 °C. Discard the supernatant.
2. Wash the cells with 300 µl PBS and centrifuge at 200 g for 10 min at 4 °C. Discard the supernatant.
3. Add 80 µl 10 mM HCl, and lyse the cells by freezing and thawing twice.
4. Add 20 µl 5% SSA and centrifuge at 8,000 g for 10 min.
5. Transfer the supernatant to a new tube, and use it for the assay. If the final concentration of SSA is over 1%, add ddH_2O to reduce the concentration of SSA from 0.5 to 1%.

Tissue (100 mg)

1. Homogenize the tissue in 0.5-1.0 ml 5% SSA.
2. Centrifuge the homogenized tissue sample at 8,000 g for 10 min.
3. Transfer the supernatant to a new tube and add ddH_2O to reduce the concentration of SSA from 0.5 to 1%. Use it for the assay.

Plasma

1. Centrifuge anticoagulant-treated blood at 1,000 g for 10 min at 4 °C.
2. Transfer the top plasma layer to a new tube and add 5% SSA equivalent to half of the volume of the plasma.
3. Centrifuge at 8,000 g for 10 min at 4 °C
4. Transfer the supernatant to a new tube, and add ddH_2O to reduce the concentration of SSA from 0.5 to 1%. Use it for the assay.

Erythrocytes

1. Centrifuge anticoagulant-treated blood at 1,000 g for 10 min at 4 °C.
2. Discard the supernatant and the white buffy layer.
3. Lyse the erythrocytes with 5% SSA equivalent to 4 times the volume of the erythrocytes.
4. Centrifuge at 8,000 g for 10 min at 4 °C.
5. Transfer the supernatant to a new tube, and add ddH_2O to reduce the concentration of SSA from 0.5 to 1%. Use it for the assay.

Erythrocytes can be isolated from the remaining sample solution after the plasma sample isolation.

Preparation of Assay Solutions

Substrate Working Solution

Add 1 ml Buffer Solution to 1 vial of Substrate, and dissolve. Substrate working solution is stable for 2 months at -20 °C.

Enzyme Working Solution

Mix Enzyme solution with pipetting before using. Take out 20 µl Enzyme solution and mix it with 4 ml Buffer solution. Enzyme working solution is stable for 2 months at 4 °C.

Coenzyme Working Solution

Add 0.7 ml ddH_2O to the Coenzyme vial and dissolve. If you don't use all of the coenzyme working solution in one day, aliquot it into microtubes and store at -20 °C. If you use all of the coenzyme working solution in one day, just add 6.3 ml Buffer solution to the vial.

The Coenzyme vial is under vacuum pressure; carefully open the cap or use a syringe to add Buffer solution.

Since the Coenzyme working solution dissolved in Buffer solution is not stable, use it in one day.

The coenzyme solution prepared with ddH_2O only is stable for 2 months at -20 °C. Dilute 10 times with Buffer solution to prepare Working solution prior to use.

GSH Standard Solutions

To prepare 200 µM GSH standard solution, add 2 ml of 0.5-1% SSA to the Standard GSH vial and dissolve. Dilute 100 µl of the 200 µM GSH standard solution with 100 µl of 0.5% SSA, and repeat using serial dilution to prepare the following GSH standard solutions:

100 µM, 50 µM, 25 µM, 12.5 µM, 6.25 µM, 3.13 µM, 1.56 µM and 0.

The Standard GSH vial is under vacuum pressure; carefully open the cap or use a syringe to add SSA. GSH powder is difficult to see. The GSH standard solutions are stable for 2 months at -20 °C.

Total Glutathione Detection - Standard Method

Detection Range: 5-100 µM

1. To each well, add 20 µl of Enzyme working solution, 140 µl of Coenzyme working solution, and 20 µl of either one of the GSH standard solutions or the sample solution.[a)]
2. Incubate the plate at 37 °C for 10 min.
3. Add 20 µl of Substrate working solution, and incubate the plate at 37 °C for 5-10 min.
4. Read the absorbance at 405 nm or 415 nm using a microplate reader.
5. Determine the concentration of GSH in the sample solution using a calibration curve[b)].

a) Adjust the concentration of SSA in the sample solution to 0.5-1% with ddH_2O before the assay. High concentrations of SSA (>1 %) interfere with the assay.

b) Since the colorimetric reaction is stable and the O.D. increases linearly over 30 min, GSH concentration can be determined by kinetic or pseudo-endpoint (no stopping reaction, quick measurement of O.D. at certain time periods between 5 and 10 min) methods.

Total Glutathione Detection - High Sensitivity Method

Detection Range: 0.5-25 µM

1. To each well, add 20 µl of Enzyme working solution, 140 µl of Coenzyme working solution, and 20 µl of either one of the GSH standard solutions[a)] or the sample solution[b)].
2. Incubate the plate at 30 °C for 10 min.
3. Add 20 µl of Substrate working solution, and incubate the plate at 37 °C for 20-40 min.
4. Read the absorbance at 405 nm or 415 nm using a microplate reader.
5. Determine the concentration of GSH in the sample solution using a calibration curve.

a) Prepare 50 mM GSH standard solution, and then prepare different concentrations of GSH standard solutions by serial dilution with 0.5% SSA as follows: 25 µM, 12.5 µM, 6.25 µM, 3.13 µM, 1.56 µM, 0.78 µM, 0.39 µM and 0.

b) Adjust the concentration of SSA in the sample solution to 0.5-1% with ddH_2O before the assay. Higher concentrations of SSA (>1%) interfere with the assay.

Determination of Total Glutathione (GSH and GSSG) Concentration

Determine the total glutathione concentration in the sample solution using the following equations. Since the values obtained by these equations are the amount of total glutathione in treated sample solutions, further calculations are necessary if the actual concentration of glutathione in cells or tissues needs to be determined.

V-3. Oxidative Stress: GSH

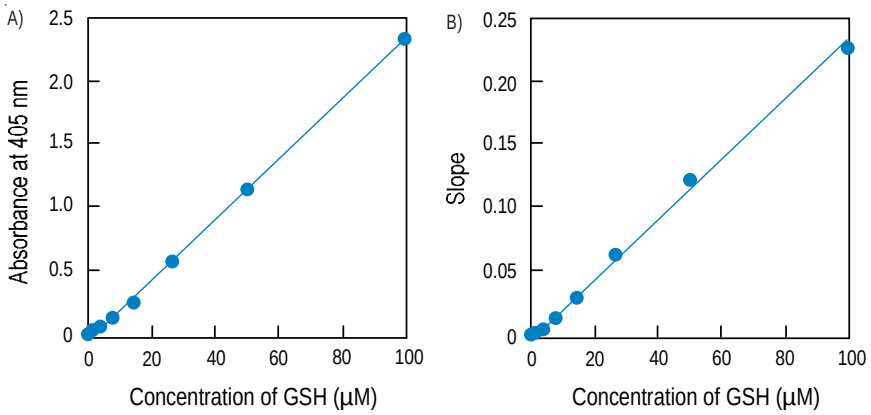


Fig. 5-10 Calibration curves prepared using pseudo-endpoint method and kinetic method
A) Calibration curve prepared using pseudo-endpoint method. 10 min incubation at room temperature.
B) Calibration curve prepared using kinetic method.

Pseudo-endpoint method:

Total glutathione = $(O.D._{sample} - O.D._{blank}) / slope^{a)}$

Kinetic method:

Total glutathione = $(Slope_{sample}{}^{b)} - Slope_{blank}{}^{b)}) / slope^{b)}$

a) The slope of the calibration curve prepared by the pseudo-endpoint or kinetic method.
b) The slope of the kinetic reaction.

FAQ

◆ Can I determine oxidized glutathione (GSSG) using this kit?

Yes. Use 2-vinylpyridine to block GSH by adding 6 µl triethanolamine and 2 mg 2-vinylpyridine to the sample solution and incubate for 30 min at room temperature. Centrifuge the solution and then use the supernatant for the assay. The detection limit of GSSG is about 1 µM in sample solution. Though 2-vinylpyridine is not easily dissolved in the buffer, it can block GSH activity completely and does not interfere with GSSG detection. For accurate determination of GSSG, use GSSG standard solution instead of GSH standard solution. The standard GSSG solution shoulded treated with 2-vinylpyridine as well. The value of GSSG obtained using this method will be half of the value of using GSH as a standard. Usually, the GSSG concentrations of biological sample solutions are very low, so even the high sensitivity method may not be enough to quantify the amount of GSSG.

◆ Do I have to dilute the sample solution prior to the assay?

If you do not know the total glutathione level of your sample, multiple dilutions may be necessary. If the total glutathione level of your sample is less than 100 µM, no dilution is necessary.

◆ What interferes with the assay?

Reducing agents (such as ascorbic acid, beta-mercaptoethanol, dithiothreitol, and cysteine) and thiol reactive compounds (such as maleimides) interfere with the glutathione assay. Therefore, they should be avoided during sample preparation.

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DTNB

5,5'-Dithiobis(2-nitrobenzoic acid) [CAS: 69-78-3]

Application: SH detection, colorimetric

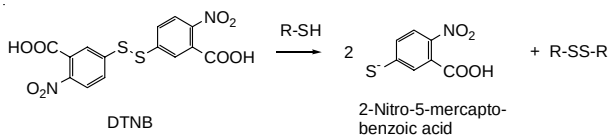
Appearance: pale yellow crystalline powder
Molar absorptivity: >12,000 at 305 nm
MW: 396.35, $C_{14}H_8N_2O_8S_2$

Ordering Information	
Product code	Unit
D029-10	1 g

Storage Condition	Shipping Condition
ambient temperature	ambient temperature

Product Description

DTNB is known as Ellman's reagent. It is used for the colorimetric determination of thiol groups in biological samples. It is fairly soluble in water. Colorless DTNB is converted to yellow 5-Mercapto-2-nitrobenzoic acid in the presence of thiol compounds (Fig. 5-11). As 5-Mercapto-2-nitrobenzoic acid has an absorption maximum at 412 nm, the absorption spectrum of DTNB does not interfere with thiol detection (Fig. 5-12).



DTNB

2-Nitro-5-mercapto-benzoic acid

Fig. 5-11 DTNB reaction with thiol compound

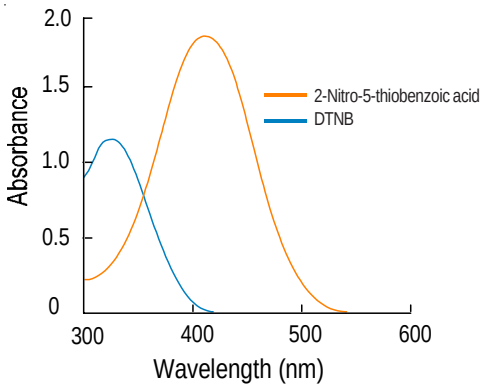


Fig. 5-12 Absorption spectra of DTNB and reduced DTNB

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2-PDS

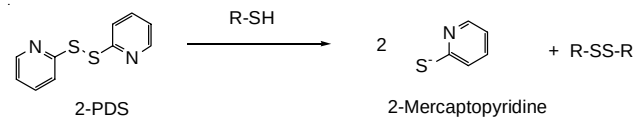
2,2'-Dithiodipyridine [CAS: 2127-03-9]

Application: SH detection, UV absorption

Appearance: white or pale yellow powder
Molar absorptivity: >14,000 at 235 nm
MW: 220.32, $C_{10}H_8N_2S_2$

Ordering Information	
Product code	Unit
P016-10	1 g

Storage Condition	Shipping Condition
ambient temperature	ambient temperature



2-PDS

2-Mercaptopyridine

Fig. 5-13 2-PDS reaction with thiol compound

V-4. Oxidative Stress: Phospholipid Peroxide

4-PDS 4,4'-Dithiodipyridine [CAS: 2645-22-9]

Application: SH detection, UV absorption

Appearance: white or pale yellow powder
Molar absorptivity: >14,000 at 235 nm
MW: 220.32, $C_{10}H_8N_2S_2$

Ordering Information

Product code	Unit
P017-10	1 g

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Product Description

2-PDS and 4-PDS are used for the photometric determination of thiol groups in biological samples. These compounds react with the thiol groups at pH 5 to form mercaptopyridine (Fig. 5-13 and 5-14). The maximum wavelengths of 2-Mercaptopyridine and 4-Mercaptopyridine are 343 nm and 324 nm, respectively.

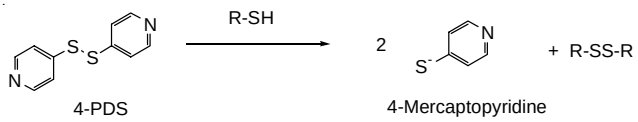


Fig. 5-14 4-PDS reaction with thiol compound

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4-PDS: 1. R. E. Humphrey, *et al.*, *Anal. Chem.*, **42**, 698 (1970); J. Haladjian, *et al.*, *J. Electroanal. Chem.*, **352**, 329 (1993); A. Andersson, *et al.*, *Clin. Chem.*, **39**, 1590 (1993); V. P. Benov, *et al.*, *Anal. Lett.*, **26**, 2061 (1993); T. Kohzuma, *et al.*, *Chem Lett.*, **12**, 2029 (1993); A. Mosca, *et al.*, *J. Vac. Sci. Technol.*, **B12**, 1486 (1994); K. Hasinaka, *et al.*, *J. Immunol. Methods*, **172**, 179 (1994); A. Cricenti, *et al.*, *J. Vac. Sci. Technol.*, **B12**, 1494 (1994); G. Pitari, *et al.*, *Eur. J. Biochem.*, **226**, 81 (1994); D. Hobara, *et al.*, *Colloids Surf.*, **A93**, 241 (1994); M. Le, G. E. Means, *Anal. Biochem.*, **229**, 264 (1995); S. Cai, *et al.*, *J. Membr. Biol.*, **158**, 147 (1997); K. Cai, *et al.*, *Biochemistry*, **38**, 7925 (1999); J. Klein-Seetharaman, *et al.*, *Biochemistry*, **38**, 7938 (1999).

DPPP Diphenyl-1-pyrenylphosphine [CAS: 110954-36-4]

Application: Phospholipid peroxide detection, fluorescent HPLC analysis

Appearance: slightly yellow powder
Purity: >97% (HPLC)
MW: 386.42, $C_{28}H_{19}P$

Ordering Information

Product code	Unit
D350-10	10 mg

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Product Description

DPPP is a non-fluorescent triphenylphosphine compound. It reacts with hydroperoxide to generate DPPPoxide that emits fluorescence at 352 nm excitation and 380 nm emission wavelenghs (Fig. 5-15). Post-column HPLC method is used to determine phospholipid peroxide in sample solutions.

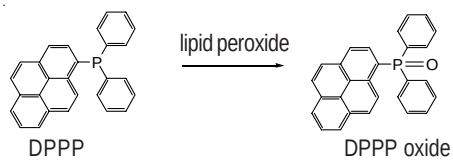


Fig. 5-15 DPPP reaction with lipid peroxidase

References

R. D. Platter, *et al.*, *J. Am. Chem. Soc.*, **54**, 511 (1977); K. Akasaka, *et al.*, *Anal. Lett.*, **20**, 797 (1987); H. Megro, *et al.*, *Methods in Enzymol.*, **186**, 157 (1990); K. Akasaka, *et al.*, *Biosci. Biotech. Biochem.*, **56**, 605 (1992); K. Akasaka, *et al.*, *J. Chromatogr.*, **617**, 205 (1993); K. Akasaka, *et al.*, *J. Chromatogr.*, **622**, 153 (1993); K. Akasaka, *et al.*, *J. Chromatogr.*, **628**, 31 (1993); K. Akasaka, *et al. Biosci. Biotech. Biochem.*, **58**, 396 (1994).

Spy-LHP

2-(4-Diphenylphosphanyl-phenyl)-9-(1-hexyl-heptyl)-anthra[2,1,9-def,6,5,10-d'e'f']diisoquinoline-1,3,8,10-tetraone

Application: Phospholipid peroxidase detection
Selective to lipid hydroperoxidase

Features: Fluometric detection

Ordering Information

Product code	Unit
S343-10	1 mg

Appearance: Reddish black crystalline powder
Purity:>90% (HPLC)
MW: 832.96, $C_{55}H_{49}N_2O_4P$

Storage Condition	Shipping Condition
0-5 °C	ambient temperature

Product Description

Spy-LHP is a newly developed fluorescent probe for live cell imaging of phospholipid peroxide. There are several detection methods available for lipid peroxides, such as iodide titration method, colorimetric method, or chemiluminometric method to determine malondialdehyde or 4-hydroxynonenal. Malondialdehyde or 4-hydroxynonenal are derivatives from lipid hydroperoxide prepared by oxidation with reactive oxygen species. Thiobarbituriic acid and 1-Methyl-2-phenylindole are used for the derivertization of malondialdehyde for the colorimetric or fluorometric analysis. Spy-LHP is a low-fluorescent compound, but is oxidized with lipid hydroperoxide to become a high fluorescent compound as indicated in Fig. 5-16. A similar product, DPPP, is oxidized by a lipid hydroperoxide and becomes a fluorescence compound which can be excited at 352 nm to emit fluorescence at 380 nm. However, the UV excitation for DPPP gives significant damage to a live cell. Since the oxidized Spy-LHP emits strong fluorescence (quantum yield: ~1) with maximum wavelength at 535 nm when excited at 524 nm, damage to a live cells is very small. Spy-LHP has two alkyl chains to improve the affinity to the lipid bilayer. Spy-LHP is highly selective to lipid hydroperoxide and does not react with hydrogen peroxide, hydroxy radicals, superoxide anion, nitric oxides, peroxynitrite, and alkylperoxy radicals.

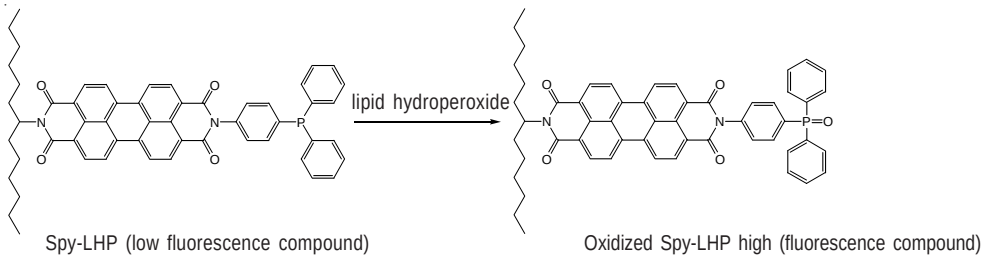


Fig. 5-16 Reaction of Spy-LHP with lipid hydroperoxide

General protocol

Reagents
0.1 mM Spy-LHP/DMSO

1. Add 0.1 mM Spy-LHP/DMSO solution to a cell culture[a)] and incubate for 15 min.
2. Remove the culture medium and wash cells with culture medium several times.
3. Add PBS buffer and analyze the cells with a microscope.

a) The final concentration of Spy-LHP should not exceed 2 μM as a final concentration. DMSO concentration should be lower than 2%.

References

N. Soh, *et al*, *Bioorg. Med. Chem. Lett.*, **16**, 2943 (2006), N. Soh, *et al.*, *Org. Biomol. Chem.*, **5**, 3762 (2007).

V-5. Oxidative Stress: Nitroguanine

Anti-Nitroguanosine Monoclonal Antibody

clone#: NO_2G52

Application: 8-Nitroguanosine, 8-Nitroguanine detection

Appearance: colorless solution
Titration: pass test Subtype: IgG1 (mouse BALB/c)
Concentration: 1 mg/ml PBS solution; 0.1% ProClin as a preservative

Storage Condition: -20 °C
Shipping Condition: with blue ice or dry ice

Ordering Information

Product code	Unit
AB02-10	50 µg

References

Y. Terasaki, *et al.*, *Am. J. Respir. Crit. Care Med.*, **174**, 665 (2006).

Anti-Nitroguanosine Polyclonal Antibody

Application: 8-Nitroguanosine, 8-Nitroguanine detection

Appearance: colorless solution
Titration: pass test Type: IgG
Concentration: 200 µg per ml PBS solution; 0.1% ProClin as a preservative
Host: Japanese rabbit

Storage Condition: -20 °C
Shipping Condition: with blue ice or dry ice

Ordering Information

Product code	Unit
AB01-10	50 µg

Product Description

8-Nitroguanosine is a nitrated base of DNA and RNA. It is formed by peroxynitrite, which is generated from nitric oxide and superoxide anion radical. It is known that a large amount of nitric oxide molecules and superoxide anion, generated by inflammation, causes nitration of guanosine. Since chemically modified nucleotides cause mutation during DNA replication, 8-nitroguanosine is thought to be one of the markers of DNA damage related to mutation and cancer. Because of its very high specificity, monoclonal antibody NO_2G52 recognizes 8-nitroguanine and 8-nitroguanosine, but it does not cross-react with normal nucleotide bases, 8-hydroxyguanine 8-hydroxydeoxy-guanosine, 3-nitrotyrosine, xanthine, or 2-nitroimidazole (Fig. 5-17).

The specificity of NO_2G52 was determined by a competitive ELISA using an 8-nitroguanosine-BSA-coated plate. As shown in the figures below, NO_2G52 has very high affinity for 8-nitroguanine and 8-nitroguanosine, and it slightly cross-reacts with 8-bromoguanosine, 8-bromoguanine, and 8-chloroguanine. Anti-Nitroguanosine polyclonal antibody also recognizes 8-nitroguanine and 8-nitroguanosine, but it does not cross-react with normal guanosine, guanine, 8-hydroxyguanine, or 3-nitrotyrosine. Since this antibody was prepared using rabbits, it can be used for immuno-histostaining of rodent tissues such as mice or rats.

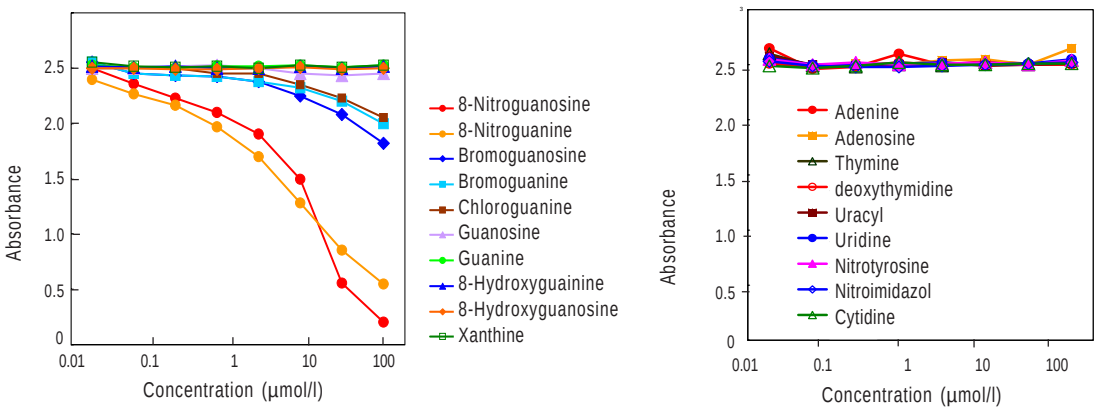


Fig. 5-17 Specificity of Anti 8-Nitroguanosine monoclonal antibody

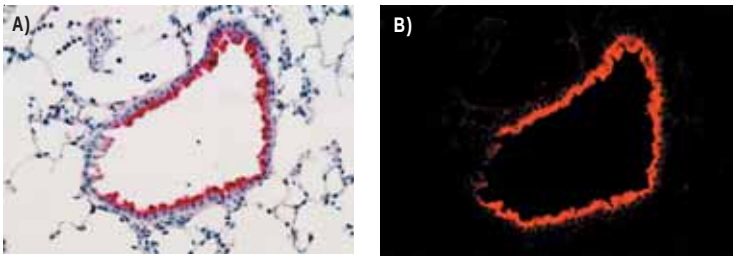


Fig 5-18 Tissue Staining with Anti-Nitroguanosine Antibody

A) Tissue section (8 days postinfection, influenza) stained with Anti-Nitroguanosine Antibody. B) The same section viewed using a confocal laser scanning microscope (Fluoroview FV300, Olympus, Nagano, Japan). Strong fluorescence, due to emission of Vector red, is evident in the cytosol.

References

T. Akaike, *et al.*, *FASEB J.*, **14**, 1447 (2000); 2. T. Akaike, *et al.*, *Proc. Natl. Acad. Sci. USA*, **100**, 685 (2003).

8-Nitroguanine (lyophilized)

8-Nitroguanine

Application: Blocking agent for immunostaining with Anti-Nitroguanosine Antibody, standard of 8-Nitroguanosine EIA

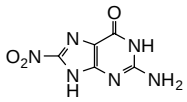
Appearance: white or slightly reddish-gray powder
Purity: >97.0% (Titration)
MW: 196.12, $C_5H_4N_6O_3$

Storage Condition: 0-5 °C
Shipping Condition: ambient temperature or with blue ice

Ordering Information

Product code	Unit
N455-10	100 µg

Chemical Structure



Product Description

8-Nitroguanine is a nitrated base of DNA and RNA. It is formed by peroxynitrite, which is generated from nitric oxide and superoxide anion radical. It is known that a large amount of nitric oxide molecules and superoxide anion, generated by inflammation, causes nitration of guanosine. Since chemically modified nucleotides cause mutation during DNA replication, 8-Nitroguanine is thought to be a marker of DNA damage related to mutation and cancer. 8-Nitroguanine (lyophilized) is made by the lyophilization of its phosphate buffered saline solution, and is used in immunohistochemistry for absorption testing. Adding 0.4 ml of distilled water to the 8-Nitroguanine powder produces a 1.2 mmol / l of 8-Nitroguanine solution. 8-Nitroguanine/ PBS solution is stable for one month at 4 °C. If an antibody pre-treated with excessive 8-Nitroguanine shows negative staining, then the subsequent positive staining with this antibody will be specific for 8-nitroguanine or 8-nitroguanosine formed in DNA or RNA.

3-Deoxyglucosone

3-Deoxy-D-erythro-hexos-2-ulose [CAS: 4084-27-9]

Application: Standard for 3-DG analysis, intermediate of AGE

Appearance: white or slightly yellowish solid
Purity: >99.0% (HPLC)
MW: 162.14, $C_6H_{10}O_5$

Storage Condition: -20 °C, protect from moisture
Shipping Condition: with blue ice or dry ice

Ordering Information

Product code	Unit
D535-08	1 mg

3-Deoxyglucosone Detection Reagents

Application: 3-DG HPLC assay, fluorometric

Ordering Information

Product code	Unit
D536-10	1 set

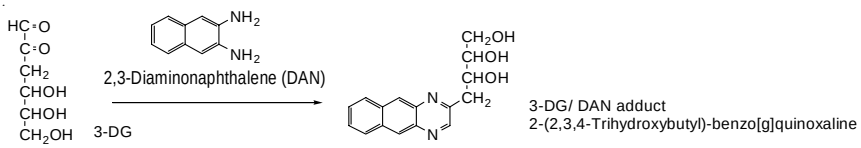
Contents of the kit

DAN ..... 10 mg x 1 3-DG/DAN adduct ..... 1 mg x 1

Storage Condition
-20 °C, protect from moisture

Shipping Condition
with blue ice or dry ice

3-DG Detection



Product Description

Advanced glycation end-products (AGEs) have been studied as one of the causes of diabetic complications. Several compounds have been identified as AGEs, including pyralline, pentosidine, imidazolone, and pyropyridine. Glyoxal and methylglyoxal are reactive dicarbonyl compounds generated by glucose self-oxidation that are known to be AGE precursors. Another dicarbonyl compound, 3-Deoxyglucosone (3-DG), is also known to be one of the AGE precursors. 3-DG is derived from the Amadori rearrangement products of proteins and sugars in early stages of the Maillard reaction. 3-DG is also derived from fructose, which is present in high levels in diabetic patients, by a self-condensation reaction. Fructose-3-phosphate has been found to enhance cross-linking reactions of lens proteins in a diabetic rat model. Therefore, 3-DG derived from fructose-3-phosphate has been studied as a possible cause of cataracts.

Dr. Miyata and others reported that the 3-DG serum level in a diabetic rat model was 918 nM (normal level: 379 nM) and it was suppressed to 695 nM after 3 weeks of feeding aminoguanidine (50 mg/kg/day), an inhibitor of protein glycation. This suggests that compounds with 3-DG quenching activity may have clinical uses. 3-DG may be involved in other diseases as well. Dr. Niwa and others reported that uremia patients had elevated 3-DG levels, and that the 3-DG levels of diabetic uremia patients were even higher. There is also evidence that 3-DG inhibits DNA synthesis, suppressing cell proliferation as a consequence. Though several roles of 3-DG have become clear, many remain unknown. Glyoxal and methylglyoxal are other reactive dicarbonyl compounds generated by glucose self-oxidation that are known to be AGE precursors.

There are two methods for determining 3-DG levels, HPLC and mass spectrometry (MS). However, there is some discrepancy between the HPLC and MS methods when measuring 3-DG levels *in vivo*. HPLC analysis is based on a fluorescent compound, 2-(2,3,4-trihydroxybutyl)-benzo[g]quinoxaline, generated by a coupling reaction between 3-DG and 2,3-diaminonaphthalene. Analogs of 2,3-diaminonaphthalene, such as 1,2-diamino-4,5-dimethoxy-benzene and 1,2-diamino-4,5-methylenedioxybenzene, can also be used. 3-DG can be utilized for AGE production or as a standard for 3-DG level detection in plasma or serum samples.

3-DG Assay Protocol

HPLC Method: Human Serum

1. Add 60% perchloric acid solution to 1 ml human serum and spin at 3,000 g for 20 min at 4 °C.
2. Dilute the supernatant with bicarbonate buffer, then add 0.1 ml of 2,3-Diaminonaphthalene/methanol solution and 25 µl of 1 ppm 3,4-hexanedione as an internal standard.
3. Incubate the mixture at 4 °C overnight.
4. Extract the mixture with 4 ml ethyl acetate, and add 4 ml methanol to the extract.
5. Analyze the mixture with reverse-phase HPLC at 267 nm excitation and 503 nm emission for fluorescent detection or at 268 nm for UV detection. Data correlates well with HbA_{1c} level.
   The normal serum 3-DG level: 12.8±5.2 ng per ml
   The serum 3-DG level of diabetic patient: 31.8±11.3 ng per ml

References

F. Hayase *et al., J. Biol. Chem.*, **264**, 3758 (1989); B. S. Szwergold *et al., Science*, **247**, 451 (1990); D. G. Dyer *et al., J. Biol. Chem.*, **266**, 11654 (1991); S. Miyata *et al., J. Clin. Invest.*, **89**, 1102 (1992); K. J. Knecht *et al., Arch. Biochem. Biophys.*, **294**, 130 (1992); T. Niwa *et al., Biochem. Biophys. Res. Commun.*, **196**, 837 (1993); S. Taneda *et al., Clin. Chem.*, **40**, 1766 (1994); H. Yamada *et al., J. Biol. Chem.*, **269 (32)**, 20275 (1994); F. Hayase *et al., Biosci. Biotechnol. Biochem.*, **58**, 1936 (1994); F. Hayase *et al., Biosci. Biotechnol. Biochem.*, **59**, 1407 (1995); T. Niwa *et al., Nephron*, **69**, 438 (1995); D. V. Zyzak *et al., Arch. Biochem. Biophys.*, **316**, 547 (1995); T. Niwa *et al., Kidney Int.*, **49**, 861 (1996); Y. Hamada *et al., Diabetes Care*, **20**, 1466 (1997); T. Niwa *et al., Kidney Int.*, **51**, 187 (1997); S. Lal *et al., Arch. Biochem. Biophys.*, **342**, 254 (1997); T. Niwa *et al, J. Chromatogr. B*, **731**, 23 (1999).

VI. Nitric Oxide Research

Introduction

Nitric oxide (NO) is a unique molecule in biological systems: it can directly interact with target molecules without the involvement of receptors. Since NO is an electrically neutral gaseous molecule, it freely crosses through cells, tissues, and organs. However, its half-life is 3 to 6 seconds, so its active period as NO is fairly short. Therefore, NO activity occurs in the NO-generating cell itself or an adjacent cell. NO is generated by NO synthetase (NOS) from L-arginine. It has been identified as an endothelial-derived relaxation factor (EDRF) and antiplatelet substance. NO also has a number of other activities: suppression of superoxide anion production from nucleophils, suppression of cell adhesive molecule expression such as VCAM-1 and secretin, suppression of cytokine secretion such as IL-8, suppression of LDL oxidation, activation of PGI2 synthetase, and so on. The most important role of NO is considered to be the activation of guanylate cyclase. The field of NO research has grown dramatically since the discovery of NOS. Despite this rapid growth, NO is still shrouded in mystery. There are many contradictory reports. For example, NO has been found to cause tissue damage in ischemia-reperfusion systems; however, there is also evidence that NO is generated to protect tissues from such damage. Another example is glutamate toxicity in a neural system: some researchers find that NO mediates the toxicity, while others report that NO protects from such damage.

There are two reasons why NO research has led to so many contradictory results. First, NO has unique chemical properties that differ from other chemical messengers and hormones. Since NO is a free radical, it is very reactive and unstable. NO is metabolized into various compounds almost immediately after it appears in a biological environment. These metabolites have different activities from NO. For this reason, it is vital to separately investigate the function of each NO metabolite in the NO pathway. Such research should specify the specific NO metabolite that is causing the observed phenomenon. Thus, reagents for NO research must be able to add pure NO or its metabolite to an experimental system without significant side effects or reactions. Second, the timing, rate, amount, and period of NO exposure are also important. The controversial results mentioned above might be caused by differences in NO exposure. The use of a pure NO solution may be the most reliable method in an experimental system. However, it is technically difficult to make a pure NO aqueous solution. The high volatility of NO makes it difficult to produce an exact solution. Moreover, even if a pure NO solution is added to a system, the NO concentration decreases very rapidly with various reactions. Therefore, it is impossible to create a continuous NO exposure state similar to *in vivo* NO synthesis by NOS.

For these reasons, NO donors, reagents that can be used to generate NO, have often been used for the addition of NO into an observed system. Several popular NO donors include nitroglycerin (GTN), isosorbide dinitrate (ISDN), sodium nitroprusside (SNP), and *S*-nitrosothiols. However, these NO donors have not yet resolved the difficulties mentioned above because they are imperfect for the generation of NO. GTN, ISDN, and SNP do not release NO spontaneously; they require thiols as cofactors for NO generation. Furthermore, the favorable conditions for these reagents are very limited, and the amount of NO released from these donors are relatively small. The release conditions might also be affected by the spatial position of samples and their kinds. Nitrate tolerance is another problem for these reagents. SNP also has the disadvantage of cyanoferrin 1 toxicity and the bioactivity of the SNP molecule itself. In contrast to GTN, ISDN and SNP, nitrosothiol is a spontaneous NO donor. However, NO release of these NO doners accelerates on the surface of the cellular membrane. A more serious issue concerning the use of nitrosothiol as the NO donor is that it may have its own NO-like activity.

Scavenging NO is another important pharmacological method for examining the NO pathway. The most commonly used NO scavengers are NOS inhibitors, including arginine derivatives such as *N*-methylarginine and *N*-nitroarginine methyl ester. These NOS inhibitors, however, scavenge the other NO-related systems as well. This makes a detailed examination of NO action difficult because it may have been caused by another NO metabolite. An ideal NO scavenger would not cut off the arginine-NO pathway by NOS inhibition, but would scavenge the NO molecule directly.

Dojindo has produced a wide variety of reagents for thorough investigation of NO bioactivity, including NO-related metabolite donors such as SIN-1, SNAP, NOCs and NORs. NOCs and NORs can be used for the spontaneous rate-controlled release of NO. Carboxy-PTIO scavenges the NO molecule without affecting its metabolites. Photoactivatable caged NO donors are also available. Dojindo also offers MGD and 2,3-Diaminonaphthalene for the detection of NO or NO_2^- and NO_3^- as metabolites of NO. Fe(II)-MGD complex easily traps NO *in vivo* or *in vitro*, and can be used to detect NO by electron spin resonance (ESR). Since 2,3-Diaminonaphthalene generates a fluorescent compound by the reaction with NO_2^-, the sensitivity of this fluorescence method is considerably higher than that of the Griess assay.

Nitric Oxide Chemistry

Properties of Nitric Oxide

NO is a free radical that has a lone electron in the 2*p*-*p* orbital. It is a highly volatile, hydrophobic, and colorless gaseous material. NO dissolves in water at a maximum of 1.9 mM, and the aqueous NO solution is fairly stable in oxygen-free conditions. NO rapidly reacts with oxygen, and produces NO_2^- and NO_3^- in aqueous conditions. The half-life of NO is shorter if the solution contains salts. Under physiological conditions, its half-life is reported to be 3 to 6 seconds. To obtain highly purified NO, the commercial NO gas has to be distilled; otherwise NO_2 and NO_3 will remain as contaminants. In the atmosphere, NO generates a brown gas, N_2O_4.

Reactions of Nitric Oxide

Since NO is a highly reactive free radical, several metabolites are produced under physiological conditions. NO reacts with oxygen (eq. 1), and the resulting NO_2 generates N_2O_4 (eq. 2) and N_2O_3 (eq. 4). In an aqueous solution, N_2O_4 is hydrolyzed to NO_2^- and NO_3^- (eq. 3), and N_2O_3 is hydrolyzed to NO_2^- (eq. 5). In most cases, the reaction ratio of equations (4) and (5) are ten times that of equation (3). Thus, most NO becomes NO_2 in a simple aqueous solution.

$$NO + 1/2O_2 \longrightarrow NO_2 \quad \text{(eq. 1)}$$
$$2NO_2 \longleftrightarrow N_2O_4 \quad \text{(eq. 2)}$$
$$N_2O_4 + H_2O \longrightarrow NO_2^- + NO_3^- + 2H^+ \quad \text{(eq. 3)}$$
$$NO + NO_2 \longleftrightarrow N_2O_3 \quad \text{(eq. 4)}$$
$$N_2O_3 + H_2O \rightarrow 2NO_2^- + 2H^+ \quad \text{(eq. 5)}$$

VI. Nitric Oxide Research

NO, NO^+, and NO^- are physiologically important substances which are similar to oxygen molecules, O_2, O_2^-, and O_2^{2-} (H_2O_2). NO coordinates with metal ions such as hemoglobin. The coordination rate constant of NO with deoxyhemoglobin is $5x10^7\ M^{-1}s^{-1}$, and its dissociation constant is $1x10^{-5}\ s^{-1}$. An interesting characteristic of NO is its ability to bind to Fe(III), unlike oxygen and carbon monoxide. However, this complex is not stable; attack with a nucleophile will release NO^+. NO also binds to many transitional metals such as Fe-S in the center of the mitochondrial electron carrier and Fe in a macrophage. Since the NO-transitional metal complex has NO^+ characteristics, highly nucleophilic material will be transformed into a nitroso compound by this complex. A NO complex with Fe(II) containing metalloenzyme has NO^- characteristics.

Metabolites of Nitric Oxide

NO^+

It is possible for NO^+ to exist in an aqueous solution, but in most cases, NO^+ exists as nitroso compounds or metal-nitrosyl compounds. These compounds behave like NO^+ in aqueous solutions. They are produced by NO_2, derived from N_2O_4 or N_2O_3, or by the neutrophilic reaction of thiols with Fe(II)-NO^+. There is evidence that the resulting NO^+ equivalents have important roles as NO^+ carriers under physiological conditions. Nitrosothiols are the most common NO^+ equivalents. Though amines may also act as nitroso compounds, they are protonated under physiological conditions and do not react with NO^+. Nitrosothiols are not produced from the reaction between thiols and NO. Thiols are first oxidized and the resulting disulfides then react with NO as shown below.

$$R\text{-}SH \longrightarrow RS^{\cdot} + H^+$$
$$RS^{\cdot} + {}^{\cdot}NO \longrightarrow RS\text{-}NO^{\cdot} + H^+ \longrightarrow RS\text{-}N\text{-}OH$$
$$2RS\text{-}N\text{-}OH \longrightarrow RSN(OH)N(SR)OH \longrightarrow R\text{-}SS\text{-}R + HON{=}NOH$$

On the other hand, NO_2 may generate nitrosothiols from thiols as shown below.

$$RSH + {}^{\cdot}NO_2 \longrightarrow RS\text{-}N(O)OH \longrightarrow RSNO + H_2O$$

However, this reaction competes with the following reaction, which produces disulfides.

$$RSH + {}^{\cdot}NO_2 \longrightarrow RS\text{-}N(O)OH \longrightarrow RS\text{-}N(OH)_2 + RS^{\cdot}$$
$$2RS^{\cdot} \longrightarrow R\text{-}SS\text{-}R$$

Furthermore, nitrosothiols generate NO by decomposition or reaction with thiol radicals.

$$RSNO \longrightarrow RS^{\cdot} + {}^{\cdot}NO$$
$$RSNO + RS^{\cdot} \longrightarrow R\text{-}SS\text{-}R + {}^{\cdot}NO$$

NO^-

NO^- becomes N_2O in aqueous solution with a rate constant of $2x10^9\ M^{-1}s^{-1}$, and rapidly forms a complex with Fe(III)heme. There are reports that NO^- is produced by Cu(I)-SOD from NO, and also by four electron oxidizations of guanidino-nitrogen by NO synthase in certain conditions.

$ONOO^-$

NO reacts with superoxide anion (O_2^-) to produce peroxynitrite ($ONOO^-$) with a rate constant $3.7x10^7\ M^{-1}s^{-1}$. It is reported that the half-life of $ONOO^-$ at pH 7.5 is 1.9 sec.

$$^{\cdot}NO + O_2^{\cdot-} \longrightarrow ONOO^-$$

This anion decomposes upon protonation to produce NO_2 and HO.

$$ONOO^- + H \longrightarrow NO_2 + {}^{\cdot}HO$$

HO is also produced by the Fenton reaction as shown below.

$$2O_2^{\cdot-} + 2H^+ \longrightarrow H_2O_2 + O_2$$
$$H_2O_2 \longrightarrow {}^{\cdot}HO + OH^-$$

There are reports that $ONOO^-$ and HO are related to epithelial cell damage and neuronal toxicity. However, in oxygen-related toxicity to the central nerve system, the toxicity decreases as NO is trapped by O_2^-. This is supported by the observation that toxicity is increased in transgenic mice that express excessive amounts of extracellular SOD. Another possible reason for the decreasing toxicity is that $ONOO^-$ is decomposed to NO^+ by transitional metals in high levels of SOD. The rate of $ONOO^-$ generation is proportional to the concentration of NO and O_2. Thus, the amount of $ONOO^-$ generated is dependent on rather small changes in the concentration of NO and O_2. There are two types of $ONOO^-$ decomposition; one generates OH and the other HNO_3. The difference between these two reactions is explained by variations in molecular conformation. In the *cis*-conformation, NO_3 is produced by an intramolecular transposition; in the *trans*-conformation, HO is produced by a homolytic cleavage. The intramolecular transposition is dominant in high pH conditions.

$$ON(O)OH \longrightarrow H^+ + NO_3^-$$
$$ONO\text{-}OH \longrightarrow {}^{\cdot}NO_2 + HO^{\cdot}$$

Experimentally, $ONOO^-$ is produced by NO and O_2^-; however, it is reported that this chemical process does not contribute to the generation of $ONOO^-$ *in vivo*. NO is stabilized in the presence of SOD. There are several possible explanations, such as the inhibition of the NO reaction by SOD quenching of O_2^-, the competitive binding of O_2^- to the NO binding site of a guanylate cyclase, or the direct reaction of NO as a substrate of SOD.

S-Nitrosothiols

Nitrosothiols are produced from thiols as they react with NO_2 or NO_2^-. They have several different biological activities including a vasorelaxant activity just like NO. Previous research found that the vasorelaxant properties of endothelium-derived relaxation factor (EDRF) are more similar to *S*-nitrosocysteine than NO; however, this does not seem to be the current majority view. Though nitrosothiol is one of the most important factors for the study of the NO pathway, only a few nitrosothiols, such as SNAP and *S*-nitrosoglutathione (GSNO), are stable enough for use as NO donors. Unfortunately, SNAP is insoluble in water. Thus, GSNO and *S*-nitrosocysteine (SNC) are the only commercially available water-soluble nitrosothiols.

VI. Nitric Oxide Research

Nitrosothiols release nitric oxide, and form disulfides as shown below.

2RSNO⟶ RSSR + 2NO

This reaction is accelerated by light and heat. If GSNO is incubated at 37 °C without light, NO will not spontaneously release. Metal ions, such as Cu(II), Cu(I), and Hg(II), also accelerate the reaction. Thus, masking reagents such as EDTA prevent the releasing reaction.

Another important characteristic of nitrosothiols is their ability to carry out nitrosation. This reaction is faster than the decomposition of RSNO itself, and proceeds readily at physiological pH levels. The reaction rate depends on the pKa of the thiol.

The vasorelaxant activities of nitrosothiols in rat aortic rings have been reported as follows:

SNAP > GSNO = SNAC (S-Nitroso-N-acetylcysteine) > CoAsNO (S-Nitroso-coenzyme A) > CYCNO (S-Nitrosocysteine)

The inhibitory potencies of nitrosothiols for the platelet aggregation have been reported as follows:

GSNO > NO > SNAP > SIN-1

Denitrosation of *S*-nitrosothiol is not spontaneous, and it needs to be catalyzed on the surface of external vascular membranes. *S*-nitroso-*L*-cysteine raises the intracellular calcium level of a PC12 cell by modifying the thiol group of a caffeine-sensitive moiety of the calcium-induced calcium release (CICR) channel. GSNO has been shown to reduce the blood pressure of anesthetized dogs (0.2 mg/kg) and monkeys (10 mg/kg) through the inhibition of the platelet aggregation.

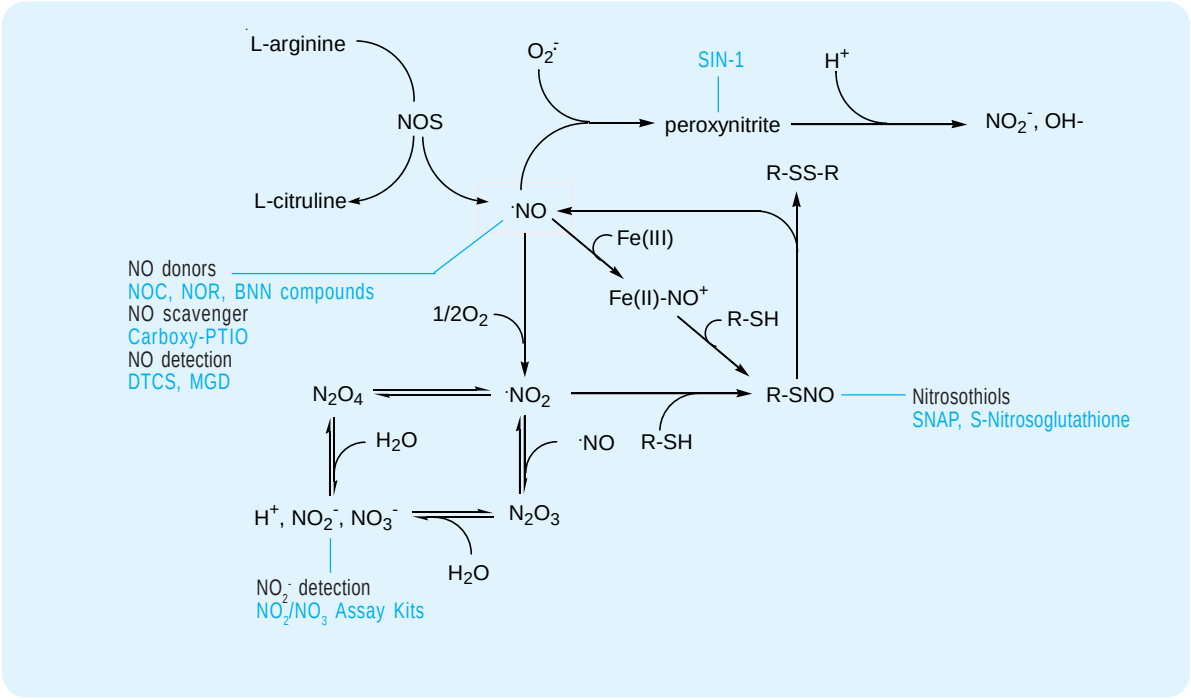


Fig. 6-1 NO metabolism

VI-1. Nitric Oxide Research: NO Donors

NOC 5

1-Hydroxy-2-oxo-3-(3-aminopropyl)-3-isopropyl-1-triazene [CAS: 146724-82-5]

Application: Spontaneous NO donor

Appearance: white powder
Purity: >90.0% (HPLC)
MW: 176.22, $C_6H_{16}N_4O_2$

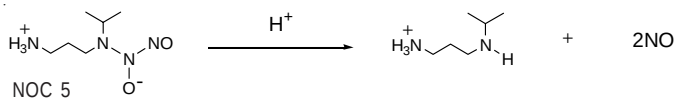
Storage Condition
-20 °C, protect from light and moisture

Shipping Condition
with blue ice or dry ice

Ordering Information

Product code	Unit
N380-10	10 mg
N380-12	50 mg

Reaction of NO release



NOC 7

1-Hydroxy-2-oxo-3-(*N*-methyl-3-aminopropyl)-3-methyl-1-triazene [CAS: 146724-84-7]

Application: Spontaneous NO donor

Appearance: white powder
Purity: >90.0% (HPLC)
MW: 162.19, $C_5H_{14}N_4O_2$

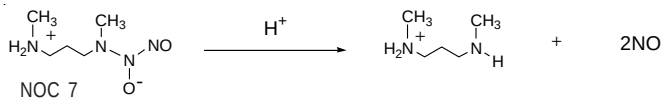
Storage Condition
-20 °C, protect from light and moisture

Shipping Condition
with blue ice or dry ice

Ordering Information

Product code	Unit
N377-10	10 mg
N377-12	50 mg

Reaction of NO release



NOC 12

1-Hydroxy-2-oxo-3-(*N*-ethyl-2-aminoethyl)-3-ethyl-1-triazene [CAS: 146724-89-2]

Application: Spontaneous NO donor

Appearance: white powder
Purity: >90.0% (HPLC)
MW: 176.22, $C_6H_{16}N_4O_2$

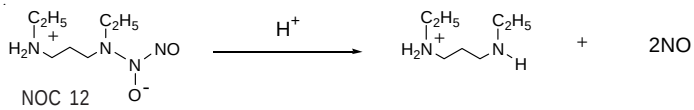
Storage Condition
-20 °C, protect from light and moisture

Shipping Condition
with blue ice or dry ice

Ordering Information

Product code	Unit
N378-10	10 mg
N378-12	50 mg

Reaction of NO release



NOC 18

1-Hydroxy-2-oxo-3,3-bis(2-aminoethyl)-1-triazene [CAS: 146724-94-9]

Application: Spontaneous NO donor

Appearance: white powder
Purity: >90.0% (HPLC)
MW: 163.18, $C_4H_{13}N_5O_2$

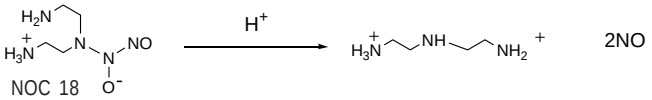
Storage Condition
-20 °C, protect from light and moisture

Shipping Condition
with blue ice or dry ice

Ordering Information

Product code	Unit
N379-10	10 mg
N379-12	50 mg

Reaction of NO release



VI-1. Nitric Oxide Research: NO Donors

Product Description of NOC Compounds

NOCs are stable NO-amine complexes that spontaneously release NO, without cofactors, under physiological conditions. The rate of NO release depends on the chemical structure of NOC. The mechanism of spontaneous NO generation by NOCs is very simple compared to other classical NO donors, such as nitroglycerin and nitropurusside, and the by-products do not interfere with cell activities. A single NOC molecule releases two NO molecules (as indicated in the reaction scheme); the release rate of the second NO molecule is very slow. NOCs can be used to add controlled amounts of pure NO to experimental systems at controlled rates with minimal side effects. The amount of NO released can be easily manipulated by altering the concentration and selection of NOC reagents. Dojindo offers four different NOCs (NOC 5, 7, 12, and 18) with different half-lifes. Stock solutions of NOC prepared in alkaline solutions, such as aqueous NaOH, are relatively stable. However,the NOC stock solution should be used within one day because it degrades about 5% per day, even at -20 °C. The release of NO begins immediately after adding the stock solution to a sample solution.

Nitric Oxide Release

1. Prepare 10 mM NOC stock solution using 0.1 M NaOH. Since the NOC stock solution is not stable, keep it on an ice bath and use it in one day.
2. Add an appropriate volume of the NOC stock solution to the sample solution in which NO is to be released. To maintain the pH of the sample solution, the volume of the NOC stock solution should not exceed 1/50 of the sample volume. The sample solution should have sufficient buffering action. NO will be released immediately after the addition of the NOC stock solution.

Table 6-1 pH Dependency of NO Release at 37 °C

NO Donor	pH	Half-life	pH	Half-life	pH	Half-life	pH	Half-life	pH	Half-life
NOC 5	7.0	12 min	7.2	20 min	7.4	25 min	7.6	42 min	7.8	66 min
NOC 7	7.0	2.2 min	7.2	3.8 min	7.4	5 min	7.6	8.2 min	7.8	12.4 min
NOC 12	7.0	40 min	7.2	1.2 hours	7.4	100 min	7.6	3 hours	7.8	4.6 hours
NOC 18	7.0	13 hours	7.2	18 hours	7.4	21 hours	7.6	34 hours	7.8	45 hours

FAQ

- **How do I prepare a stock solution?**
  Prepare 10-50 mM NOC solution with 0.1 M NaOH solution. Then add enough NOC solution to the cell culture to obtain a suitable concentration of NOC in cell culture. If the pH of the culture solution changes, use higher concentration of NOC.
- **What is the solubility of the NOC compounds?**
  NOC 5: 40 mg per 100 ml 0.1 M NaOH (2.2 M NOC 5)
  NOC 7: 70 mg per 100 ml 0.1 M NaOH (4.3 M NOC 7)
  NOC 12: 27 mg per 100 ml 0.1 M NaOH (1.5 M NOC 12)
  NOC 18: 20 mg per 100 ml 0.1 M NaOH (1.2 M NOC 18)
- **Is the stock solution stable?**
  The stock solution will lose 5% of its NOC activity per day, even when stored at -20 °C. Please prepare fresh solution prior to use and keep the solution on an ice bath during the experiment.
- **How is the half-life of NOC determined?**
  Prepare 20 mM NOC stock solution with 0.1 M NaOH. Warm PBS at 37 °C. Add 100 ml NOC solution to 1.9 ml PBS. Using a UV spectrophotometer, immediately start measuring its absorbance at the maximum wavelength of the NOC. Continue measuring until no further spectra changes are observed.
- **Can I use NOC for *in vivo* experiments?**
  Yes. Please review the papers by Shibata and colleagues (1995, 1996).
- **Is the amount of NO released *in vitro* the same as *in vivo*?**
  The amount of NO released in the solution should be the same if the pH and temperature are the same. However, the activity of NO may be different *in vivo* because of other reactive components such as thiol compounds and heme.

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VI-1. Nitric Oxide Research: NO Donors

NOR 1

(±)-(*E*)-4-Methyl-2-[(*E*)-hydroxyimino]-5-nitro-6-methoxy-3-hexenamide [CAS: 163032-70-0]

Application: Spontaneous NO donor

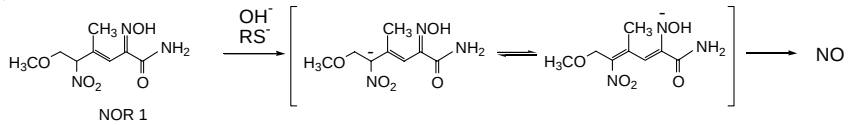
Appearance: white or slightly yellow powder
Purity: >98.0% (HPLC)
MW: 231.21, $C_8H_{13}N_3O_5$

Ordering Information	
Product code	Unit
N388-10	10 mg

Storage Condition
-20 °C, protect from light and moisture

Shipping Condition
ambient temperature

Reaction of NO release



NOR 1

half-life: 1.8 min, 1.5 mM NOR 1 in 0.1 M PBS, pH 7.4 at 37 °C

NOR 3

(±)-(*E*)-4-Ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexenamide [CAS: 163180-49-2]

Application: Spontaneous NO donor

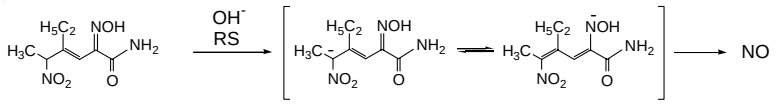
Appearance: white crystalline powder
Purity: >98.0% (HPLC)
MW: 215.21, $C_8H_{13}N_3O_4$

Ordering Information	
Product code	Unit
N390-10	10 mg

Storage Condition
-20 °C, protect from light and moisture

Shipping Condition
ambient temperature

Reaction of NO release



NOR 3

half-life: 30 min, 0.5 mM NOR 3 in 0.1 M PBS, pH 7.4 at 37 °C

NOR 4

(±)-*N*-{(*E*)-4-Ethyl-2-[(*Z*)-hydroxyimino]-5-nitro-3-hexene-1-yl}-3-pyridinecarboxamide [CAS: 162626-99-5]

Application: Spontaneous NO donor

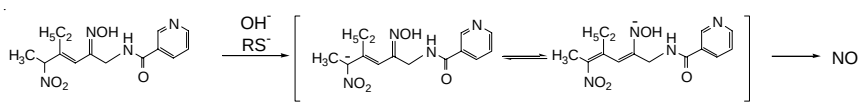
Appearance: white or slightly yellow powder
Purity: >98.0% (HPLC)
MW: 306.32, $C_{14}H_{18}N_4O_4$

Ordering Information	
Product code	Unit
N391-10	10 mg

Storage Condition
-20 °C, protect from light and moisture

Shipping Condition
ambient temperature

Reaction of NO release



NOR 4

half-life: 60 min, 0.5 mM NOR 4 in 0.1 M PBS, pH 7.4 at 37 °C

NOR 5

(±)-*N*-{(*E*)-4-Ethyl-3-[(*Z*)-hydroxyimino]-6-methyl-5-nitro-3-heptenyl}-3-pyridinecarboxamide

Application: Spontaneous NO donor

Appearance: white powder
Purity: >98.0% (HPLC)
MW: 334.37, $C_{16}H_{22}N_4O_4$

Ordering Information

Product code	Unit
N448-10	10 mg

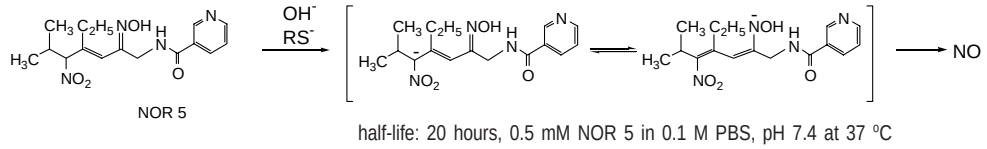
Storage Condition
-20 °C, protect from light and moisture

Shipping Condition
ambient temperature

Product Description of NOR Compounds

NORs are ideal NO donors with completely different chemical structures from the other NO donors. Although NORs do not have any ONO_2 or ONO moiety, they spontaneously release NO at a steady rate. It is also confirmed that the by-products do not possess any significant bioactivities even though the NO release mechanism of NOR has not been completely determined. NOR 3, isolated from *Streptomyces genseosporeus*, is reported to have strong vasodilatory effects on rat and rabbit aortas, and dog coronary arteries. Its activity (ED_{50}=1 nM) is 300 times that of isosorbide dinitrate (ISDN). NOR 3 also increases the plasma cyclic GMP levels, whereas ISDN does not. NOR is a potent inhibitor of platelet aggregation and thrombus formation. NOR 3 (IC_{50}=0-7 mM) effectively inhibits 100% of ADP-initiated human platelet aggregation; whereas ISDN inhibits only 32% of the total aggregation, even at 100 mM concentrations. NOR 3 has also been reported to have antianginal and cardioprotective effects in the ischemia/reperfusion system. In the rat methacholin induced coronary vasospasm model, NOR 3 suppressed the elevation of the ST segment dose-dependently and significantly at 1 mg per kg. On the other hand, ISDN suppressed it significantly at 3.2 mg per kg. The difference in the NO release rate of NOR reagents was reflected even on the *in vivo* hypotensive effects. NOR may also be used orally in a 0.5% methylcellulose suspension. NOR is relatively stable in DMSO solution. NOR 1, which has the shortest half-life, is a promising reagent for making NO standard solutions for calibration. For the preparation of the standard solution, a precisely diluted NOR 1/DMSO solution is added to the buffer solutions.

Reaction of NO release



NOR 5

half-life: 20 hours, 0.5 mM NOR 5 in 0.1 M PBS, pH 7.4 at 37 °C

Nitric Oxide Release

1. Prepare 10 mM NOR stock solution using DMSO. Since the NOR stock solution is not stable, keep it on an ice bath and use it in one day.
2. Add an appropriate volume of the NOR stock solution to the sample solution in which NO is to be released. In order to avoid possible damage to cells by DMSO, the volume of the NOR stock solution should not exceed 1/50 of the sample volume. The sample solution should have sufficient buffering action. NO will be released immediately after the addition of the NOR stock solution.

FAQ

◆ How do I prepare a stock solution?
Prepare 10-50 mM NOR solution with DMSO. The DMSO should be dried. Then add enough NOR solution to the cell culture to obtain a suitable concentration of NOR.

◆ What is the solubility of NOR compounds?
NOR 1: 100 mg per 100 ml DMSO (4.3 M)
NOR 3: 137 mg per 100 ml DMSO (6.4 M)
NOR 4: 30 mg per 100 ml DMSO (1.0 M)
NOR 5: 30 mg per 100 ml DMSO (0.9 M)

◆ Is oral administration possible?
Yes. Please review the article by Kita and colleagues *(Eur. J. Pharmacol.*, **257**, 123-130, 1994).

◆ How many NO molecules does each NOR molecule release in physiological conditions? What are the byproducts?
On average, each NOR molecule releases from 1 to 1.5 NO molecules in physiological conditions. Unfortunately, the structure of NOR byproducts remains unclear. However, the NOR byproducts have no cytotoxicity at the normal concentration for NO release experiments.

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VI-2. Nitric Oxide Research: Nitrosothiol

S-Nitrosoglutathione

N-(*N*-L-γ-Glutamyl-*S*-nitroso-L-cysteinyl)glycine [CAS: 57564-91-7]

Application: Spontaneous NO donor, nitrosothiol compound

Appearance: pink powder
Purity: >90.0% (HPLC)
MW: 336.32, $C_{10}H_{16}N_4O_7S$

Ordering Information	
Product code	Unit
N415-10	25 mg
N415-12	100 mg

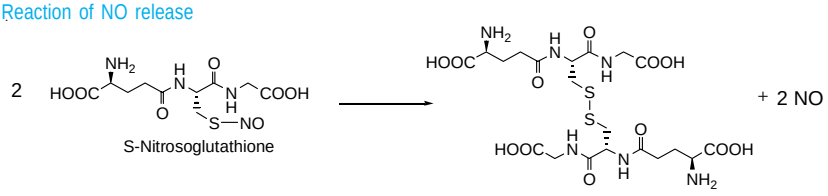
Storage Condition
-20 ºC, protect from light and moisture

Shipping Condition
ambient temperature

Product Description of *S*-Nitrosothiols

SNAP is a stable *N*-nitrosothiol commonly used as an NO donor. Nitrosothiol compounds release NO and become disulfides under specific physiological conditions. The rate of NO release by SNAP is not clear because there are many factors influencing NO production from SNAP, including light, heat, metal ions (Fe^{2+}, Cu^{2+}, Cu^{+}, Hg^{2+}), cell membrane components, and so on. While most of the *S*-nitrosothiol compounds are unstable, SNAP and *S*-Nitrosoglutathione are exceptionally stable. *S*-Nitrosoglutathione is also water-soluble. SNAP, instead of *S*-Nitrosoglutathione, should be used to add *S*-nitrosothiols in experimental systems with NO. Although *S*-nitrosothiol is a good NO donor with no nitrate tolerance, there is evidence that *S*-nitrosothiol itself has NO-like activity during guanylate cyclase activation. Another important reaction of nitrosothiol is NO transfer to other thiol compounds. Since it depends on the pKa of thiols, this transfer reaction proceeds at physiological pH levels. The relaxation efficiency of these nitrosothiol has been compared using rataprta ring samples: SNAP > *S*-Nitrosoglutathione = *S*-Nitroso-*N*-acetylcysteine > *S*-NitrosocoenzymeA ≥ *S*-Nitroso-L-cysteine. Dr. Kowaluk and others reported that the spontaneous liberation of NO from SNAP could not account for *in vitro* vascular relaxation. The spontaneous release of NO from nitrosothiol compounds may not be a key element of vascular relaxation. Metabolites of nitrosothiol generated at the cell membrane might be the essential element for relaxation.

Reaction of NO release



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SIN-1

3-(4-Morpholinyl)sydnonimine, hydrochloride [CAS: 16142-27-1]

Application: Spontaneous NO, peroxynitrite donor

Appearance: white needles or slightly yellowish-white crystalline powder
Purity: pass test (TLC)
MW: 206.63, $C_6H_{11}ClN_4O_2$

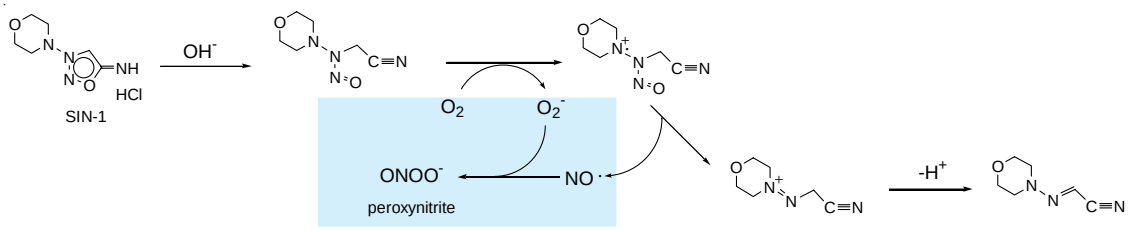
Ordering Information	
Product code	Unit
S264-10	25 mg

Storage Condition	Shipping Condition
-20 °C, protect from light	with blue ice or dry ice

Product Description

SIN-1, a metabolite of the vasodilator molsidomine, is utilized to separately estimate the effectiveness of NO and peroxynitrite with other NO donors. SIN-1 spontaneously decomposes in the presence of molecular oxygen to generate NO and superoxide. Both products bind very rapidly to form peroxynitrite (rate constant *k*: $3.7 \times 10^{-7}\ M^{-1}s^{-1}$). Therefore, SIN-1 is a useful compound that generates peroxynitrite in an efficient manner. Peroxynitrite is a very strong oxidant that generates hydroxyl and nitrosyldioxyl radicals under physiological conditions. Peroxynitrite also decomposes to generate nitrate ion quickly in acidic conditions and slowly in basic conditions. Those species have a different bioactivity from NO.

Reaction of NO release and peroxynitrite production



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VI-4. Nitric Oxide Research: NO Detection

Carboxy-PTIO

2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, sodium salt [CAS: 148819-93-6]

Application: NO quenching, NO detection by ESR
Appearance: dark blue powder
Purity: > 97.0% (TLC)
MW: 299.28, $C_{14}H_{16}N_2NaO_4$

Ordering Information

Product code	Unit
C348-10	10 mg

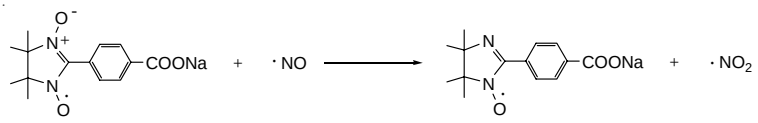
Storage Condition
-20 °C, protect from light

Shipping Condition
ambient temperature

Product Description

Carboxy-PTIO is a stable, water-soluble organic radical that reacts with NO to form NO_2. This reaction can be monitored by electron spin resonance (ESR). NO is an unstable molecule and has a complex reaction cascade for its metabolism in biological systems. Rapidly generated NO-related metabolites carry out various physiological activities. Commonly used NO scavengers such as hemoglobin trap NO; they also trap NOS inhibitors such as arginine derivatives. These NO scavengers also quench all other NO-related metabolites at the same time. In contrast, Carboxy-PTIO does not dramatically affect other NO-related product systems because it transforms NO to NO_2, which is a metabolite of NO. Thus, Carboxy-PTIO can be used to investigate the effects of NO separately from its downstream metabolites. Dr. Akaike and others showed that Carboxy-PTIO suppresses relaxation of the rat aorta ring, which is induced by acetylcholine, twice as effectively as N^G-nitroarginine. Dr. Yoshida and others reported that downstream metabolites of NO, generated by treatment with Carboxy-PTIO, have an increased antiviral activity compared to NO alone. The NO metabolites play important roles in biological systems; therefore, they should be investigated separately from NO.

Reaction of NO quenching



Carboxy-PTIO　　Carboxy-PTI

References

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2,3-Diaminonaphthalene (for NO detection)

2,3-Diaminonaphthalene [CAS: 771-97-1]

Application: NO_2 (NO metabolite) detection, fluorometric

Appearance: white or pale yellowish-brown powder
Fluorescent background: pass test
MW: 158.20, $C_{10}H_{10}N_2$

Ordering Information

Product code	Unit
D418-10	10 mg

Storage Condition
-20 °C, protect from light

Shipping Condition
ambient temperature

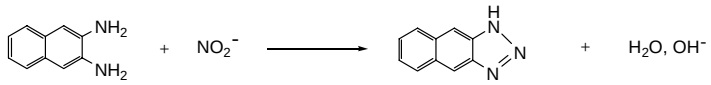
VI-4. Nitric Oxide Research: NO Detection

Product Description

The Griess assay is a simple and popular method for detecting NO concentration. 2,3-Diaminonaphthalene (DAN) is a highly sensitive alternative to the Griess assay. The DAN method is 50-100 times more sensitive than the Griess assay: While the detection limit of the Griess assay is 1 mM, the limit of the DAN method is 10-50 nM. DAN reacts with NO_2^- in acidic conditions to produce fluorescent naphthalenetriazole. The wavelength of the emission maximum of naphthalenetriazole is 410 nm. However, detection at 450 nm is recommended to avoid fluorescent blanks and increase sensitivity.

The fluorescent background of DAN is low for maximum sensitivity. The optimal reaction conditions of DAN with NO_2^- have been determined. The reaction should proceed at pH 2 at room temperature for 5 minutes, and the resulting fluorescence of naphthalenetriazole should be determined at a pH of 10 or more. DAN is a photosensitive reagent and sometimes becomes dark brown colored crystals. Since this brown product cannot be utilized for the fluorescent detection, recrystallization is necessary.

Reaction of 2,3-Diaminonaphthalene with NO_2^-



2,3-Diaminonapththalene — Naphthalenetriazole

NO_2 Assay Using 2,3-Diaminonaphthalene (DAN)

1. Dissolve 50µg DAN in 1 ml 0.62 M HCl to prepare 0.31 mM DAN solution.[a)]
2. Mix 10 µl DAN solution with 100 µl $NaNO_2$ solution (0-10 mM) or sample solution. Incubate the mixture at room temperature for 10-15 min.
3. Add 5 µl 2.8 M NaOH solution to the reaction solution.[b)]
4. Dilute 100 µl of this solution with 4 ml water, followed by fluorescent measurement with excitation wavelength at 365 nm and emission wavelength at 450 nm.
5. Prepare a calibration curve using this data where the X-axis is $NaNO_2$ concentration and the Y-axis is fluorescence intensity. Then use this calibration curve to determine the NO_2 concentration of the sample solution.

a) Acidic conditions are required for a rapid reaction.
b) Basic conditions (pH 10 or higher) are required for a high fluorescence signal.

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C. R. Sawicki, *Anal. Lett.*, **4**, 761 (1971); P. Damiani, *et al.*, *Talanta*, **8**, 649 (1986); W. R. Tracey, *et al.*, *J. Pharmacol. Exp. Ther.*, **252**, 922 (1990); J. S. Pollock, *et al.*, *Proc. Natl. Acad. Sci. USA*, **88**, 10480 (1991); T. P. Misko, *et al.*, *Anal. Biochem.*, **214**, 11 (1993); R. G. Tilton, *et al.*, *Invest. Ophthamol. Vis. Sci.*, **35**, 3278 (1994); T. A. Mayer, *et al.*, *J. Surg. Res.*, **58**, 570 (1995); Y. Kono, *et al.*, *Biochem. J.*, **312**, 947 (1995); R. Metheringham, *et al.*, *Microbiology*, **143**, 2647 (1997); A. M. Rao, *et al.*, *Brain Res.*, **793**, 265 (1998); N. Nakatsubo, *et al.*, *Biol. Pharm. Bull.*, ***21***, 1247 (1998); D. Jourd'heuil, *et al.*, *Arch. Biochem. Biophys.*, **365**, 92 (1999).

DTCS Na

N-(Dithiocarboxy)sarcosine, disodium salt, dihydrate [CAS: 13442-87-0]

Application: NO detection by ESR

Appearance: white or pale yellow powder
MW: 245.23, $C_4H_5NNa_2O_2S_2$, $2H_2O$

Storage Condition
0-5 °C, protect from light

Shipping Condition
ambient temperature

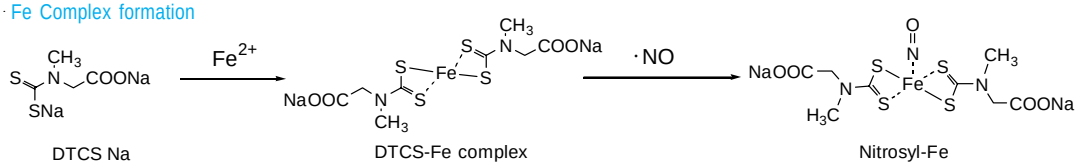
Ordering Information

Product code	Unit
D465-10	100 mg
D465-12	500 mg

Product Description

Diethyldithiocarbamate (DETC) is a good spin-trapping reagent for nitric oxide *in vivo*. However, DETC has not been widely utilized for NO detection in biological samples due to its poor water solubility. DTCS, an analog of DETC, forms a water-soluble iron(II) complex (Fe-DTCS). The Fe-DTCS complex then forms a complex with NO (NO-Fe-DTCS). Dr. Yoshimura successfully obtained two-dimensional ESR images of NO, induced by lipopolysaccharide in mouse peritoneum. DTCS sodium salt (DTCS Na) was used for this experiment because it is less toxic than ammonium salt (sodium salt LD_{50}: 1942 mg/kg; ammonium salt LD_{50}: 765 mg/kg). Since the Fe-DTCS complex is more stable than the other dithiocarbamate complexes in the air or in aqueous solutions, it could be a useful spin-trapping reagent for biochemical research. The Fe-DTCS complex should be used immediately after preparation. An excessive amount of DTCS Na (usually 5 equivalents DTCS Na to $FeSO_4$) is required to make a more stable solution. Dithiocarbamates tend to decompose under physiological conditions to form toxic carbon disulfide.

· Fe Complex formation



DTCS Na — DTCS-Fe complex — Nitrosyl-Fe

VI-4. Nitric Oxide Research: NO Detection

Preparation of Fe(II)-DTCS Complex

1. Dissolve 278 mg $FeSO_4$, $7H_2O$ (ferrous sulfate heptahydrate) with 20 ml water[a] to prepare 50 mM $FeSO_4$ solution.[b]
2. Dissolve 123 mg DTCS Na with 10 ml water[a] to prepare 50 mM DTCS solution.
3. Mix 1 ml DTCS Na solution with 8.8 ml buffer solution[a] (pH 7 or higher). Add 200 µl $FeSO_4$ solution just prior to use.[c]

a) Purge any dissolved oxygen in the water or the buffer by nitrogen gas bubbling for at least 30 min prior to dissolving $FeSO_4$.
b) The $FeSO_4$ solution can be stored at -20 °C for at least 2 months.
c) Fe(II)-DTCS complex is colorless. If the solution is brown, Fe(III)-DTCS may have formed because of dissolved oxygen in the solution. However, the brown solution can still be used for NO trapping.

Preparation of NO-Fe(II)-DTCS Complex

1. Under argon gas flow, add 200 µl of $FeSO_4$ solution to 9.4 ml buffer solution (pH 7 or higher). Then introduce NO into the solution by bubbling NO gas through a glass capillary for 15 min.
2. Add 400 ml of DTCS Na solution to the $FeSO_4$ solution, and continue to introduce NO by bubbling for another 5 min.
3. Remove excess NO with argon gas bubbling for 5 min, and store at -20 °C. The NO-Fe(II)-DTCS solution can be stored at -20 °C for at least 2 months in oxygen-free conditions.

References

U. Westenberger, *et al*, *Free Radic. Res. Commun.*, **11**, 167 (1990); M. Kelm, *et al.*, *Circ. Res.*, **66**, 1561 (1990); J. Iwamoto, *et al.*, *Respiration Physiol.*, **96**, 273 (1994); H. Kosaka, *et al.*, *Biochem. Biophys. Res. Commun.*, **184**, 1119 (1992); A. Komarov, *et al.*, *Biochem. Biophys. Res. Commun.*, **195**, 1191 (1993); C-S. Lai, *et al.*, *FEBS Lett.*, **345**, 120 (1994).

MGD

N-(Dithiocarbamoyl)-*N*-methyl-D-glucamine, sodium salt [CAS: 94161-07-6 (free acid)]

Application: NO detection with ESR

Appearance: white crystalline powder
Purity: >98.0% (HPLC)
MW: 293.34, $C_8H_{16}NNaO_5S_2$

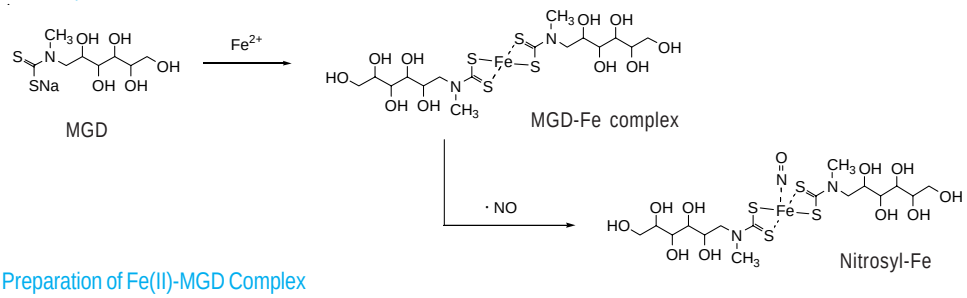
Ordering Information	
Product code	Unit
M323-12	500 mg

Storage Condition	Shipping Condition
0-5 °C, protect from light	ambient temperature

Product Description

MGD is a highly water-soluble dithiocarbamate-type chelator that generates many transitional metal complexes such as Fe and Cu. The diethyldithiocarbamate-Fe^{2+} complex has been used for NO detection by electron spin resonance (ESR). However, the poor solubility of this carbamate in an aqueous solution limits its application. Dr. Lai and others improved the technique using a water-soluble dithiocarbamate-Fe^{2+} complex, MGD-Fe^{2+}. They successfully detected *in vivo* NO of a nitroprusside-injected mouse and NO generated by an LPS injection using *in vivo* ESR. The MGD-Fe^{2+} complex is capable of NO detection under physiological conditions, and dissolved oxygen in the solution does not interfere with NO detection.

Fe Complex formation



Preparation of Fe(II)-MGD Complex

1. Dissolve 278 mg $FeSO_4$, $7H_2O$ (ferrous sulfate heptahydrate) with 20 ml water[a] to prepare 50 mM $FeSO_4$ solution.[b]
2. Dissolve 147 mg MGD with 10 ml water[a] to prepare 50 mM MGD solution.
3. Mix 1 ml MGD solution with 8.8 ml buffer solution[a] (pH 7 or higher) and then add 200 µl $FeSO_4$ solution prior to use.[c]

a) Purge any dissolved oxygen in the water or buffer by nitrogen gas bubbling for at least 30 min prior to dissolving $FeSO_4$.
b) The $FeSO_4$ solution can be stored at -20 °C for at least 2 months.
c) Fe(II)-DTCS is colorless. If the solution is brown, Fe(III)-DTCS may have formed because of dissolved oxygen in the solution. However, the brown solution can still be used for NO trapping.

VI-4. Nitric Oxide Research: NO Detection

Preparation of NO-Fe(II)-MGD Complex

1. Under argon gas flow, add 200 µl $FeSO_4$ solution to 9.4 ml buffer solution (pH 7 or higher). Then introduce NO into the solution by bubbling NO gas through a glass capillary for 15 min.
2. Add 400 µl MGD solution to the $FeSO_4$ solution and continue to introduce NO by bubbling for another 5 min.
3. Remove excess NO with argon gas bubbling for 5 min and store at -20 °C. The NO-Fe(II)-MGD solution can be stored at -20 °C for at least 2 months in oxygen-free conditions.

References

L. A. Shinobu, *et al*, *Acta Pharmacol. et Toxicol*., **54**, 189 (1984); T. Komarov, *et al., Biochem. Biophys. Res. Commun.*, **195**, 1191 (1993); C-S. Lai, *et al., FEBS Lett*., **345**, 120 (1994); P. Giboreau, *et al., J. Org. Chem.*, **59**, 1205 (1994); B. Kalyanaraman, *Methods in Enzymol*., **268(A)**, 168 (1996); H. Yokoyama, *et al., Magn. Reson. Imaging*, **15**, 249 (1997); V. D. Mikoyan, *et al.*, *Biofizika*., **42(2)**, 490 (1997).

VII. Diagnostic Analysis

Introduction

Enzymatic oxidation reactions, based on the hydrogen peroxide and peroxidase reactions, have been utilized over several decades for diagnostic analyses because of their high selectivity and sensitivity. To determine the concentration of a specific element in a biological sample, the specificity of the reaction is necessary. A large number of oxidases have been found and used in detection systems. In most cases, the enzymatic reaction of an oxidase generates hydrogen peroxide, and the concentration of hydrogen peroxide indicates the concentration of a substrate in a reaction solution. Therefore, the concentration of substrate can be determined by measuring the amount of hydrogen peroxide. The amount of hydrogen peroxide is determined by spectrophotometer using an oxidative chromogenic dye and peroxidase. Since peroxidase is one of the most commonly used enzymes for enzyme immunoassays (EIA), several oxidative chromogenic dyes are available for staining tissue sections and membrane filters. It can also be utilized for microplate assays. The stability of these oxidative chromogenic dyes, however, is sometimes low due to the oxidation by oxygen. Dojindo offers stable and water-soluble oxidative chromogenic dyes for the hydrogen peroxide (H_2O_2)-peroxidase assay. The table on page 123 indicates the characteristics of water-soluble aniline derivatives (New Trinder's Reagents) that are used for hydrogen peroxide detection.

Peroxidase Detection

Tetramethylbenzidine (TMBZ) is a commonly utilized oxidative chromogenic dyes for peroxidase detection on a membrane filter or an EIA plate. Peroxidase catalyses the reduction of hydrogen peroxide to generate two water molecules. TMBZ is the electron source for this reduction. Since the oxidized forms of TMBZ has intense colors, peroxidase can be determined colorimetrically. Horseradish peroxidase (HRP) is widely applied in EIA and diagnostic analyses. HRP (brown color) reacts with hydrogen peroxide to form Compound I (yellowish-green color). Then Compound I turns back to HRP by one electron oxidoreduction via Compound II (red color). Most of the chromogenic dyes are easily oxidized with activated oxygen by metals or light. TMBZ is a stable oxidative chromogenic dye utilized for microplate assays. Colorless TMBZ solution is turned blue or greenish-blue by the hydrogen peroxide and peroxidase oxidation reaction. Sulfuric acid (1-2 M) is sometimes added to the assay solution to enhance the color development of oxidized TMBZ. Oxidized TMBZ solution turns from blue to yellow when sulfuric acid is added, and its molar absorptivity is 2 to 3 times that of the blue oxidized TMBZ.

Hydrogen Peroxide Detection

Enzymatic oxidation reactions are highly selective and sensitive enough to measure a specific substrate in complicated mixtures such as biological samples. Due to their selectivity, oxidases have been used in various assay systems established for diagnostic analyses. There are two steps to determine the amount of substrate: 1) the oxidation reaction of the substrate with oxidase, and 2) the detection of hydrogen peroxide generated by the oxidation reaction. Aniline and phenol compounds have been used as oxidative chromogenic reagents for hydrogen peroxide detection. However, they are not stable enough to prepare assay solutions or test papers. New Trinder's Reagents, which are aniline analogs, have propylsulfonic acid (PS) or hydroxypropylsulfonic acid (OS) attached to an amino group of their aniline structure. These PS or OS groups give aniline water solubility and markedly improved its stability. Trinder's reagent requires a coupling reagent such as 4-aminoantipyrin or methylbenzothiazolinonehydrazone (MBTH), to produce a stable dye. Dojindo offers various New Trinder's Reagents for the best combination with oxidase-substrate reactions.

TMBZ

3,3',5,5'-Tetramethylbenzidine [CAS: 54827-17-7]

Application: Peroxidase detection, colorimetric

Appearance: white or pale grayish-brown crystalline powder
Purity: >99.0 % (HPLC)
MW: 240.34, $C_{16}H_{20}N_2$

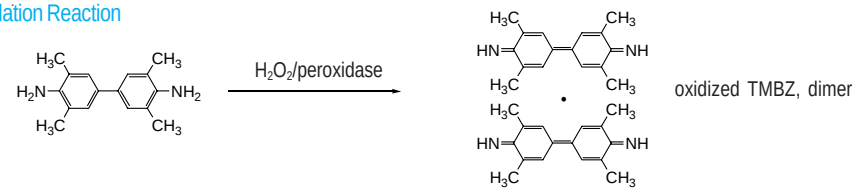
Ordering Information

Product code	Unit
T022-10	1 g
T022-12	5 g

Storage Condition
ambient temperature, protect from light, metal, and moisture

Shipping Condition
ambient temperature

Oxidation Reaction



TMBZ HCl

3,3',5,5'-Tetramethylbenzidine dihydrochloride, dihydrate [CAS: 64285-73-0]

Applicaton: Peroxidase detection, colorimetric

Appearance: white or pale yellow powder
Purity: >98.0% (Titration)
MW: 349.30, $C_{16}H_{22}Cl_2N_2 \cdot 2H_2O$

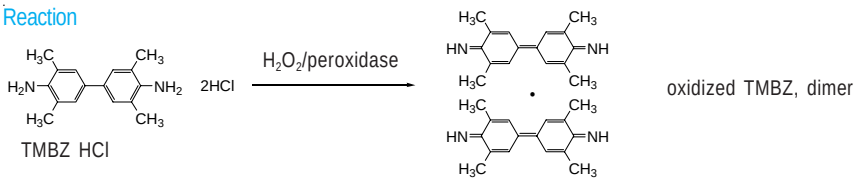
Ordering Information

Product code	Unit
T039-10	1 g

Storage Condition
ambient temperature, protect from light, metal, and moisture

Shipping Condition
ambient temperature

Oxidation Reaction



TMBZ HCl

Product Description of TMBZ Compounds

TMBZ is a chromogenic reagent utilized for peroxidase detection. It has been developed as an alternative to benzidine, which is a carcinogenic chemical. Because of the ortho methyl groups on its benzene ring, TMBZ is not metabolized into highly carcinogenic *o*-hydroxybenzidines or *o,o'*-dihydroxybenzidines. Therefore, TMBZ compounds are much less carcinogenic than benzidine. Though the TMBZ solution is colorless, it turns bluish-green (λmax: 655 nm) in the presence of hydrogen peroxide and peroxidase. The structure of this bluish-green complex is thought to be a radical form of two oxidized TMBZ molecules. TMBZ HCl is a hydrochloride form of TMBZ that is readily soluble in water (100 mg TMBZ HCl per 10 ml water).

Assay Protocol

1. Dissolve 6 mg of TMBZ with 1 ml DMSO to prepare 100X TMBZ solution.
2. Mix 5 µl of 30% hydrogen peroxide solution with 1 ml PBS to prepare 200X H_2O_2 solution.
3. Add 10 µl of 100X TMBZ solution and 5 µl of 200X H_2O_2 solution to 1 ml PBS to prepare staining solution.*
4. Add 100-200 µl of the staining solution to each well of a 96-well microplate. Incubate the plate at room temperature or at 37 °C for 5 min to 1 hour.
5. Wash the sample with PBS several times to stop the staining reaction.

* For the best results, modification of the final concentration of TMBZ and hydrogen peroxide may be necessary. The staining solution is not stable. Please prepare fresh solution prior to use.

References

TMBZ, TMBZ HCl: P. E. Thomas, *et al.*, *Anal. Biochem.*, **75**, 168 (1976); R. M. Jaffe, *et al.*, *J. Lab. Clin. Med.*, **93**, 879 (1979); S. Madersbacher, *et al.*, *J. Immunol. Methods*, **138**, 121 (1992); F. Suzuki, *et al.*, *Biomed. Res.*, **13**, 321 (1992); H. J. D. McManus, *et al.*, *J. Phys. Chem.*, **97**, 255 (1993); F. H. Pujol, *et al.*, *J. Immunoassay*, **14**, 21 (1993); F. B. Serrat, *Talanta*, **41**, 2091 (1994); R. A. Jennings, *et al.*, *Brain Res.*, **683**, 159 (1995); C. Mazzia, *et al.*, *Neuroscience*, **80**, 925 (1997); G. W. Plant, et al., Cell Transplant, **7**, 381 (1998); T. Li, et al., *Brain Res.*, **783**, 51 (1998). M. I. Romero, *et al.*, *J. Histochem. Cytochem.*, **47**, 265 (1999); A. M. Yates, *et al.*, *J. Immunoassay*, **20**, 31 (1999).

VII-1. Diagnostic Analysis: Peroxidase

SAT Blue

N,N'-Bis(2-hydroxy-3-sulfopropyl)tolidine, disodium salt, and other proprietary ingredients

Application: Peroxidase detection, colorimetric

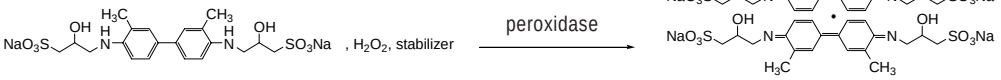
Appearance: colorless or slightly brown solution

Storage Condition	Shipping Condition
0-5 °C	ambient temperature

Ordering Information

Product code	Unit
S309-10	50 ml

Oxidation Reaction



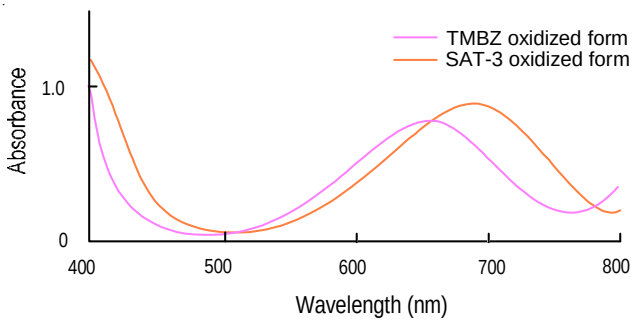
SAT-3 — oxidized SAT-3, dimer

Product Description

SAT Blue is a ready-to-use solution of SAT-3 and other ingredients. SAT Blue contains no organic solvents. SAT Blue produces an intense green dye if it is mixed with peroxidase. The maximum wavelength of oxidized SAT Blue is 675 nm (Fig. 7-1). SAT Blue is suitable for a microplate enzyme immunoassay based on the peroxidase activity detection mechanism. Like TMBZ, oxidized SAT-3 forms a yellow dye by the addition of 2 M sulfuric acid.

Assay Protocol

1. Prepare an assay plate with peroxidase activity using a peroxidase-labeled antibody or peroxidase-labeled streptavidine.
2. Wash each well 4-5 times with 200-300 µl PBST (0.05% Tween 20 PBS buffer).
3. Add 100-200 µl SAT-Blue solution to each well and incubate at room temperature or 37 °C for 15-30 min.
4. Measure the O.D. at 650 nm or 670 nm.



Condition:
50 mM phosphate buffer, pH 7.0
1 mM substrate
10 mM hydrogen peroxide
3.3 U/ml peroxidase
20 °C for 5 min incubation

Fig. 7-1 Absorption Spectrum of Oxidized Form

New Trinder's Reagents

Application: Hydrogen peroxide detection, colorimetric

Features: Highly water-soluble
Stable aniline analogs
Wide pH range in color development and oxidization reactions

Product Description

New Trinder's Reagents, which are highly water-soluble aniline derivatives, are widely used in diagnostic assays and biochemical examinations. They have several advantages over conventional chromogenic reagents in the colorimetric determination of hydrogen peroxide activity. New Trinder's Reagents are stable enough to use in both solution and test strip detection systems. New Trinder's reagents form highly stable purple or blue dyes through an oxidative coupling reaction with 4-Aminoantipyrine (4-AA) or 3-Methylbenzothiazolinone hydrazone (MBTH) in the presence of hydrogen peroxide and peroxidase (Table 7-1).The molar absorptivity of the coupled dye with MBTH is 1.5 to 2 times higher than that with 4-AA; however, 4-AA solution is more stable than MBTH solution. Enzymatic oxidization of a substrate by its oxidase produces hydrogen peroxide. The hydrogen peroxide concentration corresponds to the substrate concentration. Therefore, the amount of the substrate can be determined by the color development of an oxidative coupling reaction. Glucose, alcohol, acyl-CoA, and cholesterol are utilized for the detection of those substrates coupled with New Trinder's Reagents and 4-AA. There are 10 kinds of New Trinder's reagents available. The table below shows the maximum wavelength and molar absorptivity of each oxidized New Trinder's Reagent complex with 4-AA. Among the New Trinder's reagents, TOOS is the most frequently used to develop assay systems. However, experimentation with the different New Trinder's reagents will be necessary to develop the best detection system for a given substrate.

Table 7-1. Characteristics of Dyes Derived from New Trinder's Reagent

New Trinder's Reagent	λmax (nm), pH	Molar Absorptivity
ADOS	542, 5.5-9.5	2.72 x 10^4
ADPS	540, 5.9-9.5	2.79 x 10^4
ALPS	561, 6.5-7.5	4.13 x 10^4
DAOS	593, 5.5-9.5	1.75 x 10^4
HDAOS	583, 5.5-9.5	1.73 x 10^4
MADB	630, 5.5-9.5	1.65 x 10^4
MAOS	630, 5.5-9.5	2.25 x 10^4
TODB	550, 5.5-9.5	3.80 x 10^4
TOOS	555, 5.5-9.5	3.92 x 10^4
TOPS	550, 5.5-9.5	3.74 x 10^4

ADOS

N-Ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methoxyaniline, sodium salt, dihydrate [CAS: 82692-96-4]

Application: Hydrogen peroxide detection, colorimetric

Appearance: white or slightly grayish-yellow powder
Purity: >97.0% (Absorbance)
MW: 347.36, $C_{12}H_{18}NNaO_5S$, $2H_2O$

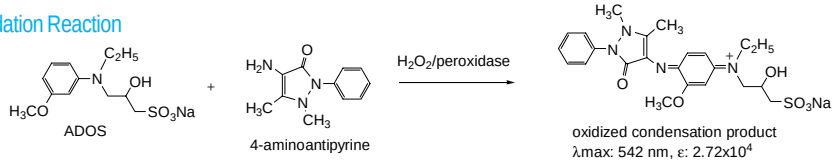
Ordering Information

Product code	Unit
OC01-10	1 g

Storage Condition
0-5 °C, protect from moisture and light

Shipping Condition
ambient temperature

Oxidation Reaction



VII-2. Diagnostic Analysis: Hydrogen Peroxide

ADPS

N-Ethyl-*N*-(3-sulfopropyl)-3-methoxyaniline, sodium salt, monohydrate [CAS: 82611-88-9]

Application: Hydrogen peroxide detection, colorimetric

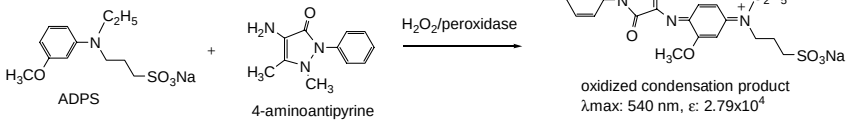
Appearance: white or slightly-brown powder
MW: 313.35, $C_{12}H_{18}NNaO_4S$, H_2O

Ordering Information	
Product code	Unit
OC02-10	1 g

Storage Condition
0-5 °C, protect from moisture and light

Shipping Condition
ambient temperature

Oxidation Reaction



ALPS

N-Ethyl-*N*-(3-sulfopropyl)aniline, sodium salt [CAS: 82611-85-6]

Application: Hydrogen peroxide detection, colorimetric

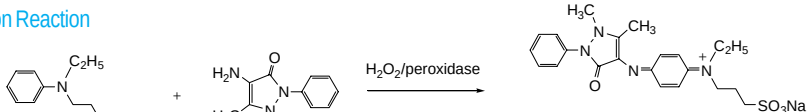
Appearance: white or slightly grayish-yellow powder
MW: 265.31, $C_{11}H_{16}NNaO_3S$

Ordering Information	
Product code	Unit
OC04-10	1 g

Storage Condition
0-5 °C, protect from moisture and light

Shipping Condition
ambient temperature

Oxidation Reaction



DAOS

N-Ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline, sodium salt [CAS: 83777-30-4]

Application: Hydrogen peroxide detection, colorimetric

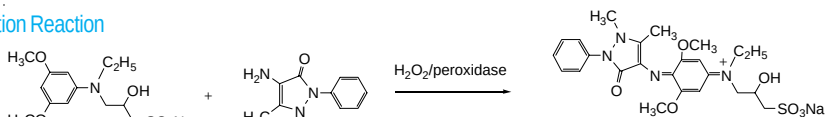
Appearance: white or slightly grayish-yellow powder
Purity: >97.0% (Absorbance)
MW: 341.36, $C_{13}H_{20}NNaO_6S$

Ordering Information	
Product code	Unit
OC06-10	1 g

Storage Condition
0-5 °C, protect from moisture and light

Shipping Condition
ambient temperature

Oxidation Reaction



HDAOS

N-(2-Hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline, sodium salt [CAS: 82692-88-4]

Application: Hydrogen peroxide detection, colorimetric

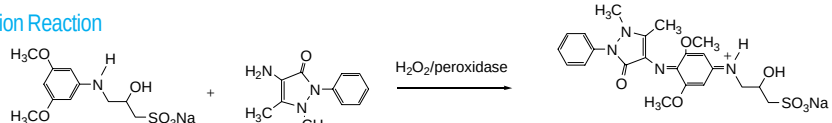
Appearance: white or off-white powder
Purity: >98.0% (Absorbance)
MW: 313.30, $C_{11}H_{16}NNaO_6S$

Ordering Information	
Product code	Unit
OC08-10	1 g

Storage Condition
0-5 °C, protect from moisture and light

Shipping Condition
ambient temperature

Oxidation Reaction



MADB

N,N-Bis(4-sulfobutyl)-3,5-dimethylaniline, disodium salt

Application: Hydrogen peroxide detection, colorimetric

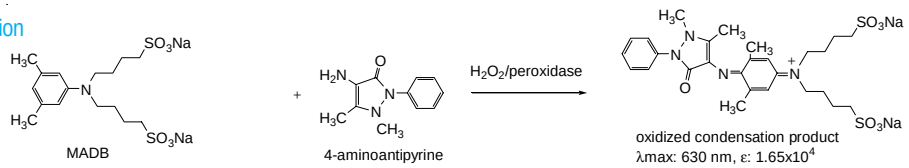
Appearance: white or slightly brown powder
Purity: >97.0% (HPLC)
MW: 437.48, $C_{16}H_{25}NNa_2O_6S_2$

Ordering Information	
Product code	Unit
OC21-10	1 g

Storage Condition
0-5 °C, protect from moisture and light

Shipping Condition
ambient temperature

Oxidation Reaction



MAOS

N-Ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3,5-dimethylaniline, sodium salt, monohydrate [CAS: 82692-97-5]

Application: Hydrogen peroxide detection, colorimetric

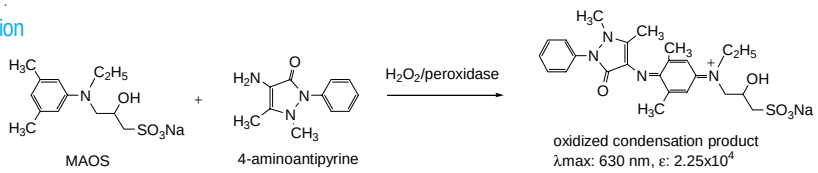
Appearance: white or slightly yellowish-pink powder
MW: 327.37, $C_{13}H_{20}NNaO_4S$, H_2O

Ordering Information	
Product code	Unit
OC11-10	1 g

Storage Condition
0-5 °C, protect from moisture and light

Shipping Condition
ambient temperature

Oxidation Reaction



TODB

N,N-Bis(4-sulfobutyl)-3-methylaniline, disodium salt

Application: Hydrogen peroxide detection, colorimetric

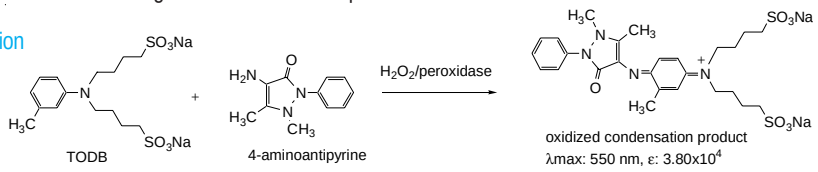
Appearance: white or slightly grayish-yellow powder
Purity: >98.0% (HPLC)
MW: 423.46, $C_{15}H_{23}NNa_2O_6S_2$

Ordering Information	
Product code	Unit
OC22-10	1 g

Storage Condition
0-5 °C, protect from moisture and light

Shipping Condition
ambient temperature

Oxidation Reaction



TOOS

N-Ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline, sodium salt, dihydrate [CAS: 82692-93-1]

Application: Hydrogen peroxide detection, colorimetric

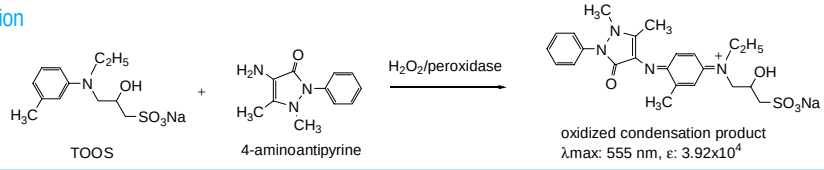
Appearance: white or slightly blue powder
Purity: >98.0% (Absorbance)
MW: 331.36, $C_{12}H_{18}NNaO_4S$, $2H_2O$

Ordering Information	
Product code	Unit
OC13-10	1 g

Storage Condition
0-5 °C, protect from moisture and light

Shipping Condition
ambient temperature

Oxidation Reaction



VII-2. Diagnostic Analysis: Hydrogen Peroxide

TOPS

N-Ethyl-*N*-(3-sulfopropyl)-3-methylaniline, sodium salt, monohydrate [CAS: 40567-80-4]

Application: Hydrogen peroxide detection, colorimetric

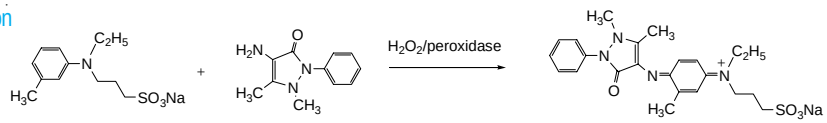
Appearance: white or slightly brown powder
Purity: >97.0% (Absorbance)
MW: 297.34, $C_{12}H_{18}NNaO_3S$, H_2O

Ordering Information	
Product code	Unit
OC14-10	1 g

Storage Condition
0-5 °C, protect from moisture and light

Shipping Condition
ambient temperature

Oxidation Reaction



Preparation of Assay Solution - TOOS

1. Dissolve 20 mg TOOS with 10 ml PBS to prepare 6.6 mM TOOS solution.
2. Dissolve 14 mg 4-aminoantipyrin (AA) with 10 ml PBS to prepare 6.6 mM 4-AA solution.
3. Prepare 2 U/ml horseradish peroxidase solution with PBS.
4. Mix the same volume of each solution together to prepare assay solution. Store the assay solution at 4 °C with protection from light.

Assay Protocol

1. Prepare sample solutions for the enzymatic oxidation reaction. The pH range of the buffer solution should be from 5.5-9.5.
2. Prepare standard solutions containing known amounts of substrate using the same buffer.
3. Add the appropriate units of oxidase to the sample solution, followed by an addition of the same volume of the assay solution.
4. Incubate the mixture at room temperature or at 37 °C for 30 min to 1 hour.
5. Measure the O.D. at 555 nm.
6. Prepare a standard curve, and determine the substrate concentration in the sample solution.

References

K. Tamaoku, *et al.*, *Chem. Pharm. Bull.*, **30**, 2942 (1982); K. Tamaoku, *et al.*, *Anal. Chim. Acta*, **136**, 121 (1982); B. C. Madsen, *et al.*, *Anal. Chem.*, 56, 2849 (1984); S. Satomura, *et al.*, *Clin. Chem.*, **31**, 1380 (1985). K. Uchida, *et al.*, *Anal. Sci.*, **33**, 181 (1987); K. S. Jonson, *et al.*, *Anal. Chim. Acta*, **201**, 83 (1987); N. Shimojo, *et al.*, *Clin. Chem.*, **35**, 1992 (1989); Y. Kayamori, *et al.*, *Clin. Biochem.*, **30**, 595 (1997).

VII-3. Diagnostic Analysis:Tetrazolium Salts

Introduction

Several tetrazolium salts have been developed to determine dehydrogenase activity. Tetrazolium salts are unique compounds with a slightly yellow color that generate intense yellow to purple colored formazan dyes when reduced. Nitro blue tetrazolium salt (NTB or NitroTB) has been widely used for dehydrogenase detection on agarose gels due to its extremely low water solubility. Tetrazolium salts have been used in a variety of quantitative assays, such as blood glucose monitoring, lactate dehydrogenase detection, and glucose-6-phosphate dehydrogenase detection. The solubility of these tetrazolium salts, however, is so poor that detergents or organic solvents are always required to solubilize their formazan dyes.

Dojindo has developed several water-soluble tetrazolium salts (WSTs) that are utilized for cell viability assays, dehydrogenase detection, superoxide detection, and reduced sugar detection. For example, WST-8 is used in Cell Counting Kit-8 and WST-1 is used in SOD Assay Kit-WST. The maximum wavelengths of formazan dyes are shown in the table. The water-solubility of WSTs ranges from 10 mM to 100 mM, except for WST-10. The water solubility of WST-10 is lower than the other WSTs. Therefore, WST-10 requires DMSO to solubilize in buffer solutaion. The spectra of formazan dyes, especially bis-type dyes, are broad and shift according to the progress of the reaction. The spectra of formazan dyes also drastically change in alkaline conditions such as at pH 11 to 12. Most of the water-soluble formazan dyes turn blue at higher pH levels. Since tetrazolium salts are not stable in light, it is necessary to protect them from light during storage.

The mechanism of the dehydrogenase detection is shown in Fig. 7-2. Dehydrogenase oxidizes its substrate and reduces its co-enzyme, β-nicotinamidoadenine-dinucleotide (NAD) or β-nicotinamidoadenine dinucleotide-phosphate (NADP).Then the electron of NADH or NADPH is transformed to the tetrazolium salt via an electron mediator, such as 1-Methoxyphenazine methosulfate (1-Methoxy PMS) or diaphorase. 1-Methoxy PMS is one of the most stable electron mediators and has no selectivity when reacting with NADH and NADPH. On the other hand, diaphorase has more specificity with NAD(P) reaction and with certain types of tetrazolium salt.

Table 7-2. Molar Absorptivity (ε) of Formazan Dyes

Tetrazolium Salt	Formazan Dye	
water-soluble type	ε	λ max
WST-1	$3.7x10^4$	438 nm
WST-3	$3.1x10^4$	433 nm
WST-4	$1.0x10^4$	530 nm
WST-5	$2.7x10^4$	550 nm
WST-8	$3.1x10^4$	460 nm
WST-9	$1.6x10^4$	490 nm
WST-10	$1.0x10^4$	530 nm
WST-11	$3.8x10^4$	474 nm
water-insoluble type	ε	λ max
INT	$1.5x10^4$	490 nm
NTB	$3.6x10^4$	530 nm
TB	$2.6x10^4$	525 nm

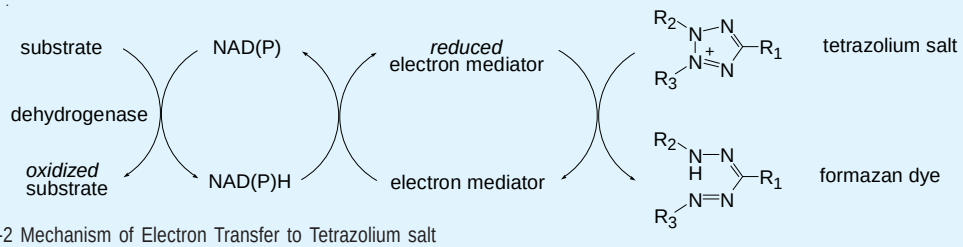


Fig. 7-2 Mechanism of Electron Transfer to Tetrazolium salt

WST-1

2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt [CAS: 150849-52-8]

Application: NADH and NADPH detection, superoxide detection

Appearance: pale yellow or pale brown powder
Purity: pass test (TLC)
MW: 651.35, $C_{19}H_{11}IN_5NaO_8S_2$

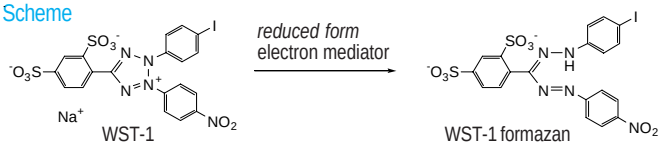
Storage Condition
0-5 ºC, protect from light

Shipping Condition
ambient temperature

Ordering Information

Product code	Unit
W201-10	100 mg
W201-12	500 mg

Reaction Scheme



VII-3. Diagnostic Analysis: Tetrazolium Salts

WST-3

2-(4-Iodophenyl)-3-(2,4-dinitrophenyl)-5-(2,4-disulfophenyl)-*2H*-tetrazolium, monosodium salt

Application: NADH and NADPH detection

Appearance: pale yellowish-brown or pale brown powder
Purity: pass test (TLC)
MW: 696.34, $C_{19}H_{10}IN_6NaO_{10}S_2$

Ordering Information

Product code	Unit
W202-10	100 mg

Storage Condition
0-5 °C, protect from light

Shipping Condition
ambient temperature

Reaction Scheme

WST-4

2-Benzothiazolyl-3-(4-carboxy-2-methoxyphenyl)-5-[4-(2-sulfoethylcarbamoyl)phenyl]-*2H*-tetrazolium [CAS: 178925-54-7]

Application: NADH and NADPH detection

Appearance: yellow or brown powder
Purity: pass test (TLC)
MW: 580.59, $C_{25}H_{20}N_6O_7S_2$

Ordering Information

Product code	Unit
W203-10	100 mg

Storage Condition
0-5 °C, protect from light

Shipping Condition
ambient temperature

Reaction Scheme

WST-5

2,2'-Dibenzothiazolyl-5,5'-bis[4-di(2-sulfoethyl)carbamoylphenyl]-3,3'-(3,3'-dimethoxy-4,4'-biphenylene)ditetrazolium, disodium salt [CAS: 178925-55-8]

Application: NADH and NADPH detection

Appearance: yellow or slightly greenish-brown powder
Purity: pass test (TLC)
MW: 1331.35, $C_{52}H_{44}N_{12}Na_2O_{16}S_6$

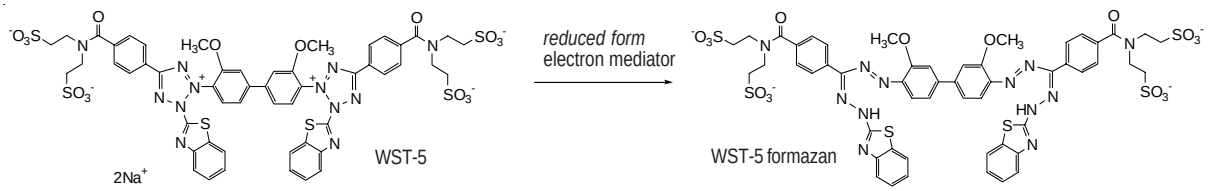
Ordering Information

Product code	Unit
W204-10	100 mg

Storage Condition
0-5 °C, protect from light

Shipping Condition
ambient temperature

Reaction Scheme



WST-9

2-(4-Nitrophenyl)-5-phenyl-3-[4-(4-sulfophenylazo)-2-sulfophenyl]-*2H*-tetrazolium, monosodium salt

Application: NADH and NADPH detection

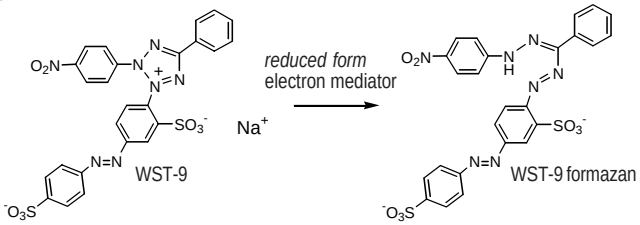
Appearance: orange or orange-brown powder
Purity: pass test (TLC)
MW: 629.56, $C_{25}H_{16}N_7NaO_8S_2$

Ordering Information	
Product code	Unit
W217-10	100 mg

Storage Condition
0-5 °C, protect from light

Shipping Condition
ambient temperature

Reaction Scheme



WST-10

2.5-Di-(4-nitrophenyl)-3-[4-(4-sulfophenylazo)-2-sulfophenyl]-*2H*-tetrazolium, monosodium salt

Application: NADH and NADPH detection

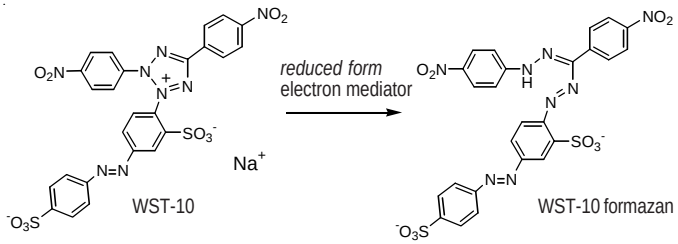
Appearance: orange or orange-brown powder
Purity: pass test (TLC)
MW: 674.56, $C_{25}H_{15}N_8NaO_{10}S_2$

Ordering Information	
Product code	Unit
W218-10	100 mg

Storage Condition
0-5 °C, protect from light

Shipping Condition
ambient temperature

Reaction Scheme



WST-11

2-(4-Nitrophenyl)-5-(2-sulfophenyl)-3-[4-(4-sulfophenylazo)-2-sulfophenyl]-*2H*-tetrazolium, disodium salt

Application: NADH and NADPH detection

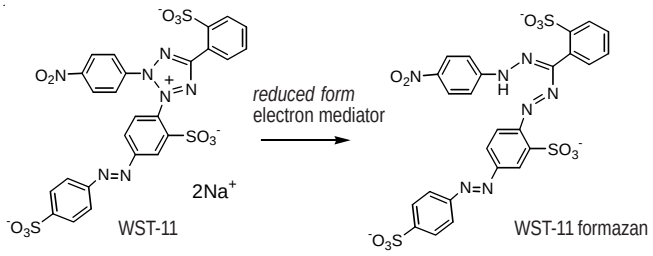
Appearance: orange or orange-brown powder
Purity: pass test (TLC)
MW: 731.60, $C_{25}H_{15}N_7Na_2O_{11}S_3$

Ordering Information	
Product code	Unit
W219-10	100 mg

Storage Condition
0-5 °C, protect from light

Shipping Condition
ambient temperature

Reaction Scheme



VII-3. Diagnostic Analysis: Tetrazolium Salts

Product Description of WSTs

Water-soluble tetrazolium salts (WSTs) were developed by introducing positive or negative charges and hydroxy groups to the phenyl ring of the tetrazolium salt. Positive charges, such as trialkylammonio groups, improve the water-solubility of the formazan dye. However, a large cation is easily precipitated out with organic anions such as carboxylate or phosphate. Though a hydroxy group also improved the water-solubility of the tetrazolium salt, the corresponding formazan dye was not sufficiently water-soluble. Dojindo's WSTs have sulfonate groups added directly or indirectly to the phenyl ring to improve water-solubility. Dojindo also offers several newly developed phenlyazo-type tetrazolium salts, which are easily reduced with NADH or other reducing agents to give orange or purple formazan dyes. Due to the phenylazo group, the color changes with heavy metal ion. Since the water solubility of Dojindo's WSTs are high, 10 mM to 100 mM solutions can be prepared, except WST-10.

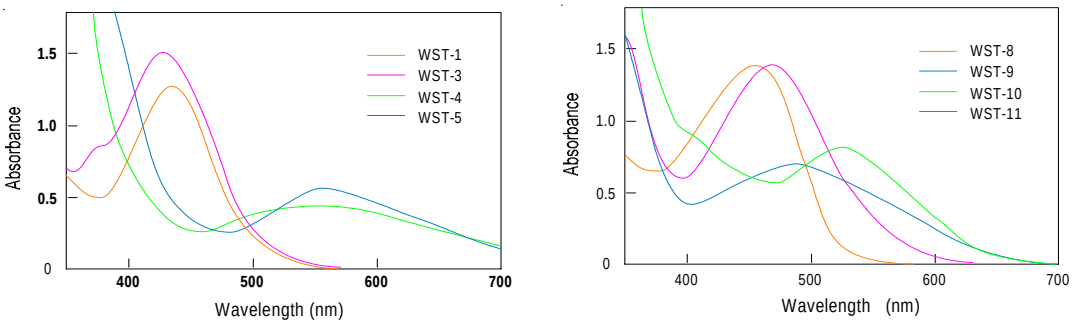


Fig 7-3 Absorption Spectra of WST Formazan Dyes

References

WST-1: M. Ishiyama, *et al.*, *Chem. Pharm. Bull*, **41**, 1118 (1993), M. Ishiyama, *et al.*, *In Vitro Toxicol*, **8**, 187 (1995), S. Shirahata, *et al.*, *Biosci. Biotech. Biochem.*, **59**, 345 (1995), K. Teruya, *et al.*, *Biosci. Biotech. Biochem.*, **59**, 341 (1995), Y. Kayamori, *et al.*, *Clin. Biochem.*, **30**, 595 (1997); W. Zhang, *et al.*, *FASEB J.*, **20**, 2496 (2006); S. Yang, *et al.*, *J. Pharmacol. Exp. Ther.*, **319**, 595 (2006); D. Inokuma, *et al.*, *Stem Cells*, **24**, 2810 (2006); T. Ishikawa, *et al.*, *Arterioscler. Thromb. Vasc. Biol.*, **26**, 1998 (2006).
WST-3: M. Ishiyama, *et al.*, *Analyst*, **120**, 113 (1995).
WST-4, 5: M. Ishiyama, *et al.*, *Anal. Sci.*, **12**, 515 (1996).

INT

2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium chloride [CAS: 146-68-9]

Application: Dehydrogenase detection, staining

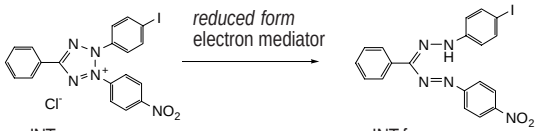
Appearance: pale yellow or yelowish-orange powder
Purity: >95.0% (Titration)
MW: 505.70, $C_{19}H_{13}ClIN_5O_2$

Ordering Information	
Product code	Unit
I003-10	1 g

Storage Condition
ambient temperature, protect from light

Shipping Condition
ambient temperature

Reaction Scheme



MTT

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide [CAS: 2348-71-2]

Application: Dehydrogenase detection, staining

Appearance: yellow or yellowish-orange powder
Purity: >97.0% (absorbance)
Molar absorptivity: >8,250 at 375 nm
MW: 414.32, $C_{18}H_{16}BrN_5S$

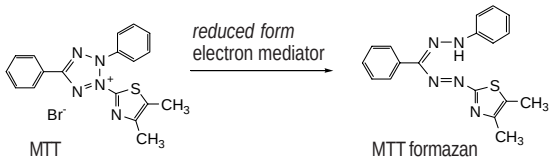
Ordering Information	
Product code	Unit
M009-08	100 mg
M009-10	1 g

Storage Condition
ambient temperature, protect from light

Shipping Condition
ambient temperature

VII-3. Diagnostic Analysis: Tetrazolium Salts

Reaction Scheme



Product Description

MTT slightly dissolves in methanol, but has very low solubility in ethanol. It barely dissolves in ether, acetone, or ethyl acetate. MTT is utilized for cell viability detection. Since MTT has a positive charge, it can pass through a cell membrane and is reduced by mitochondria to form a purple color formazan dye. Organic solvent is requried to dissovle MTT formazan dyes.

References

T. F. Slater, *et al.*, *Biochim. Biophys. Acta*, **77**, 383 (1963); T. Mosmann, *J. Immunol. Method*, **65**, 55 (1983).

Nitro-TB

3,3'-[3,3'-Dimethoxy-(1,1'-biphenyl)-4,4'-diyl]-bis[2-(4-nitrophenyl)-5-phenyl-*2H*-tetrazolium chloride] [CAS: 298-83-9]

Application: Dehydrogenase detection, staining

Appearance: yellow crystalline powder
Purity: >98.0%
Molar absorptivity: >63,000 at 257 nm
MW: 817.64, $C_{40}H_{30}Cl_2N_{10}O_6$

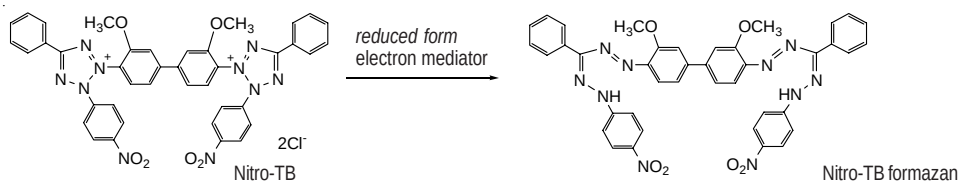
Ordering Information

Product code	Unit
N011-10	100 mg
N011-12	1 g

Storage Condition
ambient temperature, protect from light and moisture

Shipping Condition
ambient temperature

Reaction Scheme



Product Description

Nitro-TB is dissolved by hot water or hot methanol. Other organic solvents such as acetone and ether cannot solubilize Nitro-TB. Nitro-TB is easily reduced by dehydrogenase to purple formazan dye aggregates. It is used for dehydrogenase detection on agarose gels.

References

K-C. Tsou, *et al.*, *J. AM. Chem. Soc.*, **78**, 6139 (1956); J. R. Baker, *et al.*, *Clin. Chem.*, **39**, 2460 (1993).

TB

3,3'-[3,3'-Dimethoxy-(1,1'-biphenyl)-4,4'-diyl]-bis(2,5-diphenyl-*2H*-tetrazolium chloride) [CAS: 1871-22-3]

Application: Dehydrogenase detection, staining

Appearance: pale yellow or pale orange-yellow powder
Molar absorptivity: >52,500 at 254 nm
MW: 727.64, $C_{40}H_{32}Cl_2N_8O_2$

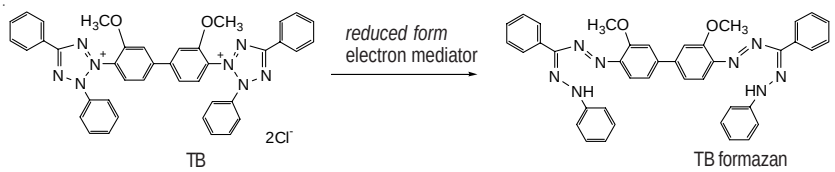
Ordering Information

Product code	Unit
T012-10	1 g

Storage Condition
ambient temperature, protect from light and moisture

Shipping Condition
ambient temperature

Reaction Scheme



VII-4. Diagnostic Analysis: Electron Mediator

Product Description

TB slightly dissolves in water, but does not dissolve easily in acetone, ethyl acetate, and ether. Other organic solvents such as methanol, ethanol, and tetrachloromethane can solubilize TB pretty well. Since TB is easily reduced with dehydrogenase to generate reddish-purple formazan dye aggregates, it is used for dehydrogenase detection in tissue samples.

References

K-C. Tsou, *et al.*, *J. Am. Chem. Soc.*, **78**, 6139 (1956); S. S. Karmarker, *et al.*, *J. Org. Chem.*, **25**, 575 (1961); J. E. Sinsheimer, *et al.*, *Anal. Chem.*, **37**, 566 (1965); E. P. Altmann, *Histochemie*, **17**, 319 (1969).

1-Methoxy PMS

1-Methoxy-5-methylphenazinium methylsulfate [CAS: 65162-13-2]

Application: Electron mediator for NAD(P)H-tetrazolium salt

Appearance: dark red or reddish-purple powder
Molar absorptivity: >2,700 at 505 nm
MW: 336.36, $C_{15}H_{16}N_2O_5S$

Ordering Information

Product code	Unit
M003-10	100 mg

Storage Condition

ambient temperature, protect from light and moisture

Shipping Condition

ambient temperature

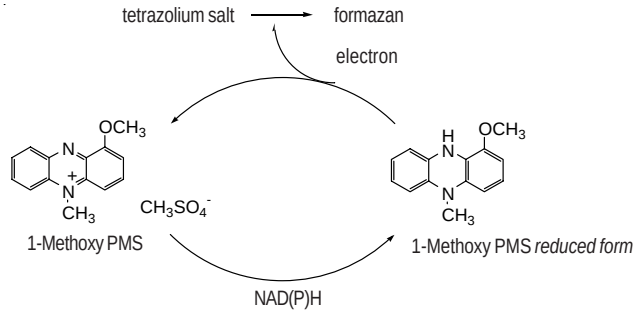


Fig. 7-4 Electron transfer system

Product Description

1-Methoxy PMS is easily dissolved by water and alcohol. Its redox potential is +63 mV. Though phenazinium methylsulfate (PMS) is commonly used as an electron carrier for NADH-tetrazolium, the stability of PMS is very poor. However, 1-Methoxy PMS solution can be stored at room temperature for over 3 months without protection from light. An electron of NAD(P)H is transfered by 1-Methoxy PMS to tetrazolium salt (Fig. 7-4). Therefore, it is a useful reagent for NAD(P)H-tetrazolium-based assay systems.

References

R. Hisada, *et al.*, *J. Biochem.*, **82**, 1469 (1977); S. Nakamura, *et al.*, *Clin. Chim. Acta*, **101**, 321 (1980); R. Hisada *et al.*, *J. Appl. Biochem.*, **3**, 535 (1981); C. J. F. V. Noorden, *J. Histochem. Cytochem*, **30**, 12 (1982); H. Tsuge, *Arch. Biochem. Biophys.*, **217**, 479 (1982); M. Rabinovitch, *J. Exp. Med.*, **155**, 415 (1982).

Protein Quantification Kit - Rapid

Application: Protein concerntration determination

Features: CBB-based colorimetric microplate assay
Wide detection range: 0.1-2,000 mg per ml
Rapid detection in 30 sec

Ordering Information

Product code	Unit
PQ01-10	500 tests
PQ01-12	2,500 tests

Contents of the Kit

500 tests

CBB solution ..... 2 bottles Standard BSA solution (4 mg/ml) ..... 1 bottle

2500 tests

CBB solution ..... 1 bottle Standard BSA solution (4 mg/ml) ..... 2 bottles

Storage Condition: 0-5 ºC

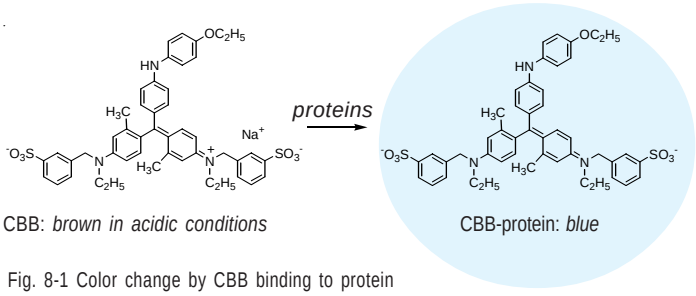
Shipping Condition: ambient temperature

Required Equipment and Materials

plate reader with 600 nm filter, 96-well clear plate, 10 µl and 200 µl adjustable pipettes, multi-channel pipette

Product Description

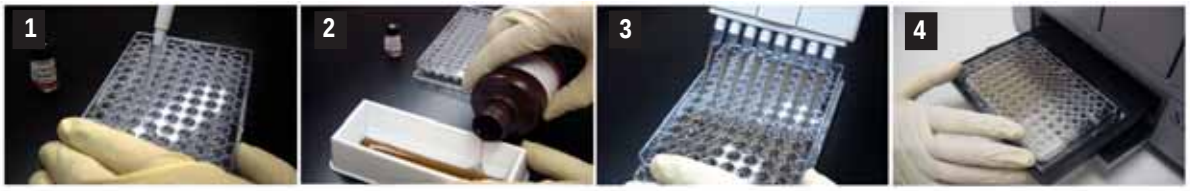
Protein Quantification Kit-Rapid (PQK-Rapid) is a complete, ready-to-use microtiter plate assay kit for the colorimetric detection of proteins. Unlike other detection methods, such as bicinchoninic acid (BCA) and Lowry methods, this assay kit does not use heavy metal ions. PQK-Rapid is based on the color change of Coomassie Brilliant Blue-G in the range of 465 to 595 nm. Coomassie Brilliant Blue-G binds to basic or aromatic amino acid residues of proteins in acidic conditions (Fig. 8-1). Since this kit requires only 30 seconds of incubation time, the measurement of protein concentration can be carried out within minutes. The detection range of PQK-Rapid is from 0.5 to 2000 µg/ml. Reducing or chelating agents such as ascorbic acid or EDTA will not interfere with the assay.



CBB: *brown in acidic conditions* CBB-protein: *blue*

Fig. 8-1 Color change by CBB binding to protein

Procedure of The Assay



1. Add 6 µl of sample or BSA solution to each well.
2. Add CBB solution to a reservoir.
3. Add 300 µl of CBB solution to each well.
4. Read O.D. at 600 nm.

Protocol: Standard Assay

1. Serially dilute the 4 mg per ml standard BSA solution with water to prepare various concentrations of BSA solution (2 mg/ml, 1 mg/ml, 500 g/ml, 250 µg/ml, 125 µg/ml, 63 µg/ml, 32 µg/ml, and 0).
2. Add 6 µl of the various concentration of BSA solution or sample solution to each well and mix.
3. Add 300 µl CBB solution to each well.
4. Incubate the plate at room temperature for 30 sec to 1 min.
5. Measure the absorbance of each well at 570-600 nm with a microplate reader.
6. Subtract the absorbance of the blank solution (0 BSA solution) from the absorbance of each well.
7. Plot the concentration of BSA on the X-axis and the absorbance value on the Y-axis to prepare a calibration curve.
8. Use the calibration curve to determine the protein concentration of the sample.

VIII. Protein Detection

Protocol: High Sensitivity Assay

1. Serially dilute 4 mg per ml standard BSA solution with water to prepare various concentrations of BSA solution (50 µg/ml, 25 µg/ml, 12.5 µg/ml, 6.3 µg/ml, 3.2 µg/ml, 1.6 µg/ml, 0.8 µg/ml, and 0).
2. Add 150 µl CBB solution to each well.
3. Add 150 µl of the various concentrations of BSA solution or sample solution to each well and mix.
4. Incubate the plate at room temperature for 30 sec to 1 min.
5. Measure the absorbance of each well at 570-600 nm with a microplate reader.
6. Subtract the absorbance of the blank solution (0 BSA solution) from the absorbance of each well.
7. Plot the concentration of BSA on the X-axis and the absorbance value on the Y-axis to prepare a calibration curve (Fig. 7-5).
8. Use the calibration curve to determine the protein concentration of the sample.

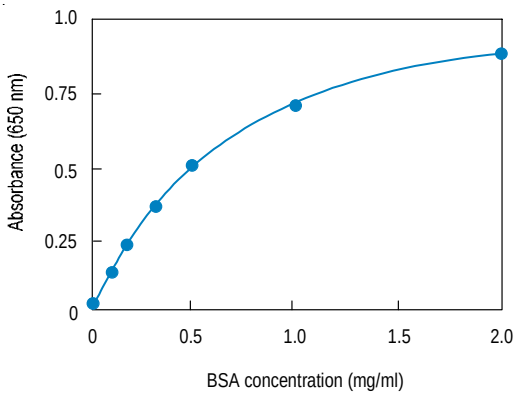


Fig. 8-2 Typical calibration curve prepared using BSA standard solution

Table 8-1 Materials That Interfere with the Assay

Chemical	Interference Concentration	Chemical	Interference Concentration	Chemical	Concentration
Detergents					
Briji 35	0.125%	Brij 56	0.025%	Brij 58	0.005%
Triton X-100	0.125%	Triton X-114	0.125%	Tween 20	0.25%
Tween 80	0.1%	SDS	0.1%	CHAPS	4%
CHAPSO	4%	MEGA 10	4%	Octylglucoside	0.5%
Organic solvents					
ethanol	10%	isopropanol	10%	DMSO	10%
Chelating agents					
EDTA	0.4 M	DTPA	0.4 M	—	
Salts					
NaCl	2 M	KCl	2 M	NaOAc	0.4 M
NaHCO3	0.1 M				
Buffers					
citrate, pH 5	0.125 M	MES, pH 6.1	0.125 M	Tris, pH 7.4	62.5 mM
PBS	no interference	HEPES, pH 7.5	0.125 M	CHES, pH 9	0.125 M
Reducing agents					
glucose	2 M	glutathione	40 mM	ascorbic acid	0.4 M
DTT	1 M	2-mercaptoethanol	1.3 M		

FAQ

◆ My sample contains detergent. Can I use this kit?
Yes, if the concentration of the detergent in the sample solution is lower than the level indicated in the table above. Dilute your sample if the concentration of the detergent in the sample is over this level.

◆ My sample contains reducing agent. Can I use this kit?
Yes, if the concentration of the reducing agent in the sample solution is lower than the level indicated in the table above. Dilute your sample if the concentration of the reducing agent in the sample is over this level.

◆ How can I determine the exact concentration of a specific protein in my sample solution?
If the sample solution contains only that protein, prepare a calibration curve using that protein instead of BSA. If the sample solution contains other proteins as well, a more specific detection method such as enzyme immunoassay should be used.

References

M. M. Bradford, *Anal. Biochem.*, **72**, 248 (1976); A. G. Splittgerber & J. Sohl, *Anal. Biochem.*, **179**, 198 (1989); S. V. Pande & S. R. Murthy, *Anal. Biochem.*, **220**, 424 (1994).

Protein Quantification Kit - Wide Range

Application: Protein concentration determination

Features: WST-based colorimetric microplate assay
Linear calibration curve from 50 µg per ml to 5 mg per ml
No use of heavy metals
Resistance to detergents

Ordering Information

Product code	Unit
PQ02-10	500 tests
PQ02-12	2,500 tests

Contents of the Kit

500 tests
WST-8 solution .....1 bottle
Standard BSA solution (10 mg/ml) ..... 1 bottle
Buffer solution .....1 bottle

2500 tests
WST-8 solution .....1 bottle
Standard BSA solution (10 mg/ml) ..... 2 bottles
Buffer solution .....1 bottle

Storage Condition: 0-5 °C
Shipping Condition: ambient temperature

Required Equipment and Materials

plate reader with 650 nm filter, 96-well clear plate, 10 µl and 200 µl adjustable pipettes, multi-channel pipette, 37 °C incubator, alminum foil

Product Description

Protein Quantification Kit-Wide Range (PQK-WR) is a complete, ready-to-use microtiter plate assay kit for the colorimetric detection of proteins. This assay kit has a much wider detection range and more linear calibration curve than the commonly used bicinchoninic acid (BCA) method. Unlike other detection methods, such as BCA and Lowry methods, this assay kit does not use any heavy metal ions. Instead, it is based on the reduction of Dojindo's water-soluble tetrazolium salt, WST-8, in basic conditions. Tetrazolium salt is easily reduced by the sugar residues of proteins and generates formazan dye (Fig. 8-3). WST-8 formazan dye is yellow at neutral pH and turns blue at higher pH levels. The maximum wavelength of the formazan dye is 650 nm at pH 12.5 or higher (Fig. 8-4). This high wavelength reduces the amount of background noise from biological samples. Furthermore, common detergents used for protein isolation have little effect on this assay kit. The detection range of PQK-WR is from 50 µg per ml to 5 mg per ml (BSA).

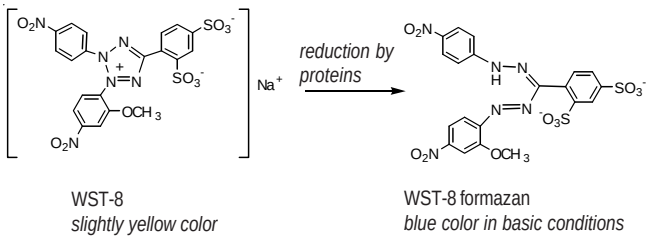


Fig. 8-3 Protein detection system with WST-8

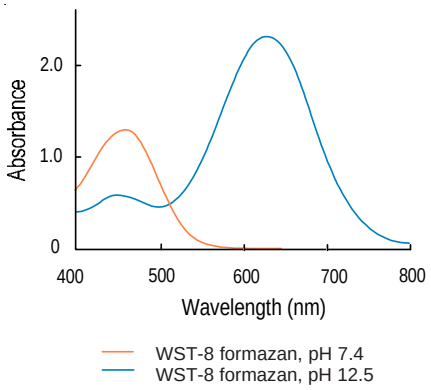


Fig. 8-4. pH dependency of WST-8 formazan

Procedure of The Assay

Add Buffer solution to each well.

Add sample or BSA solutions. Then add WST-8 solution.

Cover the plate with aluminum foil and incubate for 30 min at 37 °C.

Read O.D. at 650 nm.

VIII. Protein Detection

Protocol

1. Serially dilute the 10 mg per ml standard BSA solution[a)] with water to prepare various concentrations of BSA solution (5 mg/ml, 2.5 mg/ml, 1.25 mg/ml, 625 µg/ml, 313 µg/ml, 156 µg/ml, 78 µg/ml, and 0).
2. Add 180 µl Buffer solution to each well.
3. Add 20 µl of the various concentrations of BSA solution or sample solution to each well and mix.
4. Add 20 µl WST-8 solution to each well and mix.
5. Cover the plate with aluminum foil and incubate at 37 °C for 30 min
   WST-8 becomes light sensitive after mixing with Buffer solution; the background of the solution may increase without protection from light.
6. Measure the absorbance of each well at 630-670 nm with a microplate reader.
7. Subtract the absorbance of the blank solution (0 BSA solution) from the absorbance of each well.
8. Plot the concentration of BSA on the X-axis and the absorbance value on the Y-axis to prepare a calibration curve (Fig. 8-5).
9. Use this calibration curve to determine the protein concentration of the sample solution.

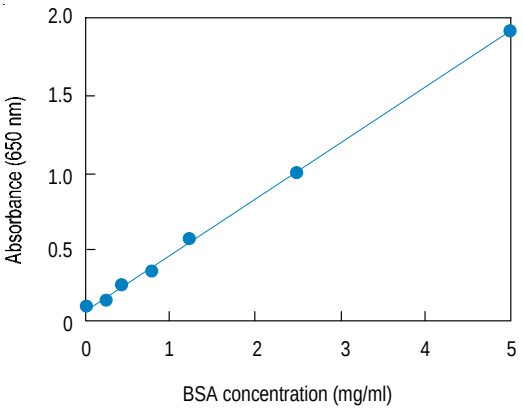


Fig. 8-5 Typical calibration curve prepared using BSA standard solution

Table 8-2 Materials That Interfere with the Assay

Chemical	Interference Concentration	Chemical	Interference Concentration	Chemical	Interference Concentration
Detergents					
Briji 35	2%	Brij 56	1%	Brij 58	1%
Triton X-100	1%	Triton X-114	1%	Tween 20	0.5%
Tween 80	0.3%	SDS	1%	CHAPS	4%
CHAPSO	2%	MEGA 10	0.5%	Octylglucoside	0.5%
Organic solvents					
ethanol	10%	isopropanol	10%	DMSO	10%
Chelating agents					
EDTA	2.5 mM	DTPA	0.625 mM	—	
Salts					
NaCl	0.5 M	KCl	1 M	NaOAc	0.2 M
$NaHCO_3$	6.25 mM				
Buffers					
citrate, pH 5	0.6 mM	MES, pH 6.1	12.5 mM	Tris, pH 7.4	2.5 mM
PBS	no interference	HEPES, pH 7.5	12.5 mM	CHES, pH 9	12.5 mM

FAQ

◆ My sample contains detergent. Can I use this kit?
Yes, if the concentration of the detergent in the sample solution is lower than the level indicated in the table above. Dilute your sample if the concentration of the detergent is over this level.

◆ My sample contains reducing agent. Can I use this kit?
No. WST-8 may be reduced by agents such as DTT and ascorbic acid, producing high background noise.

◆ How can I determine the exact concentration of a specific protein in my sample solution?
If the sample solution contains only that protein, prepare a calibration curve using that protein instead of BSA. If the sample solution contains other proteins as well, a more specific detection method such as enzyme immunoassay should be used.

References

Z. Huang, et al., J. Neurosci., **23**, 5975 (2003); T. Kodama, et al., Am. J. Pathol., **165**, 1743 (2004); R. Shibata, *et al.*, *Clin. Cancer Res.*, **12**, 5363 (2006).

CarryMax

Application: Protein delivery to viable cells

Features: Simple procedure to optimize delivery conditions
Low toxicity to cells

Ordering Information

Product code	Unit*
C462-10	50 tests

* Based on a 24-well plate..

Contents of the Kit:

PT-R (lyophilized).....	1 vial	PT-H (lyophilized).....	1 vial
Lipoform buffer	1 vial	Enhancer E.....	1 vial

Storage Condition	Shipping Condition
0-5 °C	ambient temperature

Required Equipment and Materials

10 µl, 100-200 µl and 1000 µl pipettes, CO_2 incubator, microtubes(sterile)

Product Description

CarryMax is a protein delivery kit for animal cells. This kit is designed to acheive the best performance of protein delivery to various animal cells. The kit contains two different cationic compounds for the formation of a complex with protein and material which promotes protein delivery more efficiently to viable cells. Since the kit contains two different types of delivery reagents, the protein delivery conditions can be optimized for higher delivery with a lower cytotoxicity rate. The delivery mechanism of the protein by CarryMax is similar to the gene delivery by cationic liposomes. The protein-cationic liposome complex binds on the cell membrane and then the complex is taken inside cells by endocytosis where upon the protein is released to the cytoplasm. The location of the protein delivered by this method will be concluded by various experiments. Currently, the delivered protein in the cell such as apoptosis-related proteins works in the same manner as a protein synthesized in the cell by gene expression. This delivery method will be very useful in understanding the function of the protein in the cell. The total time to deliver proteins is about 2-4 hours if no further cell culture is required. Therefore, much quicker experiments will be possible to check the function of the protein than when using a DNA transfection method. The delivered protein is stable over several hours to several days.

Protein delivery techniques are not an alternative method to DNA transfection techniques, rather they are complementary to each other. Both techniques can be used together depending on the purpose of the experiment. CarryMax can be applied to a wide variety of studies related to intracellular protein functions such as apoptosis, cell cycle regulation, cell signaling, DNA transcription, protein expression regulation, and RNA splicing. Additionally, since antibody or other foreign molecules such as peptides, biomolecules, and charged polymers can be delivered into a viable cell, therapeutic applications of CarryMax will be possible. In the near future, dual delivery of protein and DNA by CarryMax will be another possible application.

General Protocol

Preparation of Protein delivery solution

1. Add 1 ml Lipoform buffer to the PT-R* tube dissolve with a vortex mixer for 30 sec.

**If the delivery efficiency using PT-R is very small, use PT-H by following the protocol.*

2. Add 40 µl DMSO to Enhancer-E and dissolve by pipetting.
3. Mix 100 µl of PT-H solution and 5 µl of Enhancer E solution in a microtube followed by vortexing for 10 sec. to prepare Liposome solution.

Preparation of Protein-Protein delivery reagent complex

1. Add 30 µl of sterilised water solution to the tube.
2. Add 1 µg protein solution and mix with pipetting.
3. Add 4 µl Protein delivery solution to the tube and mix with pipetting.
4. Incubate 15 min at room temperature and add the solution to the cell and incubate 15 min at room temperature.

Transfection to cells

1. Change the cell culture media to fresh, serum-free.
2. Add Protein delivery complex solution to the cells.
3. Change the culture media to the media contains serum 4 hours after the addition of the Protein delivert complex solution.
4. Continue to incubate for another 4 hours or appropriate period of time depending on the experiment.

IX-1. Transfection Reagents: Protein

Amount of protein

Examples:

Recommended amount of protein	BSA	0.5 -1 µg
(Amount for 1-well of a 24-well plate)	R-Phycoerythrin	1 µg
	beta-Galactosidase	1 µg
	IgG_1	1 -2 µg

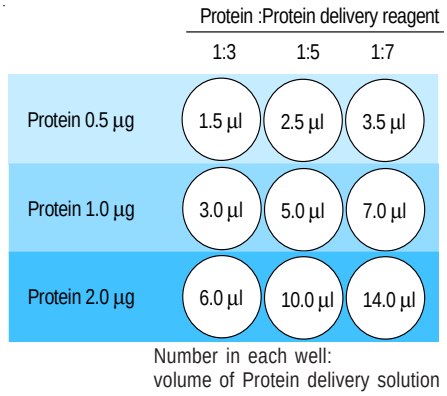


Fig. 9-1 Plate arrangement for optimum conditions for protein delivery.

If another plate is used for the protein delivery, calculate the necessary amount of protein and Protein delivery solution using the number in Table 9-1. Refer to Table 9-2 to determine the volume of each solution to prepare protein and Protein delivery reagent complex.

Table 9-1 Number to multiply the volume in each well.

96 well plate: x0.2	12 well plate: x2	6 well plate (35 mm dish): x4
60 mm dish: x8	100 mm dish: x24	

Table 9-2 Preparation conditions of the Protein-Protein delivery reagent complex.

Cell Culture condition			Prep of Protein-Protein delivery reagent complex		
plate	surface area	volume	0.9% NaCl	Protein	Protein delivery soln.
96 -well	0.3 cm^2	0.1 ml	10 µl	0.1-0.4 µg	0.3-2.8 µl
24 -well	1.9 cm^2	0.5 ml	30 µl	0.5-2.0 µg	1.5-14 µl
12 -well	3.8 cm^2	1.0 ml	60 µl	1.0-4.0 µg	3.0-28.0 µl
6 -well	9.2 cm^2	2.0 ml	120 µl	2.0-8.0 µg	6.0-56.0 µl
35 -mm	8.0 cm^2	2.0 ml	120 µl	2.0-8.0 µg	6.0-56.0 µl
60 -mm	21.0 cm^2	5.0 ml	300 µl	5.0-20.0 µg	15.0-140.0 µl
100 -mm	58.0 cm^2	15.0 ml	900 µl	15.0-60.0 µg	45.0-420.0 µl

Experimental Examples

A) R-phycoerythrin delivery to CHO cells

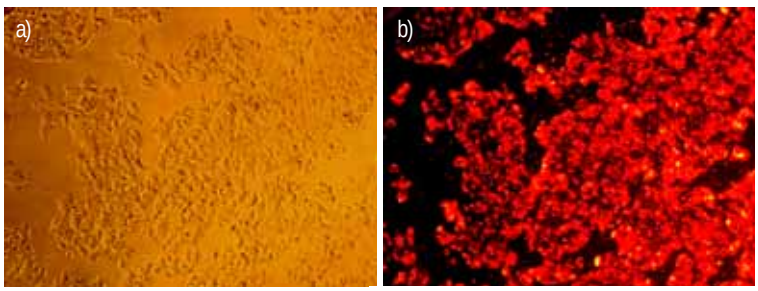
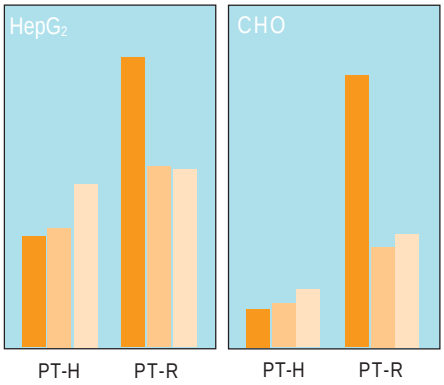


Fig. 9-2 R-Phycoerythrin-delivered CHO cells
a) Phase-contrast microscope, x100
b) Fluorescent microscope, x100 G-filter
Delivery conditions:
Complex prepared by adding 1 µg of R-Phycoerythrin and 5 µl of PT-H to CHO cells in a 24 well plate, and incubated for 4 hours.

B) R-phycoerythrin delivery to CHO cells and $HepG_2$ cells

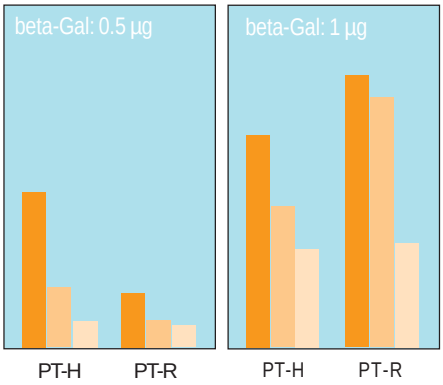


Volume of the protein delivery reagent
3 µl 5 µl 7µl

R-Phycoerythrin: 1 µg
Delivery conditions:
1. $HepG_2$ cells or CHO cells were inoculated in a 24-well plate.
2. The plate was incubated for 24 hours in a CO_2 incubator.
3. R-Phycoerythrin was delivered by following the protocol.
4. The plate was incubated for 24 hours in a CO_2 incubator.
5. R-Phycoerythrin was measured by a microplate reader with a 595 nm filter.

Fig. 9-3 Efficiency of R-Phycoerythrin delivery to $HepG_2$ and CHO cells using PT-R and PT-H under various conditions.

C) beta-Galactosidase delivery to CHO cells and $HepG_2$ cells



Volume of the protein delivery reagent
3 µl 5 µl 7µl

CHO cells
Delivery conditions:
1. CHO cells were inoculated in a 24-well plate.
2. The plate was incubated for 24 hours in a CO_2 incubator.
3. b-Gal was delivered by following the protocol.
4. The plate was incubated for 24 hours in a CO_2 incubator.
5. b-Gal was measured by enzyme assay using beta-Gal substrate.

Fig. 9-4 Efficiency of beta-Galactosidase delivery to CHO cells using PT-R and PT-H with different amounts of protein.

IX-2. Transfection Reagents: DNA, RNA

HilyMax

Application: DNA and RNA transfection to mammalian cells

Features: High transfection efficiency to a wide variety of cells
Simple protocol for maximum transfection conditions
Better response in signal transduction research
Efficient suppression in siRNA transfection

Ordering Information

Product code	Unit
H357-11	1 ml

Kit Contents:
HilyMax reagent ..... 1 vial Lipoform buffer .....1 ml x1 vial

Storage Condition	Shipping Condition
0-5 °C	ambient temperature

Required Equipment and Materials
micro plate, 10 µl, 100-200 µl, 1000 µl pipets, micro tubes, CO_2 incubator

Product Description
In order to express specific proteins in mammalian cells, there are various methods that have been developed. Calcium phosphate-precipitation method was the first method to introduce DNA to cells. However, the transfection efficiency was very poor and there was a lot of cell-to-cell variations. The second method introduced was DEAE-sephadex method. The transfection efficiency drastically improved, but still the method could not be used for all cells and required heavy metal ions to enhance transfection efficiency. The cation liposome method was then developed and this method proved to be a much better method to transfect DNA and RNA into cells. Other methods used are Magnet bead, metal particle shoot and electroporation. However, the cationoc liposome method does not require any special instruments or special skill. Therefore, many researchers are using this method. HilyMax is a newly developed gene transfection reagent that forms a liposome to be used for highly efficient gene transfection to a wide variety of cells. HilyMax can be applied to siRNA as well. Since serum in the growth medium does not interfere with the transfection using HilyMax, no exchange of the media during the transfection is required. HilyMax does not contain biological components that might interfere with the transfection.

Table 9-3. Transfection efficiency of HilyMax

Cell line	Efficiency	Cell line	Efficiency	Cell line	Efficiency
CHO	90%	HeLa	70%	HEK293	60%
NIH3T3	70%	A549	50%	L6	30%
3T3-L1	30%	K562	30%	LNCap	30%
PC3	70%	MCF-7	70%	Neuro2a	70%
MG63	20%	HC	50%	COS7	40%
HepG2	10%	Vero	40%	MDCK	20%
Jurkat	3%	UtSMC	10%		

General protocol for DNA transfection for 24-well plate*
Adherent cells: Prepare 40-90% confluent cell culture in 0.5 ml of growth medium. 24 hours preincubation may be necessary.
Non-adherent cells: Adjust the concentration of cells to 0.1-1.6x10^6 cells in 0.5 ml of growth medium and innoculate the cell suspension onto a plate. 24 hours preincubation may be necessary.
1. Add 30 µl serum-free medium[a)] to a plastic tube.
2. Add plasmid DNA (0.5-1.5 µg) to the tube and mix by gentle pipetting.
3 Add HilyMax to prepare DNA (µg):HilyMax (µl)=1:2-1:6 and mix with pipetting.
4. Incubate the tube at room temperature for 15 minutes.[b)]
5. Add DNA-HilyMax complex to each well,[c)] and incubate the plate at 37 °C in a CO_2 incubator.[d)]
6. Measure the reporter gene activity after 24 to 72 hours.

* Protocols for other plates are available in the HilyMax Technical manual.

a) Serum and antibiotics in medium interfere with the DNA-HilyMax complex formation. Opti-MEM, DMEM and MEM are confirmed in transfection use. Please examine the transfection efficiency for other media.
b) Over 30 min incubation may cause a low transfection efficiency.
c) Serum in cell culture medium does not interfere with the transfection.
d) A medium change after transfection is effective for increasing the transfection efficiency and for decreasing the cytotoxicity in some cell lines.

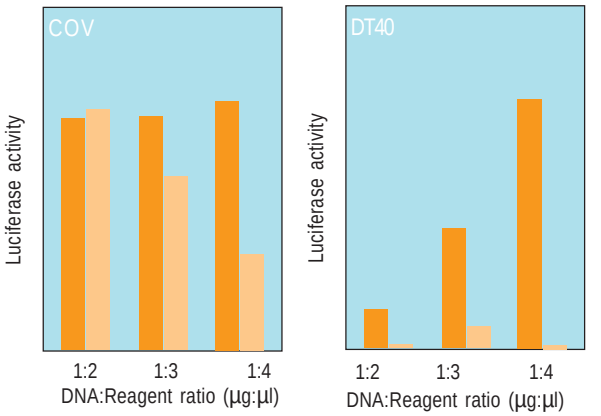


Fig. 9-5 Comparison of the transfection reagent and DNA mixing ratio. Luciferase expression vector was transfected to COV(chicken ovary, primary cell) and DT40(chicken B cell) by using HilyMax and a reagent kit from company A in the presence of serum. Luciferase assay was performed after 24 hours incubation. (Data was kindly provided from Dr. Sueyuki Kudoh, Shujitsu University, Dept. of Biological Pharmacology)

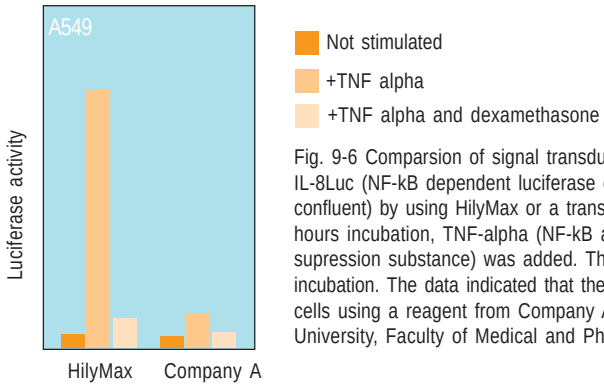


Fig. 9-6 Comparsion of signal transduction responses.
IL-8Luc (NF-kB dependent luciferase expression vector) was transfected to A549 (cell density 90%, confluent) by using HilyMax or a transfection reagent from Company A in the presence of serum. After 24 hours incubation, TNF-alpha (NF-kB activation substance) or TNF-alpha + dexamethasone (NF-kB supression substance) was added. Then luciferase acitivity of the cell culture was measured after 6-hour incubation. The data indicated that the transfected cells using HilyMax gave a higher signal than the other cells using a reagent from Company A. (Data was kindly provided from Dr. Youichiro Isohama, Kumamoto University, Faculty of Medical and Pharmaceutical Sciences, Dept. of Chemo-Pharmacological Sciences)

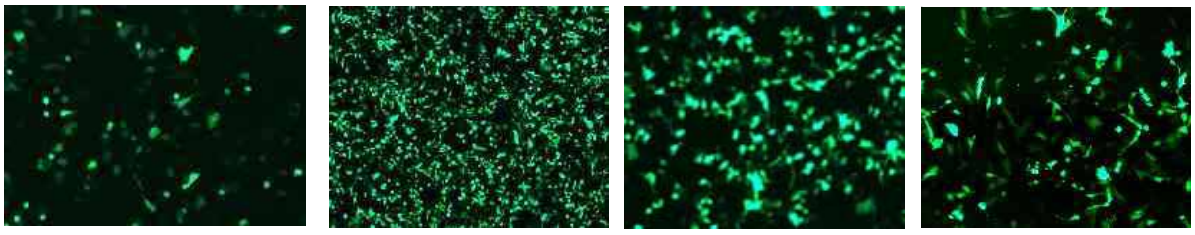


Fig. 9-7 GFP expressed cells transfected by HilyMax/ G filter, x100

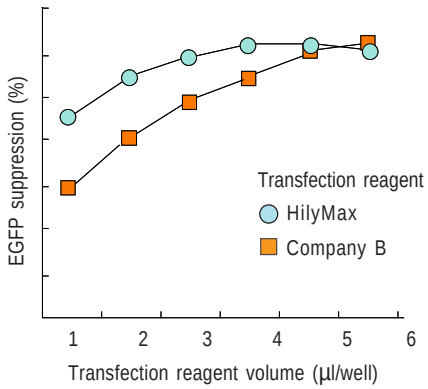
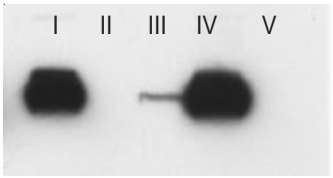


Fig. 9-8 Comparison of gene suppression by siRNA transfection. GFP siRNA was transfected to EGFP expressing CHP cells in the presence of serum. The gene suppression ratio was determined by flowcytometry after 24-hour incubation. (Data was kindly provided from Dr. Kenichi Kusumoto, Fukuoka Industrial Technology Center)



I: HilyMax
II: Transfection reagent 1 from company C
III: Transfection reagent 2 from company C
IV: Transfection reagent from comapny D
V: non-transfection

Fig. 9-9 Comparison of transfection efficiencies.
Herpes virus protein cDNA expression vector (derived from pcDNA3) 0.5 µg was transfected to 70% confluent HEK293 cells in a 6-well plate using HilyMax and 3 different transfection reagents, followed by 48-hour incubation. The protein expression was determined by western blotting. (The photo was kindly provided from Dr. Shuichi Kusano, Kagoshima University, Graduate School of Medicine and Dental Sciences)

Get *pure* DNA Kit - Cell, Tissue

Application: Genomic DNA isolation from cell and tissue

Features: No phenol or chloroform required
Short DNA isolation time
No need for spin colums or Filtration tubes
DNA recovery from a wide range of sample volumes:
up to 6 g tissue or 2x10^9 cells for 200 sample kit

Ordering Information

Product code	Unit*
GK03-20	200 samples

* 1x10^7 cells/sample, 25-30 mg tissue/sample

Contents of the Kit

Lysis buffer ..... 100 ml x 1 bottle
Precipitation solution I .....20 ml x 1 bottle
Precipitation solution II ..... 22 ml x 1 bottle
Proteinase K solution .....1 ml x 2 tubes
RNase solution ..... 0.5 ml x 1 tube

Storage Condition

0-5 °C

Shipping Condition

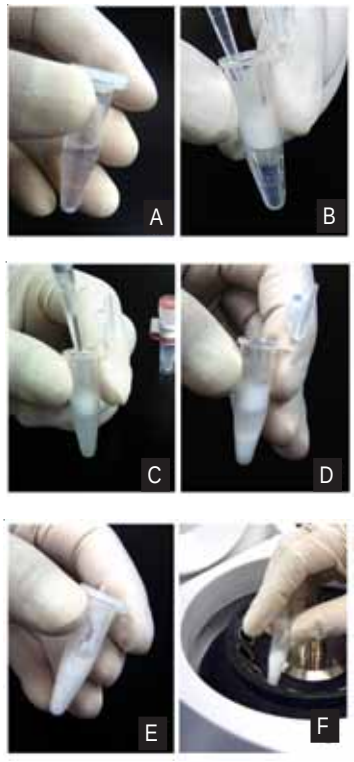
ambient temperature

Required Equipment and Materials

microcentrifuge, voltex mixer, 100 µl, 100-1000 µl adjustable pipettes, ethanol, homogenizer (for tissue), 65 °C water bath (for tissue)

Product Description

Get *pure*DNA Kit-Cell, Tissue enables isolation and purification of genomic DNA from cell cultures and tissue samples in three easy steps: sample lysis, removal of RNA and protein, and DNA recovery using ethanol precipitation. To isolate genomic DNA from 1 x 10^8 cells or 1 gram tissue sample, simply increase the volume of each solution (protocols for large samples are available at www.dojindo.com/tm). This kit does not require phenol, chloroform, centrifugal columns, or Filtration tubes. The isolated genomic DNA can be utilized for downstream experiments such as restriction enzyme digestion, ligation, PCR, and other enzymatic reactions.



Protocol for 3 x 10^6 to 1 x 10^7 cells

Photo A and B

1. Transfer cell suspension into a 1.5 ml tube and centrifuge at 1,500 rpm for 5 min.
2. Discard the supernatant and add 500 µl PBS. Vortex for 5 sec and centrifuge at 1,500 rpm for 5 min.
3. Discard the supernatant and add 250 µl Lysis buffer and 10 µl Proteinase K solution. Dissolve the cells completely by pipetting. Incubate the cell lysate at 65 °C for 10 min. Be sure that all clumps of cells dissolve before proceeding.
   ** This step is essential for high yield isolation of genomic DNA.*
4. Add 2 µl RNase solution and vortex for 5 sec. Leave the solution at room temperature for 2 min.

Photo C

5. Add 50 µl Precipitation solution I and vortex for 5 sec. White precipitate should appear immediately.

Photo D

6. Add 50 µl Precipitation solution II and vortex for 5 sec. More white precipitate should appear.

Photo E, F amd G

7. Centrifuge at 12,000-14,000 rpm for 5 min. Transfer the supernatant to a new 1.5 ml tube. To avoid contamination, do not disturb the white precipitate during the supernatant transfer. If there are white precipitates remaining in the transferred supernatant, repeat this step.

X. DNA, RNA Isolation

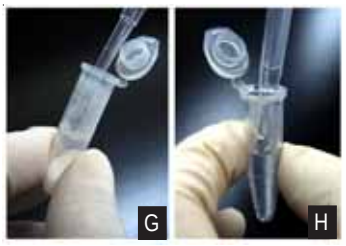


Photo H

8 Add an equal volume of ethanol to the supernatant and mix by inverting the tube several times, then vortex for 5 sec.

Photo I

9. Centrifuge at 6,000 rpm for 2 min. Carefully remove the supernatant without disturbing the precipitate. A white pellet should be visible at the bottom of the tube. To avoid contamination, do not disturb the white precipitate during the removal of supernatant.

Photo J, K

10. Add 1 ml 70% ethanol and vortex for 5 sec. Centrifuge at 6,000 rpm for 2 min and discard the supernatant. A white pellet should be visible at the bottom of the tube. Carefully remove as much of the supernatant as possible.

Photo L

11. Dry the DNA pellet using a vacuum desiccator for 10 min. Dissolve the pellet in TE buffer and use for downstream experiments. The DNA solution can be stored at 4 °C for more than one year without degradation.

Protocol for 25 to 30 mg Tissue Sample

1. Transfer 25-30 mg tissue sample into 1.5 ml tube. Add 400 µl Lysis buffer and 10 µl Proteinase K solution.
2. Homogenize the tissue sample using a homogenizer and incubate at 65 °C for 10 min.
   - *or* -Incubate at 55 °C for 2-3 hours with occasional vortexing or pipetting (no need for homogenizing).
3. Leave the tube at room temperature for 2 min. Add 2 µl RNase solution and vortex for 5 sec. Leave the tube at room temperature for 2 min.
4. Add 80 µl Precipitation solution I and vortex for 5 sec. White precipitate should appear immediately.
5. Add 80 µl Precipitation solution II and vortex for 5 sec. More white precipitate should appear. Leave the tube at room temperature for 2 min.
6. Centrifuge at 12,000-14,000 rpm for 5 min. Transfer the supernatant to a new 1.5 ml tube. To avoid contamination, do not disturb the white precipitate during the supernatant transfer. If white precipitates remain in the transferred supernatant, repeat this step.
7. Add an equal volume (~500 ug) of ethanol to the supernatant and mix by inverting the tube several times, then vortex for 5 sec.
8. Centrifuge at 6,000 rpm for 2 min. Carefully remove the supernatant without disturbing the precipitate. A white pellet should be visible at the bottom of the tube. Carefully remove as much of the supernatant as possible.
9. Add 1 ml 70% ethanol and vortex for 5 sec. Centrifuge at 6,000 rpm for 2 min, and discard the supernatant. A white pellet should be visible on the bottom of the tube. Carefully remove the supermatant as much as possible.
10. Dry the DNA pellet using a vacuum desiccator for 10 min. Dissolve the pellet in TE buffer and use for downstream experiments. The DNA solution can be stored at 4 °C over one year without degradation.

Table 10-1 Typical Amount of DNA Recovered from Given Sample Types

Sample	DNA recovery
HeLa cell (1 x 10^7 cells)	80-120 µg
HeLa cell (1 x 10^8 cells)	1-1.5 mg
HL60 cell (1 x 10^7 cells)	40-60 µg
HL60 cell (1 x 10^8 cells)	500-900 µg
Mouse liver (25-30 mg)	40-100 µg
Mouse brain (25-30 mg)	20-40 µg
Mouse kidney (25-30 mg)	50-60 µg

Sample	DNA recovery
Mouse heart (25-30 mg)	20-25 µg
Mouse tail (0.5-1 cm)	40-60 µg
Rat liver (1 g)	2-2.5 mg
Rat brain (1 g)	600-800 µg
Rat kidney (1 g)	1.8-2.3 mg
Rat heart (0.8-0.9 g)	600-800 µg
Rat tail (10 cm)	2.5-3.5 mg

The A280 nm/A260 nm ratio of the recovered DNA is between 1.7-1.9.

X. DNA, RNA Isolation

Troubleshooting

◆ No or low DNA recovery

a) Completely dissolve the tissue sample in step 2.
b) Make sure there is a DNA pellet at the bottom of the tube before discarding the supernatant in steps 8 and 9.

◆ Difficulty dissolving the tissue sample in step 2

a) Mince or cut the tissue sample into small pieces before transferring it into a tube.
b) Vortex or pipette every 30 min during the incubation. If pipetting, use a pipette tip with the point cut off, and pipette gently.

◆ Too much precipitate after centrifugation in step 6

a) Completely dissolve the tissue before adding Precipitation solution I.
b) Thoroughly mix the solution by inverting the tube several times after adding Precipitation solutions I and II.
c) Increase the centrifugation time if 12,000-14,000 rpm (10,000 g) centrifugation is difficult to achieve.

◆ Low DNA purity

a) Be sure to incubate at room temperature for 2 min after adding RNase solution in step 3.

Get *pure* DNA Kit - Agarose

Application: DNA fragment extraction from agarose gel

Features: No phenol or chloroform required
DNA isolation in 30 min
No need for colums or filtration tubes

Ordering Information

Product code	Unit*
GK01-20	200 samples

* 200 mg agarose/sample

Contents of the Kit

Gel lysis buffer ..... 65 ml x 1 bottle
Co-precipitation solution ..... 0.5 ml x 1 tube
Precipitation solution ..... 65 ml x 1 bottle

Storage Condition
0-5 °C

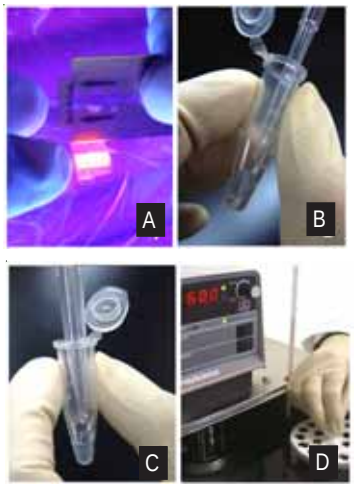
Shipping Condition
ambient temperature

Required Equipment and Materials

microcentrifuge, transiluminater or UV lamp, voltex mixer, 100 µl and 1000 µl adjustable pipettes, ethanol

Product Description

Get *pure*DNA Kit-Agarose enables isolation and purification of double-stranded DNA from agarose gel in three easy steps: lysis of agarose gel, removal of agarose gel, and DNA recovery using ethanol precipitation. Double-stranded DNA can be isolated from up to 200 mg of agarose gel using 1.5 ml tubes. To isolate DNA from a larger quantity of agarose gel, simply increase the volume of each solution. This kit does not require phenol, chloroform, centrifugal columns, or Filtration tubes. The extracted double- stranded DNA can be utilized for restriction enzyme digestion, ligation, PCR, and other enzymatic reactions.



Protocol for 100 - 200 mg Agarose Gel Slice

Photo A

1. Cut and weigh the agarose gel slice that contains double-stranded DNA fragment. Trim the edge using a razor if the weight is over 200 mg.

Photo B

2. Put the agarose gel slice into a 1.5 ml tube, and break it into several pieces using a pipette tip.

Photo C, D

3. Add 300 µl Gel lysis buffer. Incubate at 60 °C for 10 min, vortexing every 2 min. Continue incubation if agarose gel is not completely dissolved after 10 min.

X. DNA, RNA Isolation

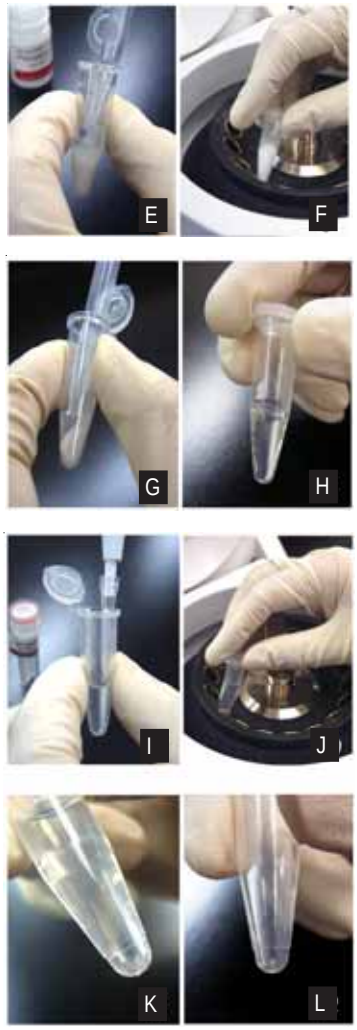


Photo E

4. Allow the tube to cool at room temperature for 2 min, and then add 300 µl Precipitation Solution. White precipitate should appear immediately. Mix the solution by inverting the tube 5 times.

Photo F

5. Centrifuge at 12,000-14,000 rpm for 5 min.

Photo G, H

6. Transfer the supernatant to a new 1.5 ml tube. To avoid contamination, do not disturb the white precipitate during the supernatant transfer. If white precipitates remain in the transferred supernatant, repeat step 5 and 6.

Photo I

7. Add 2 µl Co-precipitation solution to the supernatant, and vortex for 5 sec. Add 800 µl ethanol to the supernatant, and vortex for 5 sec. Co-precipitation solution is a DNase-free glycogen solution (20 mg/ml). If you prefer not to use this solution, you can simply omit this step. Please note that the DNA pellet may not be visible without Co-precipitation solution. Be careful not to disturb the solution during the removal of the supernatant, and do not leave the tube in the centrifuge for more than 1 min after centrifugation. Avoid using a clear tube since the DNA pellet easily peels off the wall of the tube.

Photo J

8. Centrifuge at 12,000-14,000 rpm for 3 min, and discard the supernatant. A fairly large white pellet should be visible at the bottom of the tube if you use Co-precipitation solution. Be careful not to disturb the white pellet during the removal of supernatant.

Photo K

9. Add 1 ml 70% ethanol and vortex for 5 sec. Centrifuge at 12,000-14,000 rpm for 3 min, and discard the supernatant. Repeat this step once. A white pellet should be at the bottom of the tube. Carefully remove as much of the supernatant as possible.

Photo L

10. Dry the DNA pellet using a vacuum desiccator for 10 min or leave it at room temperature for 20-30 min. Dissolve the pellet in TE buffer and use for downstream experiments. The DNA solution can be stored at 0-5 °C.

Troubleshooting

◆ No or low DNA recovery

a) Completely dissolve the agarose gel slice by vortexing in step 2. It may be difficult to see undissolved agarose gel pieces in Gel lysis buffer.

b) Make sure there is a DNA pellet at the bottom of the tube before discarding the supernatant in steps 8 and 9.

◆ Difficulty dissolving the agarose gel slice in step 3

a) Crush the gel slice in a 1.5 ml tube with a pipette tip.

b) Vortex the tube every 2 minutes during solubilization of the gel slice at 60 °C.

◆ Too much precipitate after centrifugation in step 5

a) Completely dissolve the agarose gel slice before adding Precipitation solution.

b) Thoroughly mix the solution by vortexing after adding Precipitation solution.

c) Increase the centrifugation time if 12,000-14,000 rpm (10,000 g) is difficult to achieve.

◆ Low DNA purity

a) Do not disturb the white precipitate in step 6.

b) Remove as much of the supernatant as possible in steps 8 and 9.

◆ Degradation of isolated DNA

a) Minimize the UV irradiation time during preparation of agarose gel slice in step 1.

b) Avoid using short wavelength UV light.

X. DNA, RNA Isolation

Get *pure* RNA Kit

Application: Total RNA isolation from cell, tissue and blood

Features: No phenol or chloroform required
Short RNA isolation time
No need for spin columns or Filtration tubes
RNA recovery from a wide range of sample volumes:
up to 5x10^8 cells, 1.5g tissue or 1 ml whole blood for GK04-05

Ordering Information

Product code	Unit*
GK04-05	50 samples

* 1x10^7 cells/sample, 25-30 mg tissue/sample, 200 µl whole blood/sample

Contents of the Kit

Lysis buffer ..... 13 ml x 2 bottles
DNase .....0.55 ml x 1 tube
DNase dilution buffer .....12 ml x 1 bottle
Precipitation solution I ..... 13 ml x 1 bottle
Precipitation solution II ..... 8 ml x 1 bottle

Storage Condition
0-5 °C

Shipping Condition
ambient temperature

Required Equipment and Materials
microcentrifuge, voltex mixer, 100 µl and 1000 µl adjustable pipettes, ethanol, ß-mercaptoethanol, homogenizer (for tissue)

Product Description

Get *pure*RNA Kit enables quick isolation and purification of total RNA from animal cells, tissue samples, and whole blood. This kit does not use harmful organic solvents such as phenol or chloroform. Unlike the CsCl method, this kit does not require ultracentrifugation. Get *pure*RNA Kit can be used for a wider range of sample volumes than silica-based spin column kits. The isolated RNA can be used for downstream experiments such as RT-PCR, Northern blotting, and cDNA synthesis.

Protocol for 3 x 10^6 to 1 x 10^7 Cells

1. Transfer cell suspension into a 1.5 ml tube and centrifuge at 1,500 rpm for 5 min. Discard the supernatant and add 500 µl PBS. Vortex for 5 sec and centrifuge at 1,500 rpm for 5 min.
2. Discard the supernatant and add 500 µl Lysis buffer and 5 µl ß-mercaptoethanol (not included in this kit). Completely dissolve the cells by pipetting (for 2-3 min). Make sure that all clumps of cells dissolve before proceeding.
   This step is essential for high yield RNA isolation.
3. Add 100 µl Precipitation solution I and vortex for 5 sec. White precipitate should appear immediately.
4. Centrifuge at 12,000-14,000 rpm for 5 min. Transfer the supernatant to a new 1.5 ml tube. To avoid contamination, do not disturb the white precipitate during the supernatant transfer. If white precipitate remains in the transferred supernatant, repeat this step.
5. Add an equal volume of ethanol to the supernatant and vortex for 5 sec.
6. Centrifuge at 12,000-14,000 rpm for 2 min. Carefully remove the supernatant without disturbing the precipitate. A white pellet should be visible on the side or bottom of the tube. To avoid contamination, do not disturb the white precipitate during the removal of supernatant.

7. Add 1.2 ml 70% ethanol and vortex for 5 sec.
8. Centrifuge at 12,000-14,000 rpm for 2 min and discard the supernatant. A white pellet should be visible at the side or bottom of the tube. Carefully remove as much of the supernatant as possible.
9. Prepare 20X diluted DNase working solution in a separate tube using DNase dilution buffer (e.g., 10 µl DNase + 190 µl DNase dilution buffer).

X. DNA, RNA Isolation

10. Add 200 µl DNase working solution to the tube from step 8 and dissolve the pellet.

11. Incubate at 37 °C for 15 min.

12. Add 50 µl Precipitation solution I and vortex for 5 sec. White precipitate should appear immediately.

13. Add 50 µl Precipitation solution II and vortex for 5 sec. More white precipitate should appear.

14. Centrifuge at 12,000-14,000 rpm for 5 min, then transfer the supernatant to a new 1.5 ml tube. To avoid contamination, do not disturb the white precipitate during the supernatant transfer. If white precipitates remain in the transferred supernatant, repeat this step.

15. Add an equal volume of ethanol to the supernatant and vortex for 5 sec.

16. Centrifuge at 12,000-14,000 rpm for 2 min. Carefully remove the supernatant without disturbing the precipitate. A white pellet should be visible on the side or bottom of the tube. To avoid contamination, do not disturb the white precipitate during the removal of supernatant.

17. Add 1.2 ml 70% ethanol and vortex for 5 sec.

18. Centrifuge at 12,000-14,000 rpm for 2 min and discard the supernatant. A white pellet should be visible on the side or bottom of the tube. Carefully remove as much of the supernatant as possible.

19. Dissolve the pellet in RNase-free water (DEPC-treated water) or RNase-free buffer.

Protocol for 25 to 30 mg Tissue Sample

1. Transfer 25-30 mg of chopped tissue sample pieces into 1.5 ml tube. Add 500 µl Lysis buffer and 5 µl ß-Mercaptoethanol (not included in this kit). Cool the sample in an ice bucket.
2. Homogenize the sample in the ice bucket using a homogenizer for 30-60 sec.
3. Add 100 µl Precipitation solution I and vortex for 5 sec.
4. Centrifuge at 12,000-14,000 rpm for 5 min. Transfer the supernatant to a new 1.5 ml tube. To avoid contamination, do not disturb the white precipitate during the supernatant transfer. If white precipitate remains in the transferred supernatant, repeat this step.
5. Add an equal volume of ethanol to the supernatant and vortex for 5 sec.
6. Centrifuge at 12,000-14,000 rpm for 2 min. Carefully remove the supernatant without disturbing the precipitate. A white pellet should be visible on the side or bottom of the tube. Carefully remove as much of the supernatant as possible.
7. Add 1.2 ml 70% ethanol and vortex for 5 sec.
8. Centrifuge at 12,000-14,000 rpm for 2 min and discard the supernatant. A white pellet should be on the side or bottom of the tube. Carefully remove as much of the supernatant as possible.
9. Prepare 20X diluted DNase working solution in a separate tube using DNase dilution buffer. (e.g., 10 µl DNase + 190 µl DNase dilution buffer).
10. Add 200 µl DNase working solution to the tube from step 8, and dissolve the pellet.
11. Incubate at 37 °C for 15 min.
12. Add 50 µl Precipitation solution I and vortex for 5 sec. White precipitate should appear immediately.
13. Add 50 µl Precipitation solution II and vortex for 5 sec. More white precipitate should appear.
14. Centrifuge at 12,000-14,000 rpm for 5 min. Transfer the supernatant to a new 1.5 ml tube. To avoid contamination, do not disturb the white precipitate during the supernatant transfer. If white precipitate remains in the transferred supernatant, repeat this step.
15. Add an equal volume of ethanol to the supernatant and vortex for 5 sec.
16. Centrifuge at 12,000-14,000 rpm for 2 min. Carefully remove the supernatant without disturbing the precipitate. A white pellet should be visible on the side or bottom of the tube. To avoid contamination, do not disturb the white precipitate during the removal of supernatant.
17. Add 1.2 ml 70% ethanol and vortex for 5 sec.
18. Centrifuge at 12,000-14,000 rpm for 2 min and discard the supernatant. A white pellet should be visible on the side or bottom of the tube. Carefully remove as much of the supernatant as possible.
19. Dissolve the pellet in RNase-free water (DEPC-treated water) or RNase-free buffer.

X. DNA, RNA Isolation

Protocol for 200 µl of Whole Blood

1. Transfer 200 µl whole blood into a 1.5 ml tube. Add 300 µl Lysis Buffer and 5 µl ß-Mercaptoethanol (not included in this kit). Completely dissolve the cells by pipetting for 2-3 min. Make sure that all clumps of cells dissolve before proceeding.
   This step is essential for high-yield RNA isolation.
2. Add 100 µl Precipitation solution I and vortex for 5 sec. Precipitate should appear immediately.
3. Add 100 µl Precipitation solution II and vortex for 5 sec. More precipitate should appear.
4. Centrifuge at 12,000-14,000 rpm for 5 min. Transfer the supernatant to a new 1.5 ml tube. To avoid contamination, do not disturb the white precipitate during the supernatant transfer. If white precipitates remaining in the transferred supernatant, repeat this step.
5. Add 100 µl Precipitation solution I and vortex for 5 sec. White precipitate should appear immediately.
6. Centrifuge at 12,000-14,000 rpm for 5 min, and transfer the supernatant to a new 1.5 ml tube. To avoid contamination, do not disturb the white precipitate.
7. Add an equal volume of ethanol* to the supernatant and vortex for 5 sec.
   ** Optionally, 2 µl glycogen solution (20 mg/ml, not provided) can be added as a co-precipitation solution before adding the ethanol.*
8. Add 1.2 ml 70% ethanol and vortex for 5 sec.
9. Centrifuge at 12,000-14,000 rpm for 2 min and discard the supernatant.
10. Prepare 20X diluted DNase working solution in a separate tube using DNase dilution buffer (e.g., 10 µl DNase + 190 µl DNase dilution buffer).
11. Add 200 µl DNase working solution to the tube from step 9 and dissolve the pellet.
12. Incubate at 37 °C for 15 min.
13. Add 50 µl Precipitation solution I and vortex for 5 sec. White precipitate should appear immediately.
14. Add 50 µl Precipitation solution II and vortex for 5 sec. More white precipitate should appear.
15. Centrifuge at 12,000-14,000 rpm for 5 min. Transfer the supernatant to a new 1.5 ml tube. To avoid contamination, do not disturb the white precipitate during the supernatant transfer. If white precipitate remains in the transferred supernatant, repeat this step.
16. Add an equal volume of ethanol* to the supernatant and vortex for 5 sec.
    ** Optionally, 2 µl glycogen solution (20 mg/ml, not provided) can be added as a co-precipitation solution before adding the ethanol.*
17. Centrifuge at 12,000-14,000 rpm for 2 min. Carefully remove the supernatant without disturbing the precipitate. A white pellet should be visible on the side or bottom of the tube. To avoid contamination, do not disturb the white precipitate during the removal of supernatant.
18. Add 1.2 ml 70% ethanol and vortex for 5 sec.
19. Centrifuge at 12,000-14,000 rpm for 2 min and discard the supernatant. A white pellet should be visible on the side or bottom of the tube. Carefully remove as much of the supernatant as possible.
20. Dissolve the pellet in RNase-free water (DEPC-treated water) or RNase-free buffer.

Table 10-2 RNA Recovery

Sample	RNA Recovery
HeLa (1 x 10^7 cells)	90-150 µg
Balb3T3 (1 x 10^7 cells)	90-150 µg
HL60 (1 x 10^7 cells)	30-60 µg
Mouse liver (20 mg)	60-75 µg
Mouse brain (30 mg)	15-25 µg

Sample	RNA Recovery
Mouse kidney (30 mg)	40-55 µg
Mouse heart (30 mg)	7-15 µg
Mouse liver (1 g)	3.8-4.4 mg
Mouse brain (1 g)	700-1000 µg
Mouse blood (1 ml)	14-24 µg

The $A_{260\ nm}/A_{280\ nm}$ ratio of the recovered RNA is between 2.0-2.2. TheA_{260}/A_{280} values are based on recovered RNA dissolved in TE buffer (10 mM Tris, 1mM EDTA, pH 8.0). The use of DEPC-treated water may lower the A_{260}/A_{280} values.

Troubleshooting

◆ No or low RNA recovery

Dissolve the samples compeletely with pepietting.
Make sure that the RNA pellet is on the side or bottom of the tube before discarding the supermatant.

◆ Difficulty dissolving the tissue sample

Mince or chop the tissue sample into very small pieces before transferring it to a tube.

◆ The precipitates are not packed tightly after adding Precipitation solution II

Completely dissolve the sample before adding Precipitation solution I. Mix the solution thoroughly by vortexing after adding Precipitation solutions I and II. Increase the centrifugation time if 12,000-14,000 rpm (10,000 g) centrifugation is difficult.

◆ Low RNA purity

Do not disturb the white precipitate during the supernatant transfers. Use a small volume micropipette (<20 ml) when removing the supernatants after the ethanol precipitation.

◆ Degradation of isolated RNA

Use freshly prepared samples.
Make sure that all materials and equipment are RNase-free (e.g., ethanol, microtubes, pipette tips).

XI. Self-Assembled Monolayers

Introduction

Highly stable molecular layers prepared by the self-assembling method have been used for the development of various detection systems such as electrochemical, optical, and so on. Self-assembled monolayers (SAMs) are crystalline chemisorbed organic single layers formed on a solid substrate by the spontaneous organization of molecules. Thiol compounds and gold is a well established combination[1]. Carboxylic acids, organosilicon derivatives, and diphosphonates on various metal oxide surfaces have also been explored in recent years in an effort to find a good model for such adhesive processes (Fig. 11-1). SAMs are easily modified at the single molecular and assembled levels. They are very useful research models to promote our understanding of the self-organization mechanism of molecules, molecular structure and property relationships, and phenomena at the interface between different phases. The high flexibility of SAMs have allowed us to study the electron transfer mechanism of proteins, molecular layers, and biosensors.

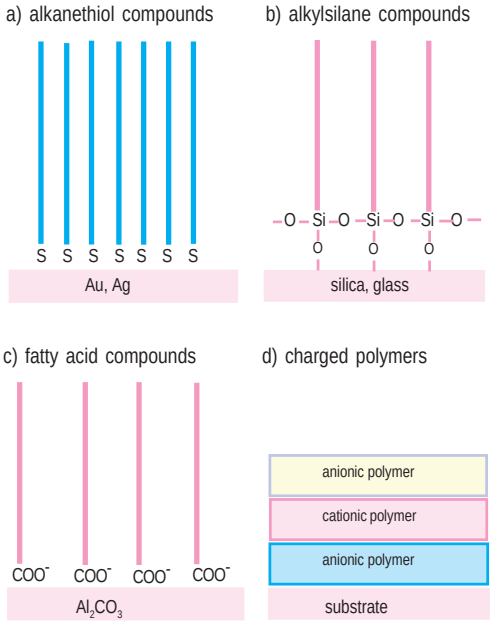


Fig. 11-1 Model of the surfaces of various SAMs

Self-Assembling Process of Alkanelthiols

The self-assembling process of alkanethiols on gold is initiated by the strong chemical interactions between the sulfur and gold surface. This interaction is a result of chemisorption that forces the thiolate molecule to adsorb to a gold lattice. The tail-to-tail interactions of the alkanethiolate molecules (created by lateral, interchain, nonbonded interactions, such as van der Waals, steric, repulsive, and electrostatic forces), are strong enough that the molecules align parallel on the gold surface and create a crystalline film[1),2)] (Fig. 11-2). Therefore, the packing and ordering of molecules is controlled by a chemisorption mechanism[3),4)]. In practice, the solid surface is simply dipped into a solution containing adsorbing molecules. Organic disulfides, thiols, and sulfides are widely used for the preparation of stable SAMs on gold surfaces. The self-assembling process and the orientation of the molecular layer on a gold surface have been investigated thoroughly using Fourier transform infrared spectroscopy (FT-IR)[5),6)], scanning tunneling microscopy (STM)[1)], atomic

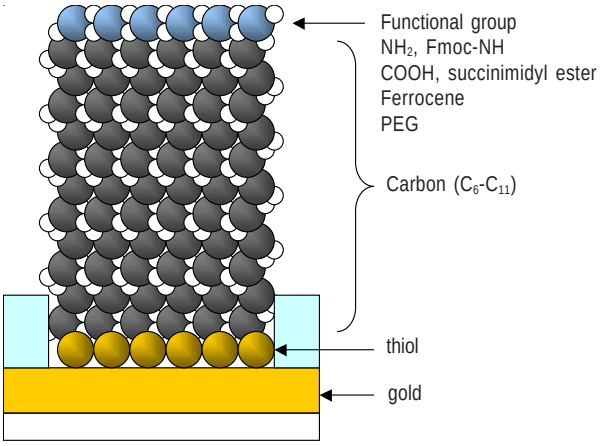


Fig. 11-2 SAM structure prepared by alkanethiol

force microscopy (AFM)[7)], X-ray photoelectron spectroscopy (XPS)[8),9)], electro-chemistry[4),10)], Raman spectroscopy[11)], ellipsometry[12),13)], and quartz crystal microbalance (QCM)[14),15)]. Please refer to the papers by Dr. Ulman for extensive reviews about the self-assembly process[3),4)].

Electrochemical Sensing by SAMs-Modified Electrodes

A common application of SAMs is in the creation of sensors with molecular recognition properties. Many groups have studied the electrochemical characteristics of alkylthiols for ion detection and molecular recognition. For example, Turyan and others used SAMs composed of ω-mercapto-carboxylic acids on mercury film and gold electrodes for very sensitive and selective analyses of cadmium(II)[16)]. Taniguchi and others created a membrane model with molecular gating by incorporating Meldola's Blue into self-assembled decanethiol monolayer-coated electrodes.[17)] Katayama and Maeda reported the electrochemical detection of cyclic AMP by a 17-mer oligopeptide-coated gold electrode. They showed that this response was dependent on the cyclic AMP concentration, but not ATP, using cyclic voltammograms of ferrocyanide/ferricyanide redox coupled with the electrode[18)]. Wang and others reviewed the behavior, utility, and advantages of an amperometric flow detector coated with unsubstituted n-alkylthiols. They indicated that a hydrophilic alkylthiol monolayer has a high selectivity toward lipophilic drugs such as chloropromazine and dipamine[19)].

Detection of Histidine-Tagged Protein Using NTA-Attached SAMs

The use of a short peptide as an affinity tag is one of the most common methods for the detection and purification of recombinant proteins. These tag proteins are mostly antibody epitopes that are detected with their antibodies[20)]. Sigal and others prepared a self-assembled monolayer that selectively binds protein with a stretch of six histidines (His-tag). They prepared two alkanethiols; one with a nitrilotriacetic acid (NTA) group that forms a tetravalent chelate with Ni(II) ion and the other with a triethyleneglycol group that is capable of avoiding nonspecific adsorption of protein (Fig. 11-3). This membrane can only recognize a His-tag protein through the nickel(II) ion chelated with NTA on the SAM. This technique is useful for immobilization of His-tag proteins for study using surface plasmon resonance (SPR)[21)].

XI. Self-Assembled Monolayers

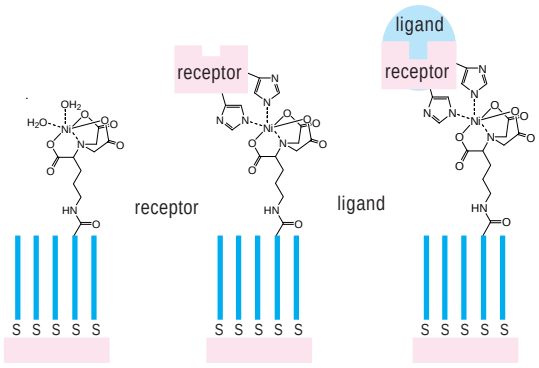


Fig. 11-3. Procedure for immobilization of receptor using His-tag.

Surface Plasmon Resonance Studies for Interactions of SAMs and Proteins

Surface plasmon resonance (SPR) is commonly used to measure the kinetics of association and dissociation of ligands and proteins in aqueous solution. It is particularly powerful for the observation of processes occurring at or near interfaces. The sensing element is a thin (40-50 nm) gold or silver film deposited on a glass surface. The back of the gold-coated glass slide is irradiated with *p*-polarized light. The reflection angles controlled to minimize the intensity of the reflection. Since the value of this reflection angle linearly depends on the amount of protein adsorbed on the surface, the protein can be monitored by SPR (Fig. 11-4)[20-22].

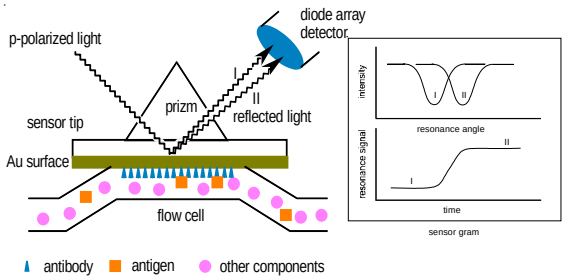


Fig. 11-4 Schematic diagram of an experimental setup for SPR measurement

Mrksich and others studied the thermodynamic and kinetic mechanism between benzenesulfonamide-attached SAM and bovine carbonic anhydrase (EC 4.2.1.1) using the SPR technique[22),23)]. Ringsdorf and others prepared protein triple layers on biotin-attached SAMs on gold surface using streptavidin and anti-chorionic gonadotropin-Fab (anti-hCG-Fab) fragments (Fig. 11-5). They investigated the hinge, or linkage region of the Fab fragment, and the second layer formation (streptavidin, Fab fragment), the third layer formation by antigen hCG, and quantification of these processing. SPR coupled with SAM is an excellent method to determine surface phenomena. The SPR method has been used for the determination of epitopes of monoclonal antibodies, development of immuno-detection systems, research on signal transfer mechanisms, and other studies.

Electrochemical Studies of Proteins on SAMs-Modified Electrodes

Protein-coated electrodes provide suitable systems for the development of devices using protein layer interfaces.[24)] Cytochrome c, ferredoxin, and myoglobin are particularly interesting proteins for interfacial electrochemical studies. For example, Niki's group has been studying diffusionless standard electron transfer rate constants of cytochrome c immobilized on a carboxylic acid-attached SAM with an electro-reflectance spectroscope[25)]. Several researchers have used electrochemical techniques for the characterization of the absorption of poly(L-lysine)-coated SAMs. Poly(L-lysine) was fixed to carboxylic acid-attached SAM through an electrostatic binding between a negative charge of carboxylate and a positive charge of amine residue. The poly(L-lysine)-coated SAMs were used to develop a detection system for glucose and for research on the electron transfer mechanism of cytochrome c[24),26),27),28)]. Nakashima's group prepared polyethylene-glycol-attached SAMs and reported that a supramolecular structure was formed by the interaction between poly(ethyleneglycol) and a-cyclodextrin[29)].

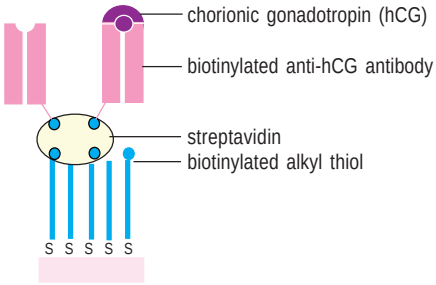


Fig. 11-5. Protein triple layer on SAM using biotinylated alkyl thiol.

DNA Sensor Using Electrochemical and Quartz Crystal Microbalance Method

Electrochemical DNA sensors may be useful devices for the sequence-specific detection and quantification of DNA or RNA in solutions. The amount of DNA or RNA in solution can be measured as an amperometric or voltammetric signal[24)]. Katayama and others prepared a calf thymus DNA-coated gold electrode to detect anti-DNA antibody. They demonstrated that this system could be useful for the development of biosensors for DNA-binding proteins[30)]. Okahata's group used frequency changes in quartz crystal microbalance (QCM) to detect one-to-one hybridization between oligonucleotides immobilized on gold electrodes of the QCM and target M13 phage DNA in aqueous solution. Thprepared a 10-mer deoxynucleotide with a mercaptopropyl group at the 5'-phosphate end whose sequence was complementary with the EcoR1 binding site of single-stranded M13 phage DNA[31),32)]. The QCM method is very sensitive because its resonance frequency decreases upon the increase of the mass on the QCM. The QCM method is useful for the study of molecular kinetics of base-pair hybridization in oligonucleotides and to detect various biological materials.

XI. Self-Assembled Monolayers

Table 11-1. Solubility data

	Methyl alcohol				Ethyl alcohol				Chloroform				Methylene chloride			
	10mM	1mM	100µM	10µM	10mM	1mM	100µM	10µM	10mM	1mM	100µM	10µM	10mM	1mM	100µM	10µM
11-AUT (A423)	S				S				S				I	I	S	
8-AOT (A424)	S				S				S				S			
6-AHT (A425)	S				S				I	S			I	S		
10-CDT (C385)	S				S				S				S			
7-CHT (C386)	S				S				S				S			
5-CPT (C387)	S				S				S				S			
11-HUT (H337)	S				S				S				S			
8-HOT (H338)	S				S				S				S			
6-HHT (H339)	S				S				S				S			
11-FUT (F246)	I	S			L	S			S				S			
8-FOT (F247)	S				L	S			S				S			
6-FHT (F269)	I	S			L	S			S				S			

	Tetrahydrofuran				Acetonitrile				Ethyl acetate				Hexane			
	10mM	1mM	100µM	10µM	10mM	1mM	100µM	10µM	10mM	1mM	100µM	10µM	10mM	1mM	100µM	10µM
11-AUT (A423)	I	I	I	S	I	I	I	I	I	I	I	I	I	I	I	I
8-AOT (A424)	L	L	S		I	L	S		I	I	L	S	I	I	I	I
6-AHT (A425)	I	L	L	S	I	I	I	I	I	I	I	I	I	I	I	I
10-CDT (C385)	S				L	L	L	L	L	L	L	L	S			
7-CHT (C386)	S				L	L	L	L	L	L	L	L	S			
5-CPT (C387)	S				L	L	L	L	L	L	L	L	S			
11-HUT (H337)	S				I	I	L	L	L	L	L	L	I	S		
8-HOT (H338)	S				L	S			L	L	L	L	S			
6-HHT (H339)	S				L	S			L	L	L	L	S			
11-FUT (F246)	S				L	L	L	L	L	L	L	L	S			
8-FOT (F247)	S				L	L	L	L	L	L	L	L	S			
6-FHT (F269)	L	L	S		S				L	L	L	L	I	S		

	Dimethylsulfoxide				N,N-Dimethylformamide				H_2O			
	10mM	1mM	100µM	10µM	10mM	1mM	100µM	10µM	10mM	1mM	100µM	10µM
11-AUT (A423)	S				S				I	S		
8-AOT (A424)	S				S				S			
6-AHT (A425)	S				S				I	S		
10-CDT (C385)	S				S				I	I	I	I
7-CHT (C386)	S				S				I	S		
5-CPT (C387)	S				S				S			
11-HUT (H337)	S				S				I	I	I	I
8-HOT (H338)	S				S				I	S		
6-HHT (H339)	S				S				S			
11-FUT (F246)	S				S				I	I	I	I
8-FOT (F247)	S				S				I	I	I	I
6-FHT (F269)	S				S				I	I	I	I

S: Soluble
L: Low Solubility
I: Insoluble

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16-Amino-1-hexadecanethiol, hydrochloride

16-Amino-1-hexadecanethiol, hydrochloride

Application: SAM preparation, amine group coating

Appearance: white or slightly yellowish powder
Purity: >95% (HPLC)
MW: 309.98, $C_{16}H_{36}ClNS$

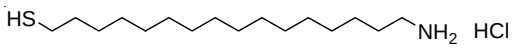
Ordering Information

Product code	Unit
A458-10	10 mg
A458-12	100 mg

Storage Condition
-20 °C, protect from light and metal

Shipping Condition
ambient temperature

Structural Formula



11-Amino-1-undecanethiol, hydrochloride

11-Amino-1-undecanethiol, hydrochloride [CAS: 143339-58-6]

Application: SAM preparation, amine group coating

Appearance: white or pale yellow powder
Purity: >90.0% (HPLC, derivatization)
MW: 239.85, $C_{11}H_{26}ClNS$

Ordering Information

Product code	Unit
A423-10	10 mg
A423-12	100 mg

Storage Condition
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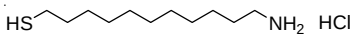
Shipping Condition
ambient temperature

Structural Formula



8-Amino-1-octanethiol, hydrochloride

8-Amino-1-octanethiol, hydrochloride

Application: SAM preparation, amine group coating

Appearance: white or slightly red crystalline powder
Purity: >90.0% (HPLC, derivatization)
MW: 197.77, $C_8H_{20}ClNS$

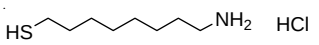
Ordering Information

Product code	Unit
A424-10	10 mg
A424-12	100 mg

Storage Condition
-20 °C, protect from light and metal

Shipping Condition
ambient temperature

Structural Formula



6-Amino-1-hexanethiol, hydrochloride

6-Amino-1-hexanethiol, hydrochloride [CAS: 31098-40-5]

Application: SAM preparation, amine group coating

Appearance: white or slightly red crystalline powder
Purity: >90.0% (HPLC, derivatization)
MW: 169.72, $C_6H_{16}ClNS$

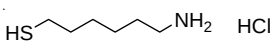
Ordering Information

Product code	Unit
A425-10	10 mg
A425-12	100 mg

Storage Condition
-20 °C, protect from light and metal

Shipping Condition
ambient temperature

Structural Formula



Amino-EG$_6$-undecanethiol, hydrochloride

20-(11-Mercaptoundecanyloxy)-3, 6, 9, 12, 15, 18-hexaoxaeicosane-1-amine, hydrochloride [CAS: 496839-01-1]

Application: SAM preparation, amine group coating, hydrophilic surface

Appearance: white or pale yellow powder
Purity: >90.0% (HPLC, derivatization)
MW: 504.16, $C_{23}H_{60}ClNO_6S$

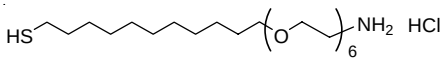
Ordering Information

Product code	Unit
A483-10	10 mg

Storage Condition
-20 °C, protect from light and metal

Shipping Condition
ambient temperature

Structural Formula



Product Description

Polyethyleneglycols (PEGs) are widely used for material modifications to improve hydrophilicity of the surface. PEG-coated materials are usually more stable under physiological conditions. Since Amino-ED$_6$-undecanethiol has 6 ethylene glycol units, 11 carbon atoms and SH group at the end, it can be used to prepare a highly oriented and hydrophilic SAM on a gold surface. This is suitable for biomaterial-labelings on the surface due to the improvement of hydrophilicity. The hydrophilic surface can prevent proteins or other biomaterials from non-specific binding. Therefore, the SAM prepared by this reagent will provide a better surface to develop biomaterial sensors or DNA/ protein microarrays. In order to prepare a Amino-ED$_6$-SAM on a gold surface, hydroxy-ED$_n$-undecanethiols (n=3, 6) are used to dilute the number of amino groups according to the density of the molecules being introduced onto the surface. There are several papers available on labeling proteins such as ovalbumin, and cytochrome C.

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Product Description

Aminoalkanethiols are utilized for the modification of a gold surface to introduce amino groups on it. Dojindo's newly developed 16-Amino-1-hexadecanethiol has a 16-carbon chain which is the longest alkanethiol available in the market. It is expected that 16-Amino-1-hexadecanethiol will form the most stable SAM on a gold surface among the aminoalkanethiol compounds because of the greater Van-der-Waals force between alkane groups. In all, there are a total 5 different aminoalkanethiols including Amino-EG$_6$-undecanethiol, hydrochloride available for gold surface modification. Amino-EG$_6$-undecanethiol is used for a hydrophilic surface preparation (for more information, see page 155). The amino group is usually modified using amine-reactive materials, such as proteins or biomaterials, to functionalize the gold surface. Several researchers have reported SAMs of short alkyl chain aminoalkanethiols, and there are an increasing number of reports of long alkyl chain compounds. Takahara and others formed a monolayer of 11-Amino-1-undecanethiol on a gold electrode and studied the effect of the terminal groups on the redox responses of ferrocene derivatives using voltammetric method. They also reported the relationship between the alkyl chain length of aminoalkanethiols and the redox behavior of 2,3-dichloro-1,4-naphtoquinone attached to the terminal amino group. Tanahashi and co-workers modified a gold surface with SAMs of several kinds of functionalized alkanethiols. They reported the effect of their terminal functional groups on apatite formation in a simulated body fluid using X-ray photoelectron spectroscopic (XPS) measurement and quartz crystal microbalance (QCM) method.

XI-1. Self-Assembled Monolayers: Aminoalkanethiol

How to Prepare SAM

1. Soak a gold-coated glass plate in Piranha solution[a] for 10-15 min. Wash the plate with purified water.[a]
2. Dissolve aminoalkanethiol compound in ethanol to prepare several μM to 1 mM solution.
3. Soak the plate in the aminoalkanethiol solution for a certain time period.[b]
4. Wash the SAM-coated plate with ethanol and then water.
5. Dry the plate under nitrogen atmosphere, if necessary.

a) Piranha solution: sulfuric acid and 30% hydrogen peroxide, 3:1. Piranha solution is a strong oxidizing agent. Extreme care is necessary when using it. Do not apply Piranha solution to resin-coated plates; it may erode the resin.

b) To prepare a SAM-coated plate with the best performance, aminoalkanethiol concentration and soaking time should be individually determined.

Application of SAM-Preparation of DNA Array (Fig. 11-6)

1. Use SF10 glass slides (Schott Glass Technologies) coated with 5 nm chromium and 45 nm gold thin film.
2. Soak the glass slide in a 100 mM 1-octadecanethiol (ODT)/ethanol solution overnight to prepare ODT SAM-coated slide.
3. Draw 500 mm x 500 mm patterns on the ODT SAM-coated slide by UV irradiation with a Hg-Xe arc lamp.[a]
4. Soak the slide in a 1 mM 11-amino-1-undecanethiol (AUT)/ethanol solution for 2 hours to form AUT SAM on the 500 mm x 500 mm photo-patterned area.
5. Drop 2 mM SPDP solution[b] onto the slide and leave the slide at room temperature.
6. Wash the slide and dry under nitrogen atmosphere.
7. Apply 1 mM thiol-DNA solution[c] to each 500 mm x 500 mm pattern and incubate at room temperature overnight.
8. Incubate the slide with a sample solution for 10 min and wash with phosphate buffer, followed by SPR imaging.

a) Irradiation time: 1-1.5 hours

b) SPDP: N-succinimidyl 3-(2-pyridyldithio)propionate. Dissolve SPDP in DMSO to prepare 50 mM solution. Dilute it 25 times with 100 mM triethanolaminebuffer, pH 7.0.

c) Dissolve thiol-DNA with 100 mM triethanolamine buffer, pH 8.0.

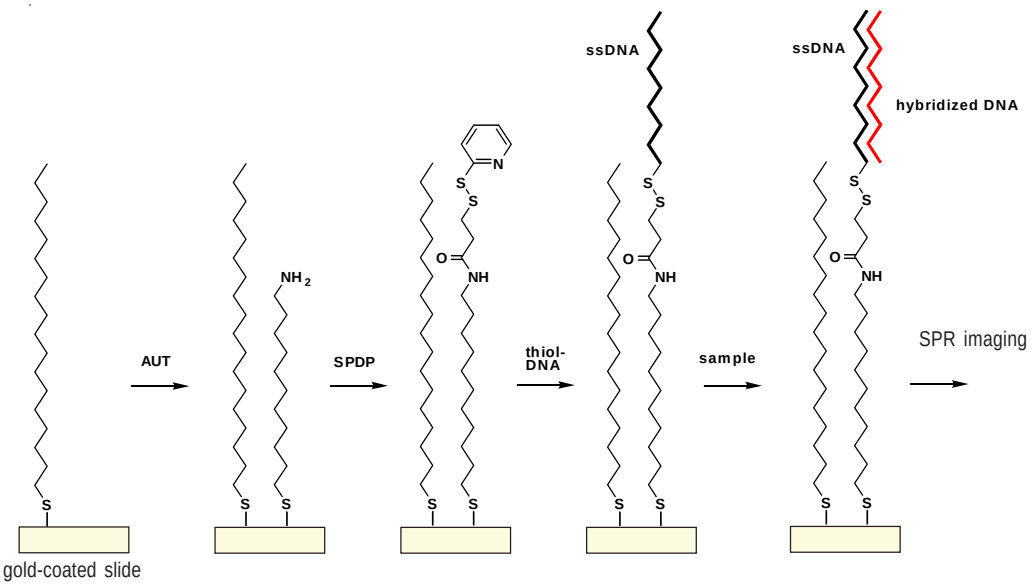


Fig. 11-6 DNA Array Preparation Scheme

References

K. Takehara, *et al*., *Electrochim. Acta*, **39**, 817 (1994); K. Takehara, *et al*., *Bull Chem. Soc. Jpn.*, **68**, 1289 (1995); F. Mukae, *et al*., *Bull. Chem. Soc. Jpn.*, **69**, 2461 (1996); S. Rubin, *et al*., *Mater. Res. Soc. Symp. Proc.*, **413**, 377 (1996); Y. Yoshimi, *et al*., *Mater. Sci. Eng. C*, **5**, 131 (1997); M. Tanahashi, *et al*., *J. Biomed. Mater. Res.*, **34**, 305 (1997); J. Tien, *et al*., *Langmuir*, **13**, 5349 (1997); Y. Li, *et al*., *Nucleic Acids Res.*, **34**, 6416 (2006).

XI-2. Self-Assembled Monolayers: Fmoc-Aminoalkanethiol

N-Fmoc-Aminoundecanethiol

Fluoren-9-ylmethyl *N*-(11-mercaptoundecyl)carbamate

Application: SAM preparation, protected amine group coating

Appearance: white or pale yellow powder
Purity: >90.0% (HPLC)
MW: 425.63, $C_{26}H_{35}NO_2S$

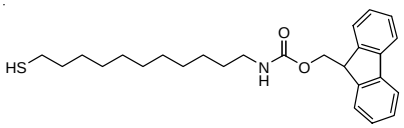
Storage Condition
-20 °C, protect from metal

Shipping Condition
with blue ice or dry ice

Ordering Information

Product code	Unit
F287-10	10 mg
F287-12	50 mg

Chemical Structure



N-Fmoc-Aminooctanethiol

Fluoren-9-ylmethyl *N*-(8-mercaptooctyl)carbamate

Application: SAM preparation, protected amine group coating

Appearance: white or pale yellow powder
Purity: >90.0%(HPLC)
MW: 383.55, C23H29NO2S

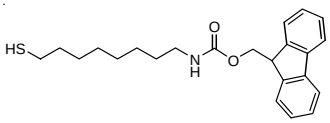
Storage Condition
-20 °C, protect from metal

Shipping Condition
with blue ice or dry ice

Ordering Information

Product code	Unit
F288-10	10 mg
F288-12	50 mg

Chemical Structure



N-Fmoc-Aminohexanethiol

Fluoren-9-ylmethyl *N*-(6-mercaptohexyl)carbamate

Application: SAM preparation, protected amine group coating

Appearance: white or pale yellow powder
Purity: >90.0% (HPLC)
MW: 355.49, $C_{21}H_{25}NO_2S$

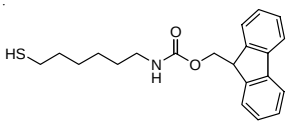
Storage Condition
-20 °C, protect from metal

Shipping Condition
with blue ice or dry ice

Ordering Information

Product code	Unit
F289-10	10 mg
F289-12	50 mg

Chemical Structure



Product Description

N-Fmoc- Aminoalkanethiols are compounds whose terminal amino group is protected by an Fmoc-group. After adsorption of *N*-Fmoc aminoalkanethiol onto a gold surface, the Fmoc-group can be removed to reproduce the amino group under mild conditions, such as 30 min immersion in 20% piperidine/acetonitrile. Brockman and coworkers protected the amino group of 11-Amino-1-undecanethiol with the Fmoc group, after making 11-Amino-1-undecanethiol SAMs, to fabricate DNA arrays with UV photopatterning and a multistep chemical modification procedure. They then used these DNA arrays to study protein-DNA interactions by surface plasmon resonance (SPR) imaging. *N*-Fmoc- Aminoalkanethiols can be used in similar methods with short-steps. The modification of a gold substrate with SAMs of *N*-Fmoc-Aminoalkanethiols may be utilized to avoid amino group-gold surface interactions and to develop a highly regulated sensor chip by photopatterning (Fig. 11-7).

XI-3. Self-Assembled Monolayers: Carboxyalkanethiol

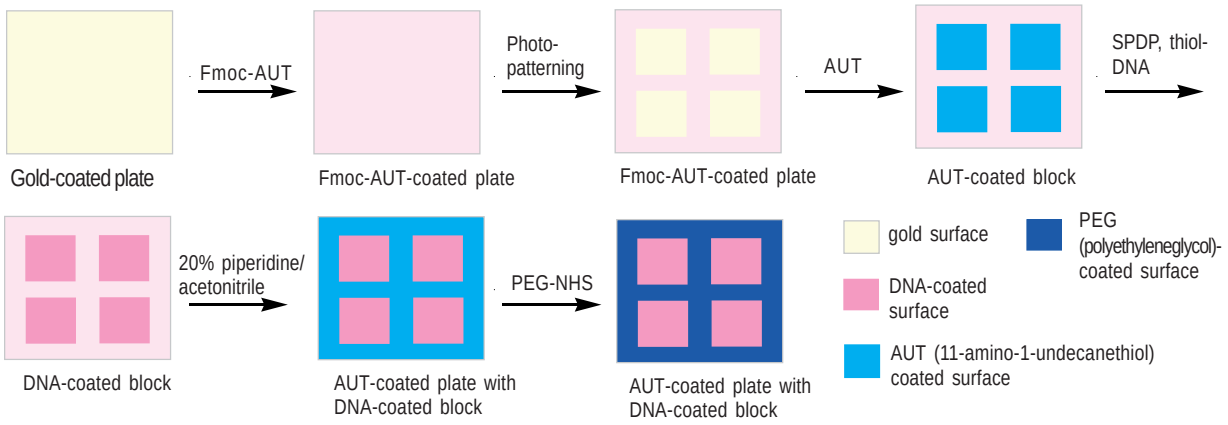


Fig. 11-7 Preparation of Multi-Element DNA Array

Reference

J. M. Brockman, *et al.*, *J. Am. Chem. Soc.*, **121**, 8044 (1999).

15-Carboxy-1-pentadecanethiol

15-Carboxy-1-pentadecanethiol

Application: SAM preparation, carboxylate coating

Appearance: white or slightly yellowish crystalline powder
Purity: >95% (HPLC)
MW: 288.49, $C_{16}H_{32}O_2S$

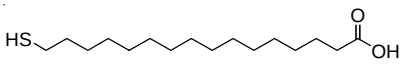
Storage Condition
-20 °C, protect from metal

Shipping Condition
ambient temperature

Ordering Information

Product code	Unit
C429-10	10 mg

Chemical Structure



10-Carboxy-1-decanethiol

10-Carboxy-1-decanethiol [CAS: 71310-21-9]

Application: SAM preparation, carboxylate coating

Appearance: white or slightly yellow powder
Purity: >97.0% (HPLC)
MW: 218.36, $C_{11}H_{22}O_2S$

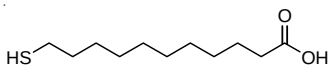
Storage Condition
0-5 °C, protect from metal

Shipping Condition
ambient temperature

Ordering Information

Product code	Unit
C385-10	10 mg
C385-12	100 mg

Chemical Structure



XI-3. Self-Assembled Monolayers: Carboxyalkanethiol

7-Carboxy-1-heptanethiol

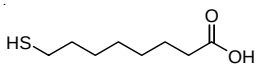
7-Carboxy-1-hepanethiol [CAS: 74328-61-3]

Application: SAM preparation, carboxylate coating

Appearance: colorless or slightly yellow liquid
Purity: >97.0% (HPLC)
MW: 176.28, $C_8H_{16}O_2S$

Storage Condition: 0-5 °C, protect from metal
Shipping Condition: ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
C386-10	10 mg
C386-12	100 mg

5-Carboxy-1-pentanethiol

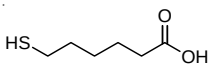
5-Carboxy-1-pentanethiol [CAS: 17689-17-7]

Purity: >97.0% (HPLC)
MW: 148.22, $C_6H_{12}O_2S$
Application: SAM preparation, carboxylate coating

Appearance: colorless or slightly yellow liquid

Storage Condition: 0-5 °C, protect from metal
Shipping Condition: ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
C387-10	10 mg
C387-12	100 mg

Product Description

Carboxyalkanethiols are utilized for the modification of a gold surface to introduce carboxylic groups on it. The carboxylic group is often converted to activated *N*-hydroxysuccinimide ester which reacts with an amine group of biomaterials. Dojindo's newly developed 15-Carboxy-1-pentadecanethiol has a 15-carbon chain which is the longest alkanethiol available in the market among carboxyalkanethiols. In all, there are a total 5 different carboxyalkanethiols including Carboxy-EG_6-undecanethiol available for gold surface modification. Malone and others fabricated a highly sensitive SPR sensor using 15-Carboxy-1-pentadecanethiol. Glenn and co- workers used carboxyalkanethiol and poly-L-lysine to create an immobilized cytochrome b5 multilayer electrode. Mizutani and others fabricated immobilized glucose oxidase multilayer electrodes in a similar manner. Both groups reported electron transfer from biomaterials to a gold surface. These kinds of multilayer film electrodes are well suited for studies of diffusion electron transfer. Frisbie and others developed a new method, chemical force microscopy, for obtaining the adhesive interactions and the friction image of patterned sample surfaces. They used atomic force microscopy (AFM) to measure the interactions and spatial mapping of chemically distinct functional groups. Frisbie and othrs formed carboxyalkanethiol monolayers on the gold surfaces of AFM cantilever tips. They used AFM to measure the adhesive and friction forces between molecularly modified probe tips and organic monolayers terminating in a lithographically-defined pattern of distinct functional groups.

References

C. D. Frisbie, *et al.*, *Science*, **265**, 2071 (1994); F. Mizutani, *et al.*, *Chem. Lett.*, 251 (1996); D. J. H. Glenn, *et al.*, *Chem. Lett.*, 399 (1996); H. Lee, *et al.*, *Chem. Mater.*, **10**, 4148 (1998); J. Kuther, *et al.*, *Thin. Solid. Films*, **327-329**, 554 (1998); K. Sasaki, *et al.*, *Appl. Phys. A: Mater. Sci. Process.*, **66**, S1275 (1998); E. Cooper, *et al.*, *Langmuir*, **14**, 4795 (1998); J. Luo, *et al.*, *Langmuir*, **14**, 3602 (1998); S. Imabayashi, *et al.*, *Langmuir*, **14**, 2348 (1998); V. Kane, *et al.*, *Langmuir*, **14**, 3303 (1998); F. P. Zamborini, *et al.*, *Langmuir*, **14**, 3279 (1998); Y. L. Cheng, *et al.*, *J. Phys. Chem. B*, **102**, 5309 (1998); A. C Templeton, *et al.*, *J. Am. Chem. Soc.*, **120**, 4845 (1998); J. Kuther, *et al.*, *J. Mater. Chem.*, **8**, 641 (1998); D. D. Schlereth, *et al.*, *J. Electroanal. Chem.*, **444**, 231 (1998); L. Scheibler, *et al.*, *Tetrahedron*, **54**, 3725 (1998); L. Scheibler, *et al.*, *Polym. Bull.*, **40**, 151 (1998); M. Pro, *et al.*, *Bull. Korean Chem. Soc.*, **19**, 122 (1998); J. L. Trevor, *et al.*, *Langmuir*, **14**, 1664 (1998); S. Heyse, *et al.*, *Biochemistry*, **37**, 507 (1998); H. Schmitt, *et al.*, *Adv. Mater.*, **10**, 475 (1998); L. A. Godinez, *et al.*, *Langmuir*, **14**, 137 (1998); K. Shimazu, *et al.*, *Chem. Lett.*, **669** (1998); J. C. DePriest, *et al.*, *Proc. SPIE-Int. Soc. Opt. Eng.*, **3491**, 772 (1998); Y. Miura, *et al.*, *Langmuir*, **15**, 1155 (1999); K. Niki, *et al.*, *J. Phys. Chem. B*, **107**, 9947 (2003).

Carboxy-EG$_6$-undecanethiol

20-(11-Mercaptiundecanoyl)-3,6,9,12,18-hexaoxaeicosanoic acid [CAS:221222-49-7]

Application: SAM preparation, carboxylate coating, hydrophilic surface

Appearance: white or pale yellow pwoder
Purity: >90.0% (HPLC)
MW: 526.73, $C_{26}H_{60}O_9S$

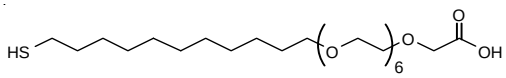
Ordering Information

Product code	Unit
C445-10	10 mg
C445-12	100 mg

Storage Condition
ambient temperature, protect from metal

Shipping Condition
ambient temperature

Chemical Structure



Product Description

Polyethyleneglycols (PEGs) are widely used for material modifications to improve hydrophilicity of the surface. PEG-coated materials are usually more stable under physiological conditions. Since Carboxy-ED$_6$-undecanethiol has 6 ethylene glycol units, 11 carbon atoms and an SH group at the end, it can be used to prepare a highly oriented and hydrophilic SAM on a gold surface which is suitable for biomaterial-labelings on the surface. This is due to the improvement of hydrophilicity. The hydrophilic surface can prevent proteins or other biomaterials from non-specific binding. Therefore, the SAM prepared by this reagent will provide a better surface to develop biomaterial sensors or DNA/ protein microarrays. In order to prepare a carboxy-ED$_6$-SAM on a gold surface, hydroxy-ED$_n$-undecanethiols (n=3, 6) are used to dilute the number of carboxyl groups according to the density of the molecules being introduced onto the surface. There are several papers available on labeling proteins such as ovalbumin and cytochrome C.

References

C. Pale-Grosdemange, *et al., Anal. Chem.*, **71**, 777 (1999); X. Qian, *et al., Anal. Chem.*, **74**, 1805 (2002); M. Kyo, *et al., Anal. Chem.*, **77**, 7115 (2005); A. Subramanian, *et al., Biosensors and Bioelectronics*, **21**, 998 (2006).

10-Carboxydecyl disulfide

10-Carboxydecyl disulfide [CAS: 23483-56-9]

Application: SAM preparation, carboxylate coating

Appearance: white or pale yellow pwoder
Purity: >97.0% (HPLC)
MW: 434.69, $C_{22}H_{42}O_4S_2$

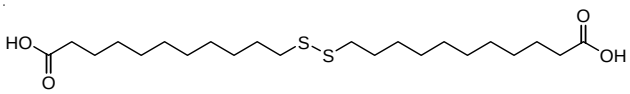
Ordering Information

Product code	Unit
C404-10	10 mg
C404-12	100 mg

Storage Condition
ambient temperature, protect from metal

Shipping Condition
ambient temperature

Chemical Structure



7-Carboxyheptyl disulfide

7-Carboxyheptyl disulfide [CAS: 107016-79-5]

Application: SAM preparation, carboxylate coating

Appearance: white or pale yellow powder
Purity: >97.0% (HPLC)
MW: 350.54, $C_{16}H_{30}O_4S_2$

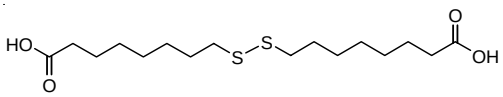
Ordering Information

Product code	Unit
C405-10	10 mg

Storage Condition
ambient temperature, protect from metal

Shipping Condition
ambient temperature

Chemical Structure



XI-4. Self-Assembled Monolayers: Carboxyalkanedisulfide

5-Carboxypentyl disulfide

5-Carboxypentyl disulfide [CAS: 92038-67-0]

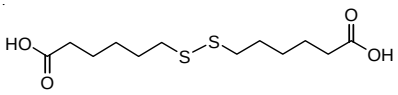
Application: SAM preparation, carboxylate coating

Appearance: white or pale yellow powder
Purity: >97.0% (HPLC)
MW: 294.43, $C_{12}H_{22}O_4S_2$

Storage Condition
ambient temperature, protect from metal

Shipping Condition
ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
C406-10	10 mg

4, 4'- Dithiodibutyric acid

4,4'-Dithiodibutyric acid [CAS: 2906-60-7]

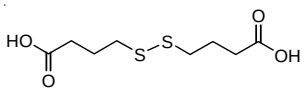
Application: SAM preparation, carboxylate coating

Appearance: white or pale yellow powder
Purity: >97.0% (HPLC)
MW: 238.33, $C_8H_{14}O_4S_2$

Storage Condition
ambient temperature

Shipping Condition
ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
D524-10	500 mg

Product Description of Carboxyallanedisulfides

Carboxyalkyldisulfides are oxidized carboxyalkanethiols. They form SAMs similar to the carboxyalkanethiols, but they are more stable than thiols. Kanayama and others formed SAMs of 4,4'-dithiodibutyric acid on gold colloids or gold electrodes, and introduced phenylboronic acid moieties to its terminus. They succeeded in recognizing various sugars by surface-enhanced Raman spectroscopy (SERS) and cyclic voltammetry (CV) using these SAMs. Takagi and co-workers fixed dinitrophenyl (DNP) groups on carboxyalkyl-disulfide SAMs. They detected an anti-DNP antibody by electrical impedance measurement. This technique could be applied to impedimetric sensing of proteins. Delamarche and his co-workers fabricated 10-carboxydecyl disulfide SAMs on a gold substrate and introduced a photoactivatable benzophenone moiety to the termini. After attaching a protein (IgG), it was tested by a variety of characterization techniques (including ellipsometry, X-ray photoelectron spectroscopy and AFM).

References

H. Taira, *et al.*, *Anal. Sci.*, **9**, 199 (1993); E. Delamarche, *et al.*, *Langmuir*, **12**, 1997 (1996); N. Kanayama, *et al.*, *Langmuir*, **16**, 577 (2000).

Dithiobis(succinimidyl undecanoate)

Dithiobis(1-succinimidyl undecanoate)

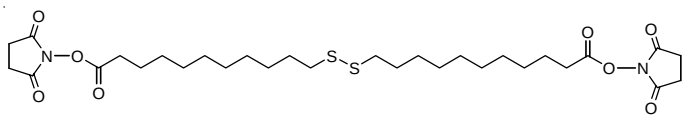
Application: SAM preparation, amine reactive group coating

Appearance: white powder
Purity: >90.0% (HPLC)
MW: 628.84, $C_{30}H_{48}N_2O_8S_2$

Storage Condition
0-5 °C, protect from moisture

Shipping Condition
ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
D537-10	10 mg
D537-12	50 mg

Dithiobis(succinimidyl octanoate)

Dithiobis(1-succinimidyl octanoate)

Application: SAM preparation, amine reactive group coating

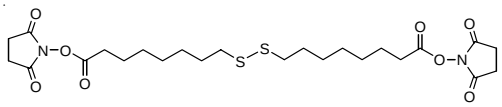
Appearance: white powder
Purity: >90.0% (HPLC)
MW: 544.68, $C_{24}H_{36}N_2O_8S_2$

Ordering Information	
Product code	Unit
D538-10	10 mg
D538-12	50 mg

Storage Condition
0-5 °C, protect from moisture

Shipping Condition
ambient temperature

Chemical Structure



Dithiobis(succinimidyl hexanoate)

Dithiobis(1-succinimidyl hexanoate)

Application: SAM preparation, amine reactive group coating

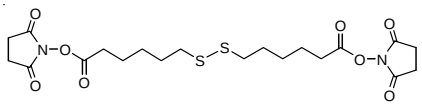
Appearance: white powder
Purity: >90.0% (HPLC)
MW: 488.58, $C_{20}H_{28}N_2O_8S_2$

Ordering Information	
Product code	Unit
D539-10	10 mg
D539-12	50 mg

Storage Condition
0-5 °C, protect from moisture

Shipping Condition
ambient temperature

Chemical Structure



Product Description of Succinimidyl Alkanedisulfides

Succinimidyl ester-terminated alkyldisulfides are amine-reactive analogs of carboxyalkyldisulfide. They are utilized for the modification of a gold surface to introduce amine-reactive sites on the surface. It is possible to use this technique for protein chips and various sensors. There is no need to use coupling agents because these compounds are already activated. Wagner and others characterized dithiobis(succinimidyl undecanoate) SAMs on a gold substrate by scanning tunneling microscopy (STM), radiolabeling and *in situ* AFM imaging. The densely packed and highly reactive surfaces allowed them to easily immobilize amino acids and proteins.

Reference

P. Wagner, *et al.*, *J. Vac. Sci. Technol. B*, **14**, 1466 (1996).

Dithiobis(C2-NTA)

3,3'-Dithiobis[*N*-(5-amino-5-carboxypentyl)propionamide-*N,N'*-diacetic acid] dihydrochloride

Application: SAM preparation, electrochemical reaction

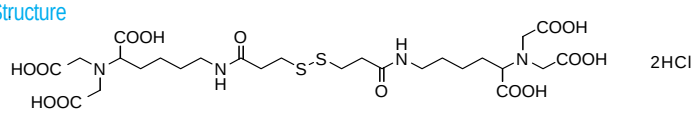
Appearance: white or slightly yellow powder
Purity: >95.0% (Titration)
MW: 771.68, $C_{26}H_{44}Cl_2N_4O_{14}S_2$

Ordering Information	
Product code	Unit
D550-10	10 mg
D550-12	50 mg

Storage Condition
ambient temperature, protect from metal

Shipping Condition
ambient temperature

Chemical Structure



Product Description

Dithiobis(C_2-NTA) is utilized for the modification of a gold surface to introduce NTA groups that can bind most heavy metal ions. It forms SAMs similar to the other alkanethiols. The SAMs prepared using Dithiobis(C_2-NTA) are highly unidirectional due to its alkyl chain interactions. Ni(II)-NTA chelates are commonly used for Histidine-tagged (His-tag) protein purification or separation. Therefore, Ni(II)-NTA-coated gold can be used for His-tag protein analyses. Dithiobis(C_2-NTA) is soluble in water and alcohol. It is also referred to as disulfide-NTA in some papers.

XI-6. Self-Assembled Monolayers: Ferrocenyl Alkanethiol

11-Ferrocenyl-1-undecanethiol

11-Ferrocenyl-1-undecanethiol [CAS: 127087-36-9]

Application: SAM preparation, electrochemical reaction

Appearance: yellow or yellowish-orange solid
Purity: >95.0% (HPLC)
MW: 372.39, $C_{21}H_{32}FeS$

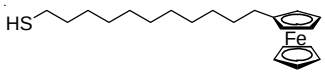
Storage Condition
-20 °C, protect from metal

Shipping Condition
with dry ice or blue ice

Ordering Information

Product code	Unit
F246-10	10 mg
F246-12	100 mg

Chemical Structure



8-Ferrocenyl-1-octanethiol

8-Ferrrocenyl-1-octanethiol [CAS: 146056-20-4]

Application: SAM preparation, electrochemical reaction

Appearance: yellow or yellowish-orange solid
Purity: >95.0% (HPLC)
MW: 330.31, $C_{18}H_{26}FeS$

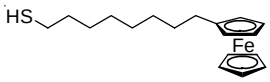
Storage Condition
-20 °C, protect from metal

Shipping Condition
with dry ice or blue ice

Ordering Information

Product code	Unit
F247-10	10 mg
F247-12	100 mg

Chemical Structure



6-Ferrocenyl-1-hexanethiol

6-Ferrrocenyl-1-hexanethiol

Application: SAM preparation, electrochemical reaction

Appearance: yellow or yellowish-orange solid
Purity: >95.0% (HPLC)
MW: 302.26, $C_{16}H_{22}FeS$

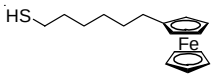
Storage Condition
-20 °C, protect from metal

Shipping Condition
with dry ice or blue ice

Ordering Information

Product code	Unit
F269-10	10 mg
F269-12	100 mg

Chemical Structure



Product Description of Ferrocenyl Alkanethiols

Ferrocenyl alkanethiols are utilized for the modification of gold surfaces to introduce electrochemically active molecules. The modified gold surface can be utilized for the development of sensitive electrochemical analyses. Rubin and others fabricated mixed SAMs of aminoalkanethiols and ferrocenyl alkanethiols with various chain lengths on a gold electrode surface. They immobilized glucose oxidase on aminoalkanethiol sites and used ferrocenyl-alkanethiol sites as electron mediators. They reported the relationship between electrical response and chain length of mixed SAMs.

Uosaki and co-workers reported the results of structural changes and the number of absorbed ferrocenyl alkanethiols during redox reaction of 11-ferrocenyl-1-undecanethiol SAMs on a gold electrode using Fourier transform infrared reflection adsorption spectroscopy (FT-IRRAS) and electrochemical quartz crystal microbalance (EQCM) method. They suggested the possibility of orientation change of the monolayer during the redox reaction of the ferrocene moiety. They also estimated this change using voltammograms and ellipsometry.

References

M. D. Porter, *et al.*, *J. Am. Chem. Soc.*, **112**, 3559 (1987); C. E. D. Chidsey, *et al.*, *J. Am. Chem. Soc*, **14**, 4301 (1990); C. E. D. Chidsey, *et al.*, *Chemtracts*, **3**, 27 (1991); K. Uosaki, *et al.*, *Langmuir*, **7**, 1510 (1991); K. Shimazu *et al.*, *Langmuir*, **8**, 1385 (1992); Y. Kajiya, *et al.*, *Chem. Lett.*, 2107 (1993); T. Ohtsuka, *et al.*, *Langmuir*, **10**, 3658 (1994); K. Shimazu, *et al.*, *J. Electroanal. Chem.*, **372**, 117 (1994); T. Kondo, *et al.*, *J. Electroanal. Chem.*, **381**, 203 (1995); J. I. Anderson, *et al.*, *Anal. Chem.*, **68**, 379R (1996); K. Chen, *et al.*, *Langmuir*, **12**, 2622 (1996); S. Rubin, *et al.*, *Mater. Res. Soc. Symp. Proc.*, **413**, 377 (1996); S. Ye, *et al.*, *Langmuir*, **13**, 3157 (1997); R. C. Sabapathy, *et al.*, *Langmuir*, **14**, 3797 (1998); J. J. Hickman, *et al.*, *Science*, **252**, 688 (1998).

16-Hydroxy-1-hexadecanethiol

16-Hydroxy-1-heptadecanethiol [CAS:114896-32-1]

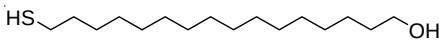
Application: SAM preparation, dilution of functional alkanethiols

Appearance: white or slightly yellowish crystalline powder
Purity: >90% (HPLC)
MW: 274.51, $C_{16}H_{34}OS$

Storage Condition
ambient temperature

Shipping Condition
ambient temperature

Structural Formula



Ordering Information

Product code	Unit
H394-10	10 mg

11-Hydroxy-1-undecanethiol

11-Hydroxy-1-undecanethiol [CAS: 73768-94-2]

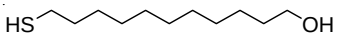
Application: SAM preparation, dilution of functional alkanethiols

Appearance: white or slightly yellow crystalline powder
Purity: >98.0% (GC)
MW: 204.37, $C_{11}H_{24}OS$

Storage Condition
-20 °C, protect from metal

Shipping Condition
ambient temperature

Structural Formula



Ordering Information

Product code	Unit
H337-10	10 mg
H337-12	100 mg

8-Hydroxy-1-octanethiol

8-Hydroxy-1-octanethiol [CAS: 33065-54-2]

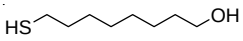
Application: SAM preparation, dilution of functional alkanethiols

Appearance: coloress or slightly yellow liquid
Purity: >98.0% (GC)
MW: 162.29, $C_8H_{18}OS$

Storage Condition
-20 °C, protect from metal

Shipping Condition
ambient temperature

Structural Formula



Ordering Information

Product code	Unit
H338-10	10 mg
H338-12	100 mg

6-Hydroxy-1-hexanethiol

6-Hydroxy-1-hexanethiol [CAS: 1633-78-9]

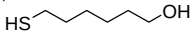
Application: SAM preparation, dilution of functional alkanethiols

Appearance: colorless or slightly yellow liquid
Purity: >98.0% (GC)
MW: 134.24, $C_6H_{14}OS$

Storage Condition
-20 °C, protect from metal

Shipping Condition
with dry ice or blue ice

Structural Formula



Ordering Information

Product code	Unit
H339-10	10 mg
H339-12	100 mg

XI-7. Self-Assembled Monolayers: Hydroxy Alkanethiol

Product Description

Hydroxyalkanethiols are utilized as dilution reagents on a gold surface to control the density of reactive groups, or as blocking agents to prevent non-specific binding of analytes on the surface. Newly developed 16-Hydroxy-1-hexadecanethiol has a 16-carbon chain which is the longest alkanethiol available in the market among hydroxyalkanethiols. In all, there are a total 6 different hydroxyalkanethiols including Hydroxy-EG_6-undecanethiol and Hydroxy-EG_3-undecanethiol available for gold surface modification. 16-Hydroxy-1-hexadecanethiol is used to prepare a homogeneous and highly oriented SAM when 16-Amino-1-hexadecanethiol or 15-Carboxy-1-pentadecanethiol is applied. Herne and his co-workers fabricated mixed SAMs of thiol-derivatized single-stranded DNA (HS-ss-DNA) and 6-Hydroxy-1-hexanethiol on a gold surface that prevented non-specific adsorption of HS-ss-DNA. Perez-Luna and others made mixed SAMs of biotin-terminated thiol and 11-hydroxy-1-undecanethiol on a gold surface. They prevented non-specific adsorption of wild type streptavidin and streptavidin mutants. Dubrovsky and coworkers controlled the non-specific adsorption of protein on the surface of gold-coated silicagel using 11-Hydroxy-1-undecanethiol. They mentioned the usefulness of gold-coated silica gel for the preparation of well-defined, surface-functionalized supports for biological assay.

Hydroxy-EG_6-undecanethiol

11-Mercaptoundecanol hexaethyleneglycol ether [CAS: 130727-44-5]

Application: SAM preparation, dilution of functional alkanethiols

Appearance: white or slightly yellow crystalline powder
Purity: >90.0% (GC)
MW: 468.69, $C_{23}H_{48}O_7S$

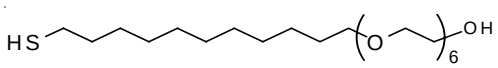
Storage Condition
-20 °C, protect from metal

Shipping Condition
ambient temperature

Ordering Information

Product code	Unit
H355-10	10 mg
H355-12	100 mg

Structural Formula



Hydroxy-EG_3-undecanethiol

11-Mercaptoundecanol hexaethyleneglycol ether [CAS: 130727-41-2]

Application: SAM preparation, dilution of functional alkanethiols

Appearance: coloress or slightly yellow liquid
Purity: >90.0% (GC)
MW: 336.53, $C_{17}H_{36}O_4S$

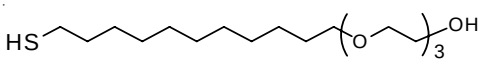
Storage Condition
-20 °C, protect from metal

Shipping Condition
ambient temperature

Ordering Information

Product code	Unit
H354-10	10 mg
H354-12	100 mg

Structural Formula



Product Description

Polyethyleneglycols (PEGs) are widely used for material modifications to improve hydrophilicity of the surface. PEG-coated materials are usually more stable under physiological conditions. Since Hydroxy-ED_n-undecanethiol has 3 or 6 ethylene glycol units, 11 carbon atoms, and an SH group at the end, it can be used to prepare a highly oriented and hydrophilic SAM on a gold surface which is suitable for biomaterial-labelings on the surface. This is due to the improvement of hydrophilicity. The hydrophilic surface can prevent proteins or other biomaterials from non-specific binding. Therefore, the SAM prepared by this reagent will provide a better surface to develop biomaterial sensors or DNA/ protein microarrays. Hydroxy-ED_n-undecanethiols (n=3, 6) are used to dilute Carboxyl -SAM or Amino-SAM according to the density of the molecules be introduced onto the surface. There are several papers available on labeling proteins such as ovalbumin, and cytochrome C.

References

C. Pale-Grosdemange, *et al.*, *Anal. Chem.*, **71**, 777 (1999); X. Qian, *et al.*, *Anal. Chem.*, **74**, 1805 (2002); M. Kyo, *et al.*, *Anal. Chem.*, **77**, 7115 (2005); A. Subramanian, *et al.*, *Biosensors and Bioelectronics*, **21**, 998 (2006).

XII. HPLC Reagents

Introduction

A large number of labeling reagents have been developed to modify, detect, or monitor certain materials. Fluorometric and colorimetric derivatizing compounds are popular labeling reagents for the analyses of biological samples such as proteins, nucleotides, peptides, and amino acids. Detection and monitoring of fluorescent-labeling materials by fluorescent microscopy or flow cytometry have become the most useful methods for analyzing the functions of materials in cells or tissues. With the development of various kinds of columns and labeling reagents, high performance liquid chromatography (HPLC) has become one of the most important tools for the analyses of non-volatile or unstable compounds in complicated matrixes such as biological samples. Several detection systems of HPLC, UV-visible, and fluorescence detection are the most common methods for the determination of purity or content of a specific compound that has UV-visible absorption or fluorescence. The detection limits of certain compounds depend on their molar absorptivity or fluorescent intensity. These detection limits can be lowered by either of two derivatization methods: One is a pre-column method and the other is a post-column method. Compounds to be derivatized should have functional groups such as amino, carboxy, sulfhydryl, hydroxyl, and aldehyde groups. Most analytes of biological samples are water-soluble and some are unstable, so labeling reactions should be carried out in mild aqueous conditions. Labeling reagents that derivatize target materials in mild conditions are necessary especially for post-column labeling. Dojindo offers a wide variety of labeling reagents for HPLC analysis.

Amine-Reactive Labeling Reagents

Amino compounds readily react with isothiocyanates, succinimidyl esters, acid chlorides, sulfonyl chlorides, and activated aryl halides under mild conditions (Fig. 12-1). The most commonly used fluorescent reagents for protein labeling and DNA sequencing are fluoresceins and rhodamines such as FITC, carboxyfluorescein succinimidyl ester, TRITC, and sulforhodamine acid chloride. Amine labeling reagents are also utilized for protein sequencing. Biotinyl-succinimide reacts with an amino group, and the biotin-labeled compound can be determined with high sensitivity by enzyme immunoassay (EIA) coupled with peroxidase or alkalinephosphatase-labeled avidin. Meares' reagents are utilized to attach chelating functions to protein.

Sulfhydryl-Reactive Labeling Reagents

Maleimido, bromoacetamido, chlorobenzofurazan, and fluorobenzofurazan compounds react with sulfhydryl groups. Maleimido compounds such as NAM are sulfhydryl-specific labeling reagents. The quenched fluorescence is recovered upon addition of sulfhydryl groups to maleimide moieties. Thus, the fluorescent intensities of maleimide compounds are greatly increased upon labeling. Bromoacetamido compounds, such as BABE or FeBABE, are readily conjugated with SH compounds to form a highly stable thioester bond. Chlorobenzofurazan and fluorobenzofurazan compounds also react with amino groups. Biotin labeling reagents and Meares' reagents for sulfhydryl group labeling are also available.

Carboxylate-Reactive Labeling Reagents

Hydrazides and alkyl halides react with carboxylic acids in the presence of a base and a condensing agent such as carbodiimide. Carboxylic acid labeling reagents are useful for the determination of free fatty acids, bile acids, or prostaglandins in biological samples.

Hydroxy-Reactive Labeling Reagents

The reactivity of hydroxyl groups are low, so highly reactive acid chloride is usually used as a labeling reagent. DMEQ-COCl reacts with primary, secondary, and even tertiary alcohols. These reactive reagents are easily hydrolyzed, so it is necessary to keep them from moisture and store them in a refrigerator or a freezer.

Aldehyde-Reactive Labeling Reagents

Aromatic diamines such as DDB react with aldehydes to produce highly fluorescent imidazole derivatives that can be used as labeling reagents for aldehyde. They also react with alpha-keto acid to produce fluorescent quinoxaline derivatives. Aldehyde Reactive Probe (ARP) and NBHA contain aminooxy groups that have highly specific aldehyde and ketone reactivity.

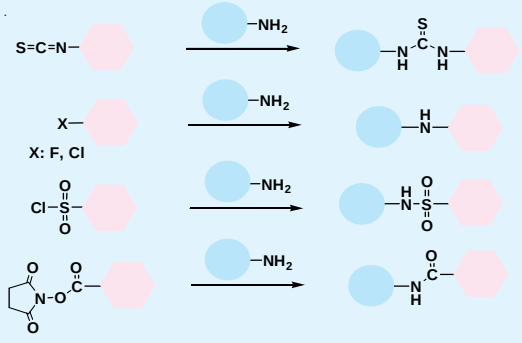


Fig. 12-1 Amine-Reactive Groups

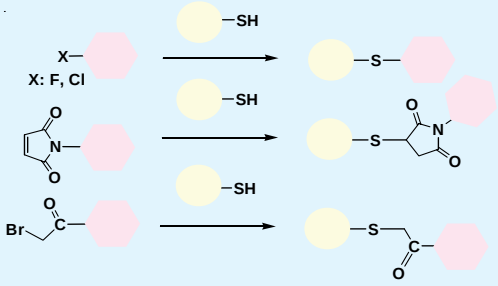


Fig. 12-2 Sulfhydryl-Reactive Groups

XII-1. HPLC Reagents: Amine Reactive/Thiol Reactive

NBD-F

4-Fluoro-7-nitrobenzofurazan [CAS: 29270-56-2]

Application: Amine compound derivatization for HPLC analysis

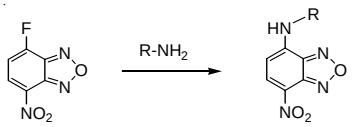
Appearance: pale yellow powder
Purity: >99.0% (HPLC)
MW: 183.10, $C_6H_2FN_3O_3$

Storage Condition	Shipping Condition
-20 °C	ambient temperature

Ordering Information

Product code	Unit
N020-10	50 mg
N020-12	100 mg

Derivatization Reaction



Product Description

NBD-F is highly reactive and can label primary and secondary amines under mild conditions (1 min reaction at 60 °C in a weak basic solution). NBD-F is a pre-labeling compound for HPLC analysis of small molecules. NBD-labeled compounds are orange with a maximum wavelength at 470 nm.The excitation and emission of the derivatized compound are 470 nm and 530 nm, respectively.

NBD Labeling Protocol

1. To prepare sample solution, mix or dissolve a sample with 50 mM borate buffer (pH 8.0) containing 20 mM EDTA.
2. Mix 300 µl of the sample solution and 100 ml of 100 mM NBD-F/acetonitrile solution in a reaction vial.
3. Heat the vial at 60 °C for 1 min and then cool it on an ice bath.
4. Add 400 ml of 50 mM HCl aqueous solution to the reaction mixture.
5. Use this mixture for HPLC analysis to determine NBD-labeled compounds.

References

K. Imai, *et al.*, *Anal. Chim. Acta*, **130**, 377 (1981); Y. Watanabe, *et al.*, *Anal. Biochem.*, **116**, 471 (1981); Y. Watanabe, *et al.*, *J. Chromatogr.*, **239**, 723 (1982); T. Toyo'oka, *et al.*, *Anal. Chim. Acta*, **149**, 305 (1983); Y. Watanabe, *et al.*, *Anal. Chem.*, **55**, 1786 (1983). Y. Watanabe, *et al.*, *J. Chromatogr.*, **309**, 279 (1984); H. Miyano, *et al.*, *Anal. Chim. Acta*, **170**, 81 (1985); H. Kotaniguchi, *et al.*, *J. Chromatogr.*, **420**, 141 (1987); K. Imai, *et al.*, *Anal. Chim. Acta*, **205**, 7 (1988); R. Kobayashi, *et al.*, *Chem. Pharm. Bull.*, **40**, 1327 (1992); K. Imai, *et al.*, *Biomed. Chromatogr.*, **7**, 177 (1993); S. Clark, *et al.*, *J. Chem. Educ.*, **70**, 593 (1993); K. Imai, *et al.*, *Biomed. Chromatogr.*, **7**, 275 (1993); S. F. Santos, *et al.*, *J. Colloid Interface Sci.*, **164**, 260 (1994); H. Ruyters, *et al.*, *J. Liq. Chromatogr.*, **17**, 1883 (1994); C. G. Bradshaw, *et al.*, *J. Med. Chem.*, **37**, 1991 (1994); E. Gazit, W. Lee, *et al.*, *Biochemistry*, **33**, 10681 (1994); K. Yoshinaga, *et al.*, *Biomed. Chromatogr.*, **8**, 297 (1994).;T. Fukushima, *et al.*, *Biomed. Chromatogr.*, **9**, 10 (1995); T. Fukushima, *et al.*, *Analyst*, **120**, 381 (1995); Y. Hui, *et al.*, *J. Pharm. Biomed. Anal.*, **14**, 131(1995); Y. H. Hui, et al., *J. Chromatogr. B. Biomed. Sci. Appl.*, **695**, 337(1997).

ABD-F

4-Fluoro-7-sulfamoylbenzofurazan [CAS: 91366-65-3]

Application: Thiol compound derivatization for HPLC analysis

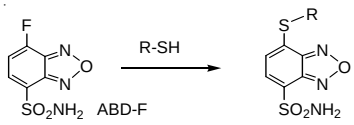
Appearance: white or slightly yellow crystalline powder
Purity: >99.0% (HPLC)
MW: 217.18, $C_6H_4FN_3O_3S$

Storage Condition	Shipping Condition
-20 °C, protect from light and moisture	ambient temperature

Ordering Information

Product code	Unit
A016-10	50 mg
A016-12	100 mg

Derivatization Reaction



Product Description

ABD-F has a benzofurazan moiety that produces a highly fluorescent compound through the reaction with a sulfhydryl group. The excitation and emission of the devivatized compound are 389 nm and 513 nm, respectively. The reaction rate of ABD-F is 30 times faster than that of SBD-F. ABD-F reactions with thiol compounds are completed within 5 minutes in aqueous conditions at 50 °C, pH 8. However, ABD-F does not react with alanine, proline, or cysteine under these conditions. Its maximum fluorescence intensity occurs at pH 2. In reverse-phase HPLC analysis, pre-labeled ABD-thiol compounds can be detected separately. The detection limits (S/N=3) are 0.6 pmol per injection for cysteine, 0.4 pmol per injection for glutathione, 1.9 pmol per injection for N-acetylcysteine, and 0.5 pmol per injection for cysteamine.

ABD Labeling Protocol

1. To prepare sample solution, mix or dissolve a sample with 100 mM borate buffer, pH 8.0 containing 2 mM EDTA.
2. Mix 500 µl of the sample solution and 500 ml of 1 mM ABD-F/100 mM borate buffer in a reaction vial.
3. Heat the vial at 50 °C for 5 min and cool it on an ice bath.
4. Add 300 ml of 100 mM HCl aqueous solution to the reaction mixture.
5. Use this mixture for HPLC analysis to determine ABD-labeled compounds; excitation: 389 nm, emission: 513 nm.

References

T. Toyo'oka, *et al.*, *Anal. Chem.*, **56**, 2461 (1984); T. Toyo'oka, *et al.*, *Anal. Chem.*, **57**, 1931 (1985); T. Toyo'oka, *et al.*, *Pept.* T. Toyo'oka, *et al.*, *Pept. Chem.*, **23rd**, 403 (1986); T. Toyo'oka, *et al.*, *Biomed. Chromatogr.*, **1**, 15 (1986); K. Imai, *et al.*, *Methods Enzymol.*, **143**, 67 (1987); B. L. Ling, *et al.*, *J. Pharm. Biomed. Anal.*, **7**, 1663 (1989); K. Imai, *et al.*, *J. Pharm. Biomed. Anal.*, **7**, 1395 (1989); B. L. Ling, *et al.*, *J. Pharm. Biomed. Anal.*, **7**, 1671 (1989); B. L. Ling, *et al.*, *J. Chromatogr.*, **502**, 230 (1990); B. L. Ling, *et al.*, *J. Chromatogr.*, **514**, 189 (1990); S. Uzu, *et al.*, *Analyst*, **115**, 1477 (1990); T. Toyo'oka, *et al.*, *Analyst*, **116**, 609 (1991); B. L. Ling, *et al.*, *J. Chromatogr.*, **553**, 433 (1991); S. Uzu, *et al.*, *Analyst*, **116**, 1353 (1991); T. Toyooka, *et al.*, *Analyst*, **117**, 727 (1992). K. Imai, *et al.*, *Biomed. Chromatogr.*, **7**, 56 (1993). M. J. Treuheit, *et al.*, *Anal. Biochem.*, **212**, 138 (1993). T. Hiratsuka, *J. Biol. Chem.*, **268**, 24742 (1993); P. E. Cornwell, *et al.*, *J. Chromatogr. Biomed. Appl.* **617**, 136 (1993); S. Takeda., *et al.*, *Trans. Mater. Res. Soc. Jpn.*, **15A**, 509 (1994); T. Fukushima, *et al.*, *Biomed. Chromatogr.*, **9**, 10 (1995); S. Takeda, *et al.*, *J. Biochem.*, **117**, 267 (1995); S. H. Kang, *et al.*, *J. Pharm. Biomed. Anal.*, **15**, 1435 (1997); S. L. Lauren, *et al.*, *J. Chromatogr.*, **798**, 47 (1998); C. C. Chin, *et al.*, *Anal. Biochem.*, **233**, 181 (1999).

NAM

N-(9-Acridinyl)maleimide [CAS: 49759-20-8]

Application: Thiol compound derivatization for HPLC analysis

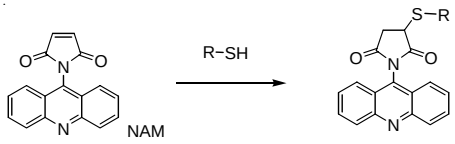
Appearance: yellow crystalline powder
Purity: >96.0% (HPLC)
MW: 274.27, $C_{17}H_{10}N_2O_2$

Storage Condition	Shipping Condition
0-5 °C	ambient temperature

Ordering Information

Product code	Unit
N018-08	10 mg

Derivatization Reaction



Product Description

NAM is a labeling reagent for sulfhydryl groups that forms highly fluorescent compounds. It reacts with thiol compounds in aqueous solutions at pH 3-10, and can be used to detect picomolar levels of cysteine per injection by HPLC. The sensitivity of NAM is approximately 100 times that of DTNB. The excitation and emission of the devivatized compound are 365 nm and 435-440 nm, respectively.

References

H. Takahashi, *et al.*, *Agric. Biol. Chem.*, **43**, 1439 (1979); H. Meguro, *et al.*, *Anal. Lett.*, **16**, 1625 (1983); K. Akasaka, *et al.*, *Anal. Lett.*, **18**, 357 (1985); T. Kamata, *et al.*, *Agric. Biol. Chem.*, **55**, 1989 (1991); T. Yamamoto, *Biochim. Biophys. Acta*, **1163**, 227 (1993); T. Kamata, *et al.*, *Anal. Sci.*, **9**, 867 (1993); T. Konno, *et al.*, *Anal. Sci.*, **9**, 871 (1993); T. Kamata, *et al.*, *Biosci., Biotechnol., Biochem.*, **58**, 881 (1994); T. Kamata, *et al.*, *Biosci., Biotechnol., Biochem.*, **58**, 878 (1994); S. Takeda, *et al.*, *J. Biochem.*, **117**, 267 (1995).

SBD-F

4-Fluoro-7-sulfobenzofurazan, ammonium salt [CAS: 84806-27-9]

Application: Thiol compound derivatization for HPLC analysis

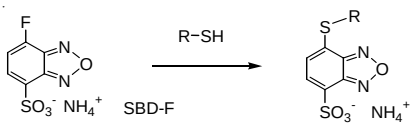
Appearance: pale yellow or pale yellowish-brown crystalline powder
Purity: >98.0% (HPLC)
MW: 235.19, $C_6H_6FN_3O_4S$

Storage Condition	Shipping Condition
-20 °C	ambient temperature

Ordering Information

Product code	Unit
S013-10	50 mg
S013-12	100 mg

Derivatization Reaction



XII-3. HPLC Reagents: Carboxylate Reactive

Product Description

SBD-F is a water-soluble reagent that reacts with sulfhydryl groups to produce highly fluorescent compounds. The detection limit of thiol compounds such as glutathione, cysteine, N-acetylcysteine, CoA and BSA by HPLC analysis is in the range of 100-500 pmol per injection.The excitation and emission of the derivatized compound are 385 nm and 515 nm, respectively.

References

K. Imai, *et al., Anal. Biochem.*, **128**, 471 (1983); K. Imai, *et al., J. Chromatogr.*, **282**, 495 (1983); T. Toyo'oka, *et al., Analyst*, **109**, 1003 (1984); T. Sueyoshi, *et al., J. Biochem.*, **97**, 1811 (1985); T. Toyo'oka, *et al., Chim. Acta*, **205**, 29 (1988); J. B. Ubbink, *et al., J. Chromatogr.*, **565**, 441 (1991); B. L. Ling, *et al., J. High Resolut. Chromatogr.*, **14**, 169 (1991); B. L. Ling, *et al., J. Chromatogr.*, **553**, 433 (1991); T. Toyo'oka, *et al., J. Chromatogr.*, **588**, 61 (1991); P. B. Young, *et al., J. Liq. Chromatogr.*, **17**, 3553 (1994); I. Daskalakis, *et al., Biomed. Chromatogr.*, **10**, 205 (1996); T. Araki, *et al., Arch. Biochem. Biophys.*, **335**, 118 (1996;. N. P. Dudman, *et al., Clin. Chem.*, **42**, 2028 (1996); S. H. Kang, *et al., J. Pharm. Biomed. Anal.*, **15**, 1435 (1997); S. Miyairi, *et al., Anal. Biochem.*, **258**, 168 (1998); T. Oe, *et al., J. Chromatogr. B. Biomed. Sci. Appl.*, **708**, 285 (1998); I. Fermo, *et al., J. Chromatogr. B. Biomed. Sci. Appl.*, **719**, 31 (1998); C. M. Pfeiffer, *et al., Clin. Chem.*, **45**, 1261(1999).

Br-DMEQ

3-Bromomethyl-6,7-dimethoxy-1-methyl-1,2-dihydroquinoxaline-2-one [CAS: 100595-07-1]

Application: Carboxylic acid derivatization for HPLC analysis

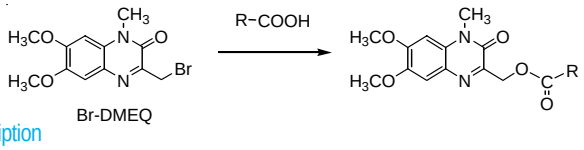
Appearance: yellow needles
Purity: >96.0% (HPLC)
MW: 313.15, $C_{12}H_{13}BrN_2O_3$

Storage Condition	Shipping Condition
-20 °C	ambient temperature

Ordering Information

Product code	Unit
B036-10	10 mg

Derivatization Reaction



Br-DMEQ

Product Description

Br-DMEQ is a highly fluorescent labeling reagent for carboxylates such as fatty acids and prostaglandins. Quinoxalinone compounds are formed by a reaction between 1,2-diamino-4,5-dimethoxybenzene and pyruvic acid. Br-DMEQ also reacts with pyrimidine bases of nucleic acids. Potassium carbonate and 18-crown-6 are necessary for carboxylate labeling with Br-DMEQ. The HPLC detection limit of saturated fatty acids with 3 to 20 carbons is 0.3 to 1 femtomoles per injection. The excitation and emission wavelength of labeled materials are 370 nm and 450 nm, respectively.

DMEQ Labeling Protocol

1. Dissolve a sample with acetonitrile to prepare sample solution.
2. Mix 500 µl of the sample solution, 250 µl of 3.8 mM Br-DMEQ/acetonitrile, and 250 µl of 0.8 mM 18-crown-6/acetonitrile in a reaction vial.
3. Heat the vial at 80 °C for 20 min and then cool it in water bath.
4. Use the mixture for HPLC analysis to determine DMEQ-labeled compounds; excitation: 370 nm, emission: 450 nm.

References

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Br-Mmc

4-Bromomethyl-7-methoxycoumarin [CAS: 35231-44-8]

Application: Carboxylic acid derivatization for HPLC analysis

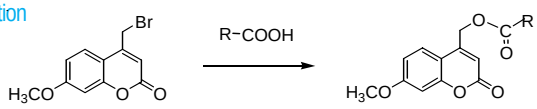
Appearance: pale yellow or light yellow crystalline powder
MW: 269.09, $C_{11}H_9BrO_3$

Storage Condition	Shipping Condition
ambient temperature	ambient temperature

Ordering Information

Product code	Unit
B023-10	100 mg

Derivatization Reaction



Br-Mmc

Product Description

Br-Mmc, a coumarin derivative, is a blue fluorescent labeling reagent for carboxylates. This reagent is used for the determination of trace fatty acids in medicines or agricultural chemicals by TLC or HPLC. Potassium carbon is necessary for the labeling. The reaction is completed within 40 to 60 min in acetone reflux. Br-Mmc does not react with pyruvic acid, trichloroacetic acid, fumaric, acid or acetylenedicarboxylic acid. The excitation and emission wavelength of labeled materials are 360 nm and 410 nm, respectively.

References

W. Dünges, *Chromatographia*, **9**, 624 (1976); W. Dünges, *Anal. Chem.*, **49**, 442 (1977); J. Turk, *et al., Prostaglandins*, **16**, 291 (1978); S. Okuyama, *et al., Chem. Lett.*, 461 (1979). C. J. Knight, *Biochem.* J., **274**, 45 (1991); H. Cohen, *et al., J. Liq. Chromatogr.*, **14**, 313 (1991); K. Berger, *et al., Dtsch. Lebensm.-Rundsch.*, **87**, 137 (1991); X. He, *et al., Chin. Chem. Lett.*, **2**, 953 (1991); V. L. McGuffin, *et al., J. Microcolumn Sep.*, **3**, 513 (1991); M. R. L. Stratford, *et al., Int. J. Radiat. Oncol., Biol., Phys.*, **22**, 485 (1992); M. T. French, *et al., J. Pharm. Biomed. Anal.*, **10**, 23 (1992); J. H. Wolf, *et al., J. Pharm. Biomed. Anal.*, **10**, 99 (1992); E. H. J. M. Jansen, *et al., J. Liq. Chromatogr.*, **15**, 2247 (1992); S. Guldutuna, *et al., Clin. Chim. Acta*, **214**, 195 (1993); R. Ben Gueddour, *et al., Anal. Lett*, **26**, 429 (1993); J. Baranowska-Kortylewicz, *et al., Bioconjugate Chem.*, **4**, 300 (1993); A. Moreno, *et al., Comun. Jorn. Com. Esp. Deterg.*, **24**, 45 (1993); T. Furuta, *et al., J. Chem. Soc., Perkin Trans. 1*, **24**, 3139 (1993); R. Ben Gueddour, *et al., Talanta*, **41**, 485 (1994); M. Li, *et al., Bioconjugate Chem.*, **5**, 454 (1994); R. Abushufa, *et al., Clin. Chem.*, **40**, 1707 (1994); J. C. Marr, *et al., Nat. Toxins*, **2**, 302 (1994); M. Slebioda, *et al., Chem. Anal.(Warsaw)*, **39**, 439 (1994); T. Furuta, *et al., J. Org. Chem.*, **60**, 3953 (1995); C. Aromdee, *et al., J Chromatogr B Biomed Appl.*, **677**, 313 (1996); K. Wang, *et al., J. Am. Soc. Mass Spectrom.*, **9**, 970 (1998); J. K. Robinson, *et al., J. Chromatogr. B Biomed. Sci. Appl.*, **731**, 179 (1999).

DMEQ-COCl

3-Chlorocarbonyl-6,7-demethoxy-1-methyl-2(*1H*)-quinoxalinone [CAS: 104077-15-8]

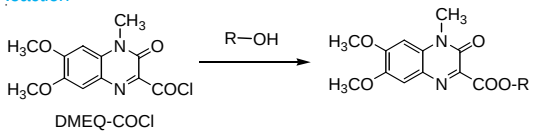
Application: Alcohol derivatization for HPLC analysis

Appearance: reddish-orange or orange powder
Purity: >95.0% (HPLC, derivatization)
MW: 282.68, $C_{12}H_{11}ClN_2O_4$

Ordering Information	
Product code	Unit
D049-10	10 mg

Storage Condition	Shipping Condition
-20 °C, protect from moisture	with dry ice or blue ice

Derivatization Reaction



DMEQ-COCl

Product Description

DMEQ-COCl is a labeling reagent for primary and secondary alcohols. In organic solvents such as benzene and acetonitrile, this reagent readily reacts with alcohols to yield highly fluorescent derivatives. The HPLC detection limits of benzylalcohol, n-hexanol, and cyclohexanol are 2-3 femtomoles per injection. Steroids that have primary and secondary alcohols can be detected as fluorescent DMEQ derivatives. However, *tert*-alcohols, hydroxycarboxylic acids, and phenols do not react under the same labeling conditions. DMEQ-COCl also reacts with amines; as little as 0.3 pmol per ml of β-phenylethylamine has been detected in human serum. The excitation and emission wavelengths of the labeled materials are 400 nm and 500 nm, respectively.

References

T. Iwata, *et al., J. Chromatogr.*, **362**, 209 (1986); J. Ishida, *et al., Anal. Biochem.*, **184**, 86 (1990); J. Ishida, *et al., Anal. Biochem.*, **195**, 168 (1991).

DDB

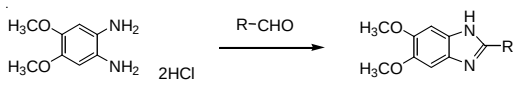
1,2-Diamino-4,5-dimethoxybenzene, dihydrochloride [CAS: 131076-14-7]

Application: Aromatic aldehyde derivatization
Appearance: white or slightly pink crystalline powder
Purity: >98.0% (Titration)
MW: 241.11, $C_8H_{14}Cl_2N_2O_2$

Ordering Information	
Product code	Unit
D034-10	50 mg

Storage Condition	Shipping Condition
-20 °C, protect from moisture	with dry ice or blue ice

Derivatization Reaction



XII-5. HPLC Reagents: Aldehyde Reactive

Product Description

DDB reacts with aromatic aldehydes in acidic conditions to give strong fluorescent benzimidazole. This reagent, as well as DTAN, can be used for the detection of aromatic aldehydes. DDB is soluble in aqueous solutions. The excitation and emission wavelength of the labeled materials are 338 nm and 402 nm, respectively.

References

M. Nakamura, *et al.*, *Anal. Chim. Acta*, **134**, 39 (1982); M. Nakamura, *et al.*, *Chem. Pharm. Bull.*, **31**, 2910 (1983); T. Iwata, *et al.*, *Chem. Pharm. Bull.*, **33**, 3499 (1985); S. Hara, *et al.*, *J. Chromatogr.*, **377**, 111 (1986); H. Masui, *et al.*, *Inorg. Chem.*, **32**, 258 (1993).

MDB

1,2-Diamino-4,5-methylenedioxybenzene, dihydrochloride [CAS: 38608-07-0]

Application: Alpha-keto acid derivatization
Appearance: white or slightly pink crystalline powder
Purity: >98.0% (Titration)
MW: 225.07, $C_7H_{10}Cl_2N_2O_2$

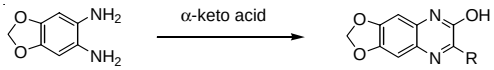
Ordering Information

Product code	Unit
M021-10	50 mg
M021-12	100 mg

Storage Condition
-20 °C, protect from moisture

Shipping Condition
with dry ice or blue ice

Derivatization Reaction



MDB 2HCl

Product Description

Phenylenediamine (OPD) reacts with α-keto acids to form highly fluorescent quinoxaline derivatives. MDB is an *o*-phenylenediamine analogue. It is the best labeling reagent for α-keto acids because of its reactivity and sensitivity. In HPLC analysis, more than ten different a-keto acids, such as α-keto glutaric acid, pyruvic acid, and *p*-hydroxyphenyl-pyruvic acid, can be separately detected at a time, even at concentrations of only a few femtomoles per injection. The sensitivity of MDB is almost 150 times as high as OPD. The excitation and emission wavelength of the labeled materials are 367 nm and 445 nm, respectively.

References

M. Nakamura, *et al.*, *Chem. Pharm. Bull.*, **35**, 687 (1987); S. Hara, *et al.*, *Anal. Chim. Acta*, **215**, 267 (1988); T. Kremmer, *et al.*, *Biochim. Clin.*, **11**, 995 (1992); A. Lagana, *et al.*, *Anal. Biochem*, **215**, 266 (1993); E. Fujii, *et al.*, *J. Chromatogr., B: Biomed. Appl.*, **660**, 265 (1994); B. K. Singh, *et al.*, *J. Liq. Chromatogr.*, **17**, 4469 (1994); A. P. Bishop, *et al.*, *Free Radical Biol. Med.*, **18**, 617 (1995); P. G. Stanton, *et al.*, *J. Biochem. Biophys. Methods*, **30**, 37 (1995); C. Sato, *et al.*, *Anal. Biochem.*, **261**, 191 (1998); C. Sato, *et al.*, *Anal. Biochem.*, **266**, 102 (1999); T. Hayakawa, *et al.*, *J. Oral. Sci.*, **41**, 9 (1999).

XIII. Detergents

Introduction

The phospholipid bilayer is the basic structure of the cell membrane. The most important functions of cells include transportation of substances, energy exchange, and transmission of information. These functions are conducted at the cell membrane by membrane proteins.

In membrane biochemistry research, membrane proteins are solubilized and purified to study their structure and function. Proteins bound to cell membranes have hydrophobic sites buried within the phospholipid bilayers and hydrophilic sites facing toward the water layer. Detergents are utilized to isolate large insoluble molecules like proteins. Detergents interact with the hydrophobic sites of proteins, which are then solubilized in the water layer. In this way, membrane proteins can be separated. It is important to choose a detergent that does not disrupt the bioactivities of target proteins. A detergent requires the following characteristics to be suitable for membrane protein isolation:

1) Sufficient protein solubilization capability
2) No denaturing or inactivation of proteins
3) No interference with protein activities
4) No precipitation at 4 °C
5) Appropriate CMC and micelle size
6) No absorption in the UV region
7) No toxicity
8) Availability of detergent detection methods
9) Non-ionic detergent if ion exchange chromatography is used

In the past, polyoxyethylene ether non-ionic detergents were widely used. These detergents, however, had several problems such as denaturation of proteins and low critical micelle concentrations (CMC) value, which cannot be separated easily by dialysis. *n*-Octyl-β-D-glucoside, *n*-Octyl-β-D-thioglucoside, CHAPS, and CHAPSO eliminate these problems and are widely used today. Most of the current detergents are non-ionic, and easily applied to ion exchange chromatography purification. Deoxy-BIGCHAP is a non-ionic detergent possessing deoxycholic acid and a gluconamide polar group. It has a high CMC value of 1.4 mM, and can be easily separated by dialysis. Because its UV absorbance is low, it can be used for the determination of proteins. Deoxy-BIGCHAP has been used for the extraction of opioid receptors from neuroblastoma or hybrid cells of glyoma. It has also been applied to adenylate cyclase or acetyltransferase. These detergents are also widely utilized to solubilize chromophores or to stabilize enzymes in diagnostic analyses and biochemical assays.

Trials of various kinds of detergents are needed to find the appropriate detergent for each study. Dojindo's Detergent Screening Sets, which contain assorted packages of detergents, are available for use in these trials.

Critical Micelle Concentration (CMC)

Detergents are amphipathic compounds, with both lipophobic and lipophilic sites, that will form micelles above a critical concentration that is specific to each detergent. This is called the critical micelle concentration (CMC). The solubilizing abilities of detergents increase dramatically above their CMC values. After extracting membrane proteins, detergents can be easily removed by dilution, and then dialysis.

Detergent	MW	CMC
CHAPS	614.88	8 mM
CHAPSO	630.88	8 mM
BIGCHAP	878.06	2.9 mM
DeoxyBIGCHAP	862.06	1.4 mM
Octylglucoside	292.37	25 mM
Heptylthioglucoside	294.41	30 mM
Octylthioglucoside	308.44	9 mM
Decylmaltoside	482.57	1.8 mM
Dodecylmaltoside	510.62	0.17 mM
Nonylthiomaltoside	484.60	2.4 mM
MEGA-8	321.41	-
MEGA-9	335.44	25 mM
MEGA-10	349.46	7 mM
Sucrose monocholate	732.85	4.7 mM
Sodium cholate	448.57	14 mM

BIGCHAP

N,N-Bis(3-D-gluconamidopropyl)cholamide [CAS: 86303-22-2]

Appearance: white powder
Purity: >98.0% (HPLC)
MW: 878.06, $C_{42}H_{75}N_3O_{16}$

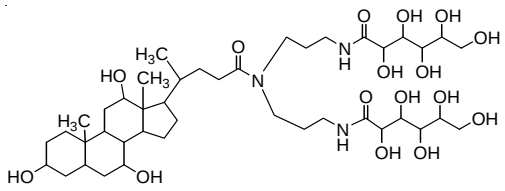
Storage Condition
ambient temperature

Shipping Condition
ambient temperature

Ordering Information

Product code	Unit
B043-10	1 g
B043-12	5 g

Chemical Structure



XIII. Detergents

deoxy-BIGCHAP

N,N-Bis(3-D-gluconamidopropyl)deoxycholamide [CAS: 86303-23-3]

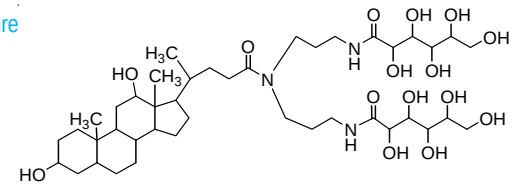
Appearance: white powder
Purity: >95.0% (HPLC)
MW: 862.06, $C_{42}H_{75}N_3O_{15}$

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Ordering Information

Product code	Unit
D045-08	500 mg

Chemical Structure



Product Description of BIGCHAPs

BIGCHAP and deoxy-BIGCHAP are non-ionic detergents based on a cholic acid and a bisglucon-amidopropyl group. The CMC values are 2.9 mM and 1.4 mM, respectively. BIGCHAPS and deoxyBIGCHAP are easily removed by dialysis, and their absorption in the UV region is very low.

References

L. J. Hjelmeland, *et al.*, *Anal. Biochem.*, **130**, 485 (1983); V. Lakashmi, *et al.*, *J. Steroid Biochem.*, **22**, 331 (1985); H. Buehler, *et al.*, *Biochim. Biophys. Acta*, **1075**, 206 (1991); F. S. Bonelli, *et al.*, *Biochim. Biophys. Acta*, **1166**, 92 (1993); A. Aigner, *et al.*, *Biochem. J.*, **317**, 213 (1996); S. Abe, *et al.*, *Forensic Sci. Int.*, **91**, 19 (1998).

CHAPS

3-[(3-Cholamidopropyl)dimethylammonio]propanesulfonic acid [CAS: 75621-03-3]
Licenced under U.S. Patent 4,372,888

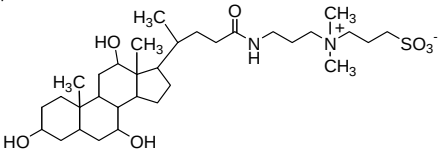
Appearance: white powder
Purity: >97.0% (HPLC)
MW: 614.88, $C_{32}H_{58}N_2O_7S$

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Ordering Information

Product code	Unit
C008-10	1 g
C008-12	5 g
C008-14	25 g

Chemical Structure



CHAPSO

3[(3-Cholamidopropyl)dimethylammonio]-2-hydroxypropanesulfonic acid [CAS: 82473-24-3]
Licenced under U.S. Patent 4,372,888

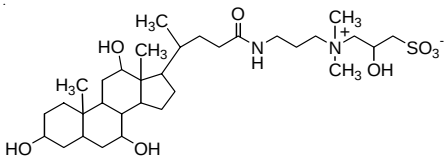
Appearance: white powder
Purity: >96.0% (HPLC)
MW: 630.88, $C_{32}H_{58}N_2O_8S$

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Ordering Information

Product code	Unit
C020-10	1 g
C020-12	5 g

Chemical Structure



Product Description of CHAPS and CHAPSO

CHAPS and CHAPSO are zwitter ionic detergents, and have cholic acid and sulfobetaine moieties in their structures. Their low background absorbance in the UV region is an attractive feature for the UV monitoring of membrane proteins. The CMC values of both CHAPS and CHAPSO are 8 mM.

References
CHAPS: W. F. Simonds, *et al.*, *Proc. Natl. Acad. Sci. USA.*, **77**, 4623 (1980); L. M. Hjelmeland, *Proc. Natl. Acad. Sci USA.*, **77**, 6368 (1980); A. J. Bitonti, *et al.*, *Biochemistry*, **21**, 3650 (1982); B. Rivnay, *et al.*, *Biochemistry*., **21**, 6922 (1982); T. Murayama, *et al.*, *Biomed. Res*., **14**, 71 (1993); B. Lundberg, *J. Pharm. Sci*., **83**, 72 (1994); S. Caglio, *et al.*, *Electrophoresis (Weinheim, Fed. Repub. Ger.)*, **15**, 209 (1994); W. Hu, *et al.*, *Anal. Chim. Acta*, **285**, 335 (1994); S. P. Radko, *et al.*, *Anal. Biochem*., **223**, 82 (1994); R. R. O. Loo, *et al.*, *Protein Sci*., **3**, 1975 (1994); D. B. Wetlaufer, *et al.*, *Protein Sci*., **4**, 1535 (1995); D. Apanovitch, *et al.*, *Biochim. Biophys. Acta*, **1291**, 16 (1996); I. Ibarrola, *et al.*, *Biochim. Biophys. Acta*, **1284**, 41 (1996); E. Boadu, *et al.*, *Biochemistry*, **36**, 10954 (1997); S. Parvathy, *et al.*, *Biochem. J*., **327**, 37 (1997); W. J. Arion, *et al.*, *J. Biol. Chem*., **273**, 6223 (1998); C. Hannam, *et al.*, *Anal. Biochem*., **258**, 246 (1998); P. Riccio, *et al.*, *Neuroreport*, **9**, 2769 (1998); K. Aoyagi, *et al.*, *J. Clin. Microbiol*., **37**, 1802 (1999); L. H. Conlan, *et al.*, *Biotechniques*, **27**, 955 (1999); C. Yu, *et al.*, *J. Biol. Chem*., **274**, 36139 (1999).
CHAPSO: W. F. Simonds, *et al.*, *Proc. Natl. Acad. Sci. USA*, **77**, 4623 (1980); L. M. Hjelmeland, *Proc. Natl. Acad. Sci. USA*, **77**, 6368 (1980); D. S. Liscia, *et al.*, *J. Biol. Chem*., **257**, 9401 (1982); W. Gaertner, *et al.*, *Photochem. Photobiol*., **54**, 1047 (1991); W. Hu, *et al.*, *Anal.Chem*., **65**, 2204 (1993); H. H. Hassanain, *et al.*, *Anal. Biochem*., **213**, 162 (1993); P. Banerjee, *et al.*, *Chem. Phys. Lipids*, **77**, 65 (1995); Y. Chen, *et al.*, *Biochemistry*, **35**, 3227 (1996); N. A. Wilson, *et al.*, *J. Immunol*., **161**, 4777 (1998); M. B. Jones, *et al.*, *Anal. Biochem*., **268**, 126 (1999); G. Q. Chen, *et al.*, *Biochemistry*, **38**, 15380 (1999).

Sodium cholate (purified)

Cholic acid, sodium salt, monohydrate [CAS: 361-09-1]

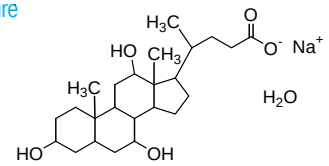
Appearance: white powder
Purity: >98.0% (HPLC)
MW: 448.57, $C_{24}H_{39}NaO_5 \cdot H_2O$

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Ordering Information

Product code	Unit
C321-10	5 g
C321-12	25 g

Chemical Structure



Product Description

Sodium cholate is an anionic detergent with 14 mM CMC. Since three hydroxy groups are located on a steroid ring and one carboxylic group is at the terminus of the molecular structure, there are no definitive lipophilic or hydrophilic regions in the molecule. In general, cholate detergents can be easily removed by dialysis because of their fairly small micelle sizes.

n-Decyl-β-D-maltoside

n-Decyl-β-D-maltopyranoside [CAS: 82494-09-5]

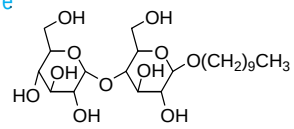
Appearance: white powder
Purity: >98.0% (GC)
MW: 482.57, $C_{22}H_{42}O_{11}$

Storage Condition: 0-5 °C
Shipping Condition: ambient temperature

Ordering Information

Product code	Unit
D382-10	1 g
D382-12	5 g

Chemical Structure



n-Dodecyl-β-D-maltoside

n-Dodecyl-β-D-maltopyranoside [CAS: 69227-93-6]

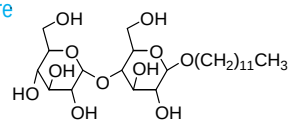
Appearance: white powder
Purity: >98.0% (GC)
MW: 510.62, $C_{24}H_{46}O_{11}$

Storage Condition: 0-5 °C
Shipping Condition: ambient temperature

Ordering Information

Product code	Unit
D316-10	1 g
D316-12	5 g

Chemical Structure



XIII. Detergents

Product Description

n-Dodecyl-β-D-maltoside is a non-ionic detergent. It has a glyco-chain in its lipophilic site that is similar to the one in *n*-Octyl-β-D-glucoside. Using this detergent, Dr. Van Aken and others succeeded in solubilizing the active cytochrome oxidase from mitochondria. Its CMC value is 0.17 mM.

References

T. VanAken, *et al.*, *Methods Enzymol.*, **125**, 27 (1986); G. Zardeneta, *et al.*, *Anal. Biochem.*, **218**, 392 (1994); D. H. W. Kastrau, *et al.*, *Eur. J. Biochem.*, **222**, 293 (1994); M. Yeager, *Acta Crystallogr., Sect. D: Biol. Crystallogr.*, **D50**, 632 (1994); A. Musatov, *et al.*, *Biochemistry*, **33**, 13005 (1994); J. Stoeckel, *et al.*, *J. Biol. Chem.*, **269**, 29571 (1994); Van Erum, M., *et al.*, *Eur. J. Biochem.*, **227**, 150 (1995); X. Grandier-Vazeille, *et al.*, *Anal. Biochem.*, **242**, 248 (1996); M. Dong, *et al.*, *Anal. Biochem.*, **247**, 333 (1997); L. Heginbotham, *et al.*, *Biochemistry*, **36**, 10335 (1997); E. Tamai, *et al.*, *Protein Expr. Purif.*, **10**, 275 (1997); M. Dong, *et al.*, *Biochim. Biophys. Acta*, **1371**, 317 (1998); O. Lambert, *et al.*, *Biophys. J.*, **74**, 918 (1998); J. Knol, *et al.*, *Biochemistry*, **37**, 16410 (1998); D. Orlowski, *et al.*, *Cancer Biochem. Biophys.*, **16**, 85 (1998); M. Beck, *et al.*, *FEBS Lett.*, **436**, 304 (1998); M. Putman, *et al.*, *Biochemistry*, **38**, 1002 (1999); N. J. Fraser, *et al.*, *Biochemistry*, **38**, 9684 (1999); Y. Gerchman, *et al.*, *J. Biol. Chem.*, **274**, 24617 (1999); A. Poetsch, *et al.*, *Biochem. Biophys. Res. Commun.*, **265**, 520 (1999); U. Ahting, *et al.*, *J. Cell. Biol.*, **147**, 959 (1999); J. K. Hill, *et al.*, *J. Neurosci.*, **26**, 9944 (2006); M. Iwamoto, *et al.*, *J. Biol. Chem.*, **281**, 28379 (2006); S. Tanaka, *et al.*, *J. Biol. Chem.*, **282**, 13379 (2007); S. Narita, *et al.*, *J. Biol. Chem.*, **282**, 13372 (2007); A. Seki, *et al.*, *Biophys. J.*, **92**, 2559 (2007).

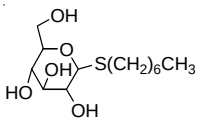
n-Heptyl-β-D-thioglucoside

n-Heptyl-β-D-thioglucopyranoside [CAS: 85618-20-8]

Appearance: white waxy solid
Purity: >98.0% (HPLC)
MW: 294.41, $C_{13}H_{26}O_5S$

Storage Condition	Shipping Condition
ambient temperature	ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
H015-10	1 g
H015-12	5 g

Product Description

n-Heptyl-β-D-thioglucoside is an effective non-ionic detergent for the solubilization of membrane proteins such as *n*-Octyl-β-D-glucoside. Though *n*-Octyl-β-D-glucoside is degraded by β-glycosidase, thioether is not. This means that *n*-Heptyl-β-D-thioglucoside is effective for the solubilization of samples with β-glycosidase activity. The CMC value of *n*-Heptyl-β-D-thioglucoside is 30 mM. It is soluble in aqueous solutions at 4 °C making it suitable for the solubilization of membrane proteins at low temperatures.

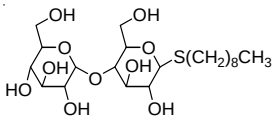
n-Nonyl-β-D-thiomaltoside

n-Nonyl-β-D-thiomaltopyranoside [CAS: 148565-55-3]

Appearance: white power
Purity: >98.0% (GC)
MW: 484.60, $C_{21}H_{40}O_{10}S$

Storage Condition	Shipping Condition
0-5 °C	ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
N373-10	1 g

Product Description

n-Nonyl-β-D-thiomaltoside is a newly developed detergent whose lipophilic moiety is maltose. This reagent is suitable for isolating proteins inside of cell membranes. The activity of protein isolated using *n*-Nonyl-β-D-thiomaltoside is higher than those isolated using *n*-Octyl-β-D-thioglucoside or *n*-Heptyl-β-D-thioglucoside. As the CMC value of this detergent is 2.4 mM, it is easy to remove from protein solutions by dialysis.

Reference

S. Izawa, *et al.*, *J. Biochem.*, **113**, 573 (1993).

n-Octyl-β-D-glucoside

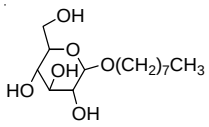
n-Octyl-β-D-glucopyranoside [CAS: 29836-26-8]

Appearance: white powder
Purity: >98.0% (GC)
MW: 292.37, $C_{14}H_{28}O_6$

Ordering Information	
Product code	Unit
O001-10	1 g
O001-12	5 g

Storage Condition: 0-5 °C
Shipping Condition: ambient temperature

Chemical Structure



Product Description

n-Octyl-β-D-glucoside has definite advantages over the conventional non-ionic detergents as a membrane protein solubilizer. It can be easily separated by dialysis, and has very little interaction with membrane proteins. Its CMC value is 25 mM. It is strongly recommended for studies of ATPase or NADH dehydrogenase.

References

C. Baron, *et al.*, *Biochim. Biophys. Acta*, **382**, 276 (1975); W. J. Schneider, *et al.*, *Proc. Natl. Acad. Sci. USA*, **76**, 5577 (1979); S. Horiuchi, *et al.*, *Biol. Chem.*, **260**, 475 (1985); A. Levitzki, *Biochim. Biophys. Acta*, **822**, 127 (1985); A. Malley, *et al.*, *J. Immunol. Methods*, **178**, 31 (1995); J. T. Smith, *et al.*, *Electrophoresis*, **15**, 1248 (1994); J. T. Smith, *et al.*, *J. Chromatogr.*, **685**, 131 (1994); M. Matsumoto, *et al.*, *J. Chromatogr.*, **663**,217 (1995); E. J. Weinman, *et al.*, *Am. J. Med. Sci.*, **312**, 47 (1996); P. Boulanger, *et al.*, *Biochemistry*, **35**, 14216 (1996); O. Vinogradova, *et al.*, *Biophys. J.*, **72**, 2688 (1997); C. H. Yun, *et al.*, *J. Biol. Chem.*, **272**, 19725 (1997); Y. T. Hsu, *et al.*, *J. Biol. Chem.*, **273**, 10777 (1998); D. E. Cohen, *et al.*, *Biochemistry*, **37**, 14798 (1998); S. S. Iyer, *et al.*, *J. Biol. Chem.*, **274**, 2350 (1999); L. H. Conlan, *et al.*, *Biotechniques*, **27**, 955 (1999).

n-Octyl-β-D-thioglucoside

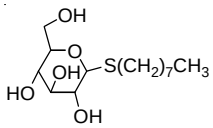
n-Octyl-β-D-thioglucopyranoside [CAS: 85618-21-9]

Appearance: white powder
Purity: >98.0% (GC)
MW: 308.44, $C_{14}H_{28}O_5S$

Ordering Information	
Product code	Unit
O003-10	1 g
O003-12	5 g

Storage Condition: 0-5 °C
Shipping Condition: ambient temperature

Chemical Structure



Product Description

n-Octyl-β-D-thioglucoside is similar to *n*-Octyl-β-D-glucoside. After solubilization, it can be separated from phospholipid or protein by dialysis. It does not interfere with enzyme activities. It is more stable than *n*-Octyl-β-D-glucoside and is not affected by esterase. Its CMC value is 9 mM.

References

S. Saito, *et al.*, *Biochem. J.*, **222**, 829 (1984); T. Tsuchiya, *et al.*, *J. Biochem.*, **96**, 1593 (1984); M. R. Wenk, *et al.*, *Biophys. J.*, **73**, 2565 (1997).

n-Octyl-β-D-maltoside

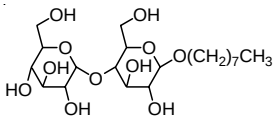
n-Octyl-β-D-maltopyranoside [CAS: 82494-08-4]

Appearance: white powder
Purity: >98.0% (GC)
MW: 454.51, $C_{20}H_{38}O_{11}$

Ordering Information	
Product code	Unit
O393-10	500 mg

Storage Condition: 0-5 °C
Shipping Condition: ambient temperature

Chemical Structure



References

T. Tsukihara, *et al.*, *Science*, **269**, 1069 (1995), S. Iwata, *et al.*, *Nature*, **376**, 660 (1995).

XIII. Detergents

3-Oxatridecyl-α-D-mannoside

3-Oxatridecyl-α-D-mannopyranoside

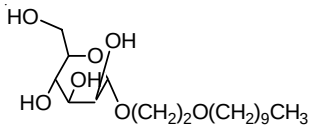
Appearance: white powder
Purity: >98.0% (GC)
MW: 364.47, $C_{18}H_{36}O_7$

Storage Condition: 0-5 °C
Shipping Condition: ambient temperature

Ordering Information

Product code	Unit
O401-10	500 mg

Chemical Structure



MEGA-8

n-Octanoyl-*N*-methyl-D-glucamine [CAS: 85316-98-9]

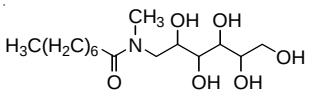
Appearance: white powder
Purity: >98.0% (HPLC)
MW: 321.41, $C_{15}H_{31}NO_6$

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Ordering Information

Product code	Unit
M014-10	1 g
M014-12	5 g

Chemical Structure



MEGA-9

n-Nonanoyl-*N*-methyl-D-glucamide [CAS: 85261-19-4]

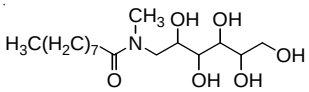
Appearance: white powder
Purity: >98.0% (HPLC)
MW: 335.44, $C_{16}H_{33}NO_6$

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Ordering Information

Product code	Unit
M015-10	1 g
M015-12	5 g

Chemical Structure



MEGA-10

n-Decanoyl-*N*-methyl-D-glucamide [CAS: 85261-20-7]

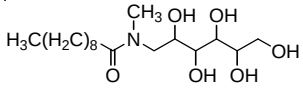
Appearance: white powder
Purity: >98.0% (HPLC)
MW: 349.46, $C_{17}H_{35}NO_6$

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Ordering Information

Product code	Unit
M016-10	1 g
M016-12	5 g

Chemical Structure



Product Description

Glucamide detergents are non-ionic, transparent in the UV region, and possess relatively high CMC values. Therefore, they are ideal for use as membrane protein solubilizers. The CMC values for these detergents are between 7 mM and 25 mM.

References
J. E. K. Hildreth, *Biochem. J.*, **207**, 363 (1982); M. Hanatani, *et al.*, *J. Biochem.*, **95**, 1349 (1984); I. F. Luescher, *et al.*, *J. Immunol. Methods*, **135**, 233 (1990); C, Morissette, *et al.*, *Appl. Environ. Microbiol.*, **57**, 836 (1991); R. A. Farrell, *et al.*, *J. Inorg. Biochem.*, **49**, 9 (1993); T. Nishiki, *et al.*, *Biochim. Biophys. Acta*, **1158**, 333 (1993); B. Lundberg, *J. Pharm. Sci.*, **83**, 72 (1994); D. Little, *et al.*, *Biochem. J.*, **304**, 951 (1994); J. T. Smith, *et al.*, *J. Chromatogr.*, **685**, 131 (1994); J. T. Smith, *et al.*, *Anal. Chem.*, **66**, 1119 (1994); P. Banerjee, *et al.*, *Chem. Phys. Lipids*, **77**, 65 (1995); B. G. Sundquist, *et al.*, *Vaccine*, **14**, 892 (1996); J. . Sowadski, *et al.*, *J. Bioenerg. Biomembr.*, **28**, 7 (1996); I. Abusugra, *et al.*, *Vet. Immunol. Immunopathol.*, **59**, 31 (1997); G. Hollerer-Beitz, *et al.*, *Receptors Channels*, **5**, 61 (1998).

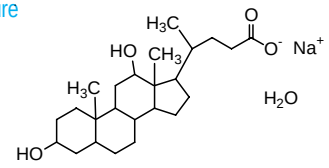
Sodium deoxycholate (for protein crystallization)

Deoxycholic acid, sodium salt, monohydrate [CAS: 145224-92-6]

Appearance: white powder
Purity: >98.0% (HPLC)
MW: 432.57, $C_{24}H_{39}NaO_4 \cdot H_2O$

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
D520-10	1 g
D520-12	5 g

Detergent Screening Set (first choice-II)

Contents of the Set

CHAPS	200 mg x 1
n-Dodecyl-β-D-maltoside	200 mg x 1
n-Octyl-β-D-glucoside	200 mg x 1
Sodium cholate (purified)	200 mg x 1
MEGA-8	200 mg x 1

Storage Condition: 0-5 °C
Shipping Condition: ambient temperature

Ordering Information

Product code	Unit
DS06-10	1 set

Detergent Screening Set (for crystallization)

Contents of the Set

n-Decyl-β-D-maltoside	200 mg x 1
n-Dodecyl-β-D-maltoside	200 mg x 1
n-Octyl-β-D-glucoside	200 mg x 1
n-Octyl-β-D-maltoside	200 mg x 1
MEGA-10	200 mg x 1

Storage Condition: 0-5 °C
Shipping Condition: ambient temperature

Ordering Information

Product code	Unit
DS05-10	1 set

Product Description

Detergent Screening Sets are used to find the best detergent for an application such as enzyme stabilization, specific membrane protein isolation, assay development, protein crystallization, protein refolding and so on. Detergent Screening Set (first choice-II) contains commonly used detergents for membrane protein isolation. Detergent Screening Set (for crystallization) contains detergents commonly used for protein crystallization.

XIV. Good's Buffers

Introduction

In biological experiments, it is important to maintain the pH of the solutions used. Buffers, mixtures of appropriate weak acids, and their conjugate bases, are usually used. Most biological reactions occur at a neutral pH, from 6 to 8; the buffer needs to be effective in this range. Furthermore, the acids and bases used in the buffer should not produce chelates with metal ions, which are essential in biological systems. For these reasons, Dr. Good developed several aminoethane and aminopropane sulfonic acids that are now widely used for biological research and analyses. Good's buffers have the following characteristics:

1) High water-solubility
2) Low cell membrane permeability
3) Consistent acid-base dissociation constants
4) Low metal chelating capability
5) High chemical stability
6) Low absorption spectra in UV and visible region

Table 14-1 Active pH Range of Good's Buffers

Good's Buffer	pKa (20 °C)	pH
MES	6.15	5.5-7.0
Bis-Tris	6.46	5.7-7.3
ADA	6.60	5.8-7.4
PIPES	6.80	6.1-7.5
ACES	6.90	6.0-7.5
MOPSO	6.95	6.2-7.4
BES	7.15	6.6-8.0
MOPS	7.20	6.5-7.9
TES	7.50	6.8-8.2
HEPES	7.55	6.8-8.2
TAPSO	7.70	7.0-8.2
POPSO	7.85	7.2-8.5
HEPPSO	7.90	7.4-8.6
EPPS	8.00	7.5-8.5
Tricine	8.15	7.8-8.8
Bicine	8.35	7.7-9.1
TAPS	8.40	7.7-9.1
CHES	9.50	8.6-10.0
CAPS	10.40	9.7-11.1

Preparation of Good's Buffer Solution

ADA, PIPES, POPSO

Solution A: 0.1 M solution

ADA	19.02 g + NaOH 4.0 g /1 L
PIPES	30.24 g + NaOH 4.0 g /1 L
POPSO	39.85 g + NaOH 4.0 g /1 L

Solution B: 0.1 M NaOH solution

NaOH	4.0 g /1 L

Solution A	Solution B				
25 ml	0 ml	5 ml	10 ml	15 ml	20 ml
ADA	5.8	6.6	6.9	7.3	7.8
PIPES	5.6	6.4	6.8	7.2	7.7
POPSO	6.4	7.3	7.7	8.1	8.5

Bis-Tris

Solution A: 0.1 M Bis-Tris
Solution B: 0.1 M HCl solution

Solution A	Solution B			
25 ml	0 ml	5 ml	10 ml	15 ml
Bis-Tris	9.5	7.1	6.6	6.1

Other Good's Buffers

Solution A: 0.1 M Good's buffer solution
Solution B: 0.1 M NaOH solution

Solution A	Solution B				
25 ml	0 ml	5 ml	10 ml	15 ml	20 ml
ACES	4.6	6.6	7.0	7.3	7.7
BES	3.8	6.6	7.0	7.4	8.0
Bicine	5.1	7.8	8.2	8.6	10.4
CAPS	6.8	10.0	10.5	10.8	11.2
CHES	5.9	9.0	9.4	9.7	10.1
EPPS	5.2	7.3	7.8	8.2	8.8
HEPES	5.3	7.0	7.4	7.7	8.1
HEPPSO	5.7	7.6	7.8	8.2	8.6
MES	3.7	5.6	6.0	6.4	8.4
MOPS	3.8	6.6	7.0	7.4	8.8
MOPSO	3.9	6.2	6.6	7.0	7.4
TAPS	4.6	7.8	8.3	8.6	9.0
TAPSO	4.7	7.0	7.4	7.8	8.2
TES	4.4	6.7	7.4	7.7	8.2
Tricine	4.9	7.5	7.9	8.3	8.6

ACES

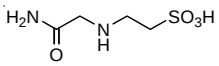
N-(2-Acetamido)-2-aminoethanesulfonic acid [CAS: 7365-82-4]

Appearance: white crystalline powder
Purity: >99.0% (Titration)
MW: 182.20, $C_4H_{10}N_2O_4S$

Storage Condition
ambient temperature

Shipping Condition
ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
GB01-10	25 g
GB01-12	100 g

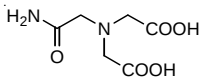
ADA

N-(2-Acetamido)iminodiacetic acid [CAS: 26239-55-4]

Appearance: white crystalline powder
Purity: >99.0% (Titration)
MW: 190.15, $C_6H_{10}N_2O_5$

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
GB02-10	25 g
GB02-12	100 g

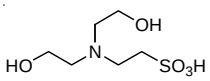
BES

N,*N*-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid [CAS: 10191-18-1]

Appearance: white crystalline powder
Purity: >99.0% (Titration)
MW: 213.25, $C_6H_{15}NO_5S$

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
GB03-10	25 g
GB03-12	100 g

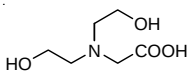
Bicine

N,*N*-Bis(2-hydroxyethyl)glycine [CAS: 150-25-4]

Appearance: white crystalline powder
Purity: >99.0% (Titration)
MW: 163.17, $C_6H_{13}NO_4$

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
GB04-10	25 g
GB04-12	100 g

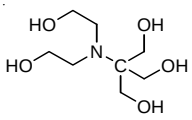
Bis-Tris

Bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane [CAS: 6976-37-0]

Appearance: white crystalline powder
Purity: >99.0% (Titration)
MW: 209.24, $C_8H_{19}NO_5$

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
GB05-10	25 g
GB05-12	100 g

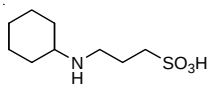
CAPS

N-Cyclohexyl-3-aminopropanesulfonic acid [CAS: 1135-40-6]

Appearance: white crystalline powder
Purity: >99.0% (Titration)
MW: 221.32, $C_9H_{19}NO_3S$

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
GB06-10	25 g
GB06-12	100 g

XIV. Good's Buffers

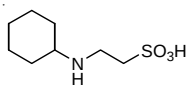
CHES

N-Cyclohexyl-2-aminoethanesulfonic acid [CAS: 103-47-9]

Appearance: white crystalline powder
Purity: >99.0% (Titration)
MW: 207.29, $C_8H_{17}NO_3S$

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
GB07-10	25 g

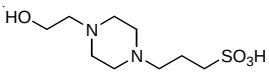
EPPS

3-[4-(2-Hydroxyethyl)-1-piperazinyl]propanesulfonic acid [CAS: 16052-06-5]

Appearance: white crystalline powder
Purity: >99.0% (Titration)
MW: 252.33, $C_9H_{20}N_2O_4S$

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
GB09-10	25 g

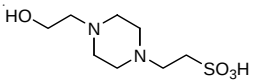
HEPES

2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid [CAS: 7365-45-9]

Appearance: white crystalline powder
Purity: >99.0% (Titration)
MW: 238.31, $C_8H_{18}N_2O_4S$

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
GB10-10	25 g
GB10-12	100 g

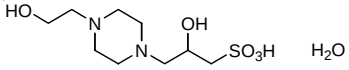
HEPPSO

2-Hydroxy-3-[4-(2-hydroxyethyl)-1-piperazinyl]propanesulfonic acid, monohydrate [CAS: 68399-78-0]

Appearance: white crystalline powder
Purity: >99.0% (Titration)
MW: 286.35, $C_9H_{20}N_2O_5S$, H_2O

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
GB11-10	25 g

MES

2-Morpholinoethanesulfonic acid, monohydrate [CAS: 145224-94-8]

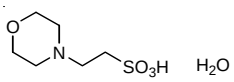
Appearance: white crystalline powder
Purity: >99.0% (Titration)
MW: 213.25, $C_6H_{13}NO_4S$, H_2O

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Ordering Information

Product code	Unit
GB12-10	25 g
GB12-12	100 g

Chemical Structure



MOPS

3-Morpholinopropanesulfonic acid [CAS: 1132-61-2]

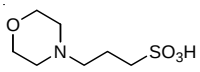
Appearance: white crystalline powder
Purity: >99.0% (Titration)
MW: 209.26, $C_7H_{15}NO_4S$

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Ordering Information

Product code	Unit
GB13-10	25 g
GB13-12	100 g

Chemical Structure



MOPSO

2-Hydroxy-3-morpholinopropanesulfonic acid [CAS: 68399-77-9]

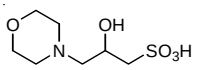
Appearance: white crystalline powder
Purity: >99.0% (Titration)
MW: 225.26, $C_7H_{15}NO_5S$

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Ordering Information

Product code	Unit
GB14-10	25 g

Chemical Structure



PIPES

Piperazine-1,4-bis(2-ethanesulfonic acid) [CAS: 5625-37-6]

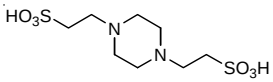
Appearance: white crystalline powder
Purity: >99.0% (Titration)
MW: 302.37, $C_8H_{18}N_2O_6S_2$

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Ordering Information

Product code	Unit
GB15-10	25 g
GB15-12	100 g

Chemical Structure



PIPES sesquisodium

Piperazine-1,4-bis(2-ethanesulfonic acid), sesquisodium salt, monohydrate

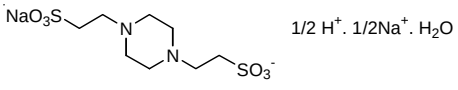
Appearance: white crystalline powder
Purity: >99.0% (Titration)
MW: 353.36, $C_8H_{16.5}N_2Na_{1.5}O_6S_2$, H_2O

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Ordering Information

Product code	Unit
GB25-10	25 g

Chemical Structure



XIV. Good's Buffers

POPSO

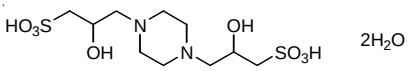
Piperazine-1,4-bis(2-hydroxy-3-propanesulfonic acid), dihydrate [CAS: 68189-43-5]

Appearance: white crystalline powder
Purity: >99.0% (Titration)
MW: 398.45, $C_{10}H_{22}N_2O_8S_2$, $2H_2O$

Ordering Information	
Product code	Unit
GB16-10	25 g

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



TAPS

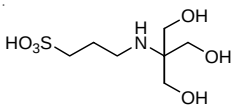
N-Tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid [CAS: 29915-38-6]

Appearance: white crystalline powder
Purity: >99.0% (Titration)
MW: 243.28, $C_7H_{17}NO_6S$

Ordering Information	
Product code	Unit
GB17-10	25 g
GB17-12	100 g

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



TAPSO

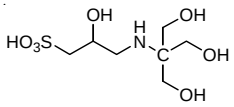
2-Hydroxy-*N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid [CAS: 68399-81-5]

Appearance: white crystalline powder
Purity: >99.0% (Titration)
MW: 259.28, $C_7H_{17}NO_7S$

Ordering Information	
Product code	Unit
GB20-10	25 g

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



TES

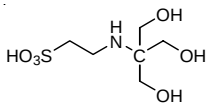
N-Tris(hydroxymethyl)methyl-2-aminoethenesulfonic acid [CAS: 7365-44-8]

Appearance: white crystalline powder
Purity: >99.0% (Titration)
MW: 229.25, $C_6H_{15}NO_6S$

Ordering Information	
Product code	Unit
GB18-10	25 g
GB18-12	100 g

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



Tricine

N-[Tris(hydroxymethyl)methyl]glycine [CAS: 5704-04-1]

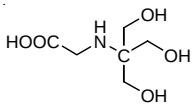
Appearance: white crystalline powder
Purity: >99.0% (Titration)
MW: 179.17, $C_6H_{13}NO_5$

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Ordering Information

Product code	Unit
GB19-10	25 g
GB19-12	100 g

Chemical Structure



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XV-1. Ion Detection: Intracellular Fluorescent Probes

Introduction

Signal transduction is one of the most important functions carried out by living cells. Information outside of a cell is usually transmitted into the cell in the form of various chemical stimulations. A living cell has many different receptors on its surface. The information is transferred to the intracellular spaces through the activation of such receptors. The signal then changes to another form to process the information in order to cause a suitable cellular response. Second, messengers play an important role in the information transfer to the inside of the cell. The change in ion concentration is a major step that helps second messengers to relay information. The monitoring and control of the intracellular ion concentration is extremely important for the understanding second messengers, and intracellular signal transduction. Dojindo offers a wide selection of fluorescent intracellular ion probes for the calcium, proton, zinc, and chloride ions.

Fluorescent Calcium Indicators

Calcium is one of the most important messengers in living cells. It carries various messages to help muscle contraction, nerve cell signal transmission, hormone secretion, immune cell activation and so on. Dr. Tsien first developed fluorescent probes for calcium monitoring in viable cells. Calcium indicators selectively bind to calcium ions. Their fluorescent signal intensities or wavelengths change because each intracellular calcium probe has a calcium selective chelator and a fluorescent moiety in its molecule. EGTA is a good chelator with selectivity for calcium ion 10^5 times higher than for magnesium ion. However, an EGTA molecule has two highly basic aliphatic amines that are protonated at neutral pH. This means that a deprotonation step is involved in the calcium binding process, which causes a change in the fluorescent signal. To avoid pH dependence of the fluorescent signal in calcium detection, Dr. Tsien changed the aliphatic amines of EGTA to aromatic amines, thereby transforming EGTA into a new calcium chelator, BAPTA. In designing calcium probes, the BAPTA structure is required as the calcium-binding moiety. To prepare a calcium probe, which shifts its fluorescent wavelength and changes its fluorescent intensity upon calcium chelation, the selection of a suitable fluorescent part is necessary. When the fluorophore is a stilbene type molecule, which is the case for Fura 2 and Indo 1, the lone electron pair on the aromatic nitrogen of BAPTA structure conjugates the fluorophore. If the calcium ion binds to indicators, donation of the lone electron pair on the aromatic nitrogen to the calcium ion causes a change in the fluorescent signaling. In this case, the calcium concentration may be measured by ratiometry. When xanthene is combined with BAPTA, as is the case of Fluo 3 and Rhod 2, the signal wavelength does not change; however, the signal intensity increases dramatically. This is because the conjugation of the lone electron pair on the BAPTA nitrogen is not involved in the excitation state of xanthene. Instead, the lone pair conjugation contributes to the resonance form, which is non-fluorescent. Thus, this type of calcium indicator is practically non-fluorescent. Once the calcium complex is formed, the contribution of the lone electron pair disappears and the original xanthene fluorescent signal is markedly recovered (as shown in Fig.15- 1). Xanthene has a high quantum yield and long wavelength fluorescence, thus Fluo 3 has an emission and excitation maximum of 528 nm and 490 nm, respectively. For Rhod 2, they are 581 nm and 557 nm, respectively. These excitation wavelengths are convenient in using an argon laser microscope. Therefore, this type of probe is gaining its popularity with the progress in the laser microscopy, especially confocal laser microscopy.

Chelators for Calcium Experiments

Dojindo offers chelators for experiments using fluorescent calcium indicators. Fluorescent calcium probes bind to some transition metal cations very tightly, and this causes a signal artifact. The zinc ion makes a fluorescent complex with calcium probes just as the calcium ion does. TPEN, a transition metal ion selective chelator, is often used to cancel such an artifact. We also offer calcium selective chelators, such as EGTA, BAPTA and BAPTA-AM, to control the intra- or extra-cellular calcium concentrations. EGTA is convenient for creating the calcium buffer used in calcium calibration.

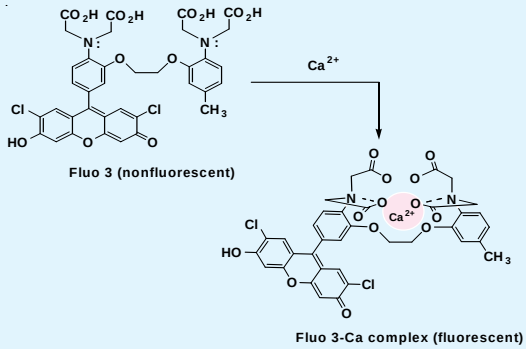


Fig. 15-1 Calcium complex formation

Fluorescent Zinc Indicators

Zinc is an essential metal ion, and its level is reported to be 28.5 mg per g tissue in humans. However, since most zinc ions are strongly combined with proteins, the concentration of free zinc ions in a cell is at the millimolar level or lower. DNA binding proteins combine with DNA through several motifs called zinc fingers, zinc twists, ring fingers, or zinc clusters. Therefore, a shortage of zinc ions influences DNA transcription, and may have some relationship with the development of cancer. Furthermore, zinc ions are thought to have a role in controlling gestation and sexual activity. Zinc ion regulates the activities of several enzymes. Some tissues and organs, such as brain, spermatazoa and salivary glands, contain a large amount of zinc ions. Staining of such tissues with TSQ has demonstrated that zinc ions play very important roles in neuronal cell death, endocrine, and other physiological phenomena. The role of intracellular zinc ions in apoptosis has also been discussed for several decades. TSQ, Zinquin ethyl ester and Dansylaminoethyl-cyclen are useful probes for these studies.

Fluorescent pH Probes

Changes in proton concentration (pH) are often coupled with many cellular events. For example, the Na/H antiporter causes intracellular pH changes. The release of some cellular materials and motility of bacterial flagella are caused by pH fluctuations around the cellular membrane. Monitoring of the intra- or extra-cellular pH is useful in understanding the cell signal transduction. If the proton dissociation affects the fluorescent signal of the parent molecule, the molecule can be used to monitor the pH around its pKa value. Thus, to monitor the pH of biological samples, a fluorescent molecule that has pKa value around 7 is needed. Carboxyfluorescein is a classical pH indicator for physiological pH fluctuation since its pKa value is 6.3. However, its pKa value is still a little too low for the accurate monitoring of biological pH changes. Carboxyfluorescein also tends to leak from cells. Dojindo offers effective fluorescent pH probes that have good intracellular retention rates and suitable pKa values for this purpose.

XV-1. Ion Detection: Intracellular Fluorescent Probes

Probe Loading (AM Method)

Most intracellular probes have some hydrophilic groups, carboxylates, in their molecules. Their high water solubility does not allow passage through the cell membrane because it is a hydrophobic barrier against the extracellular environment. Dr. Roger Y. Tsien developed acetoxymethyl ester (AM ester) of those probes to deal with this issue. The ester renders the molecules hydrophobic and eases the transfer of the probes into the cell membrane. The acetoxymethyl ester probe is easily and rapidly hydrolyzed into a hydroxymethyl ester by intracellular ubiquitous esterases. The hydroxymethyl ester spontaneously hydrolyzes and produces a parent carboxylate (Fig. 15-2). Thus, the original ion probe molecules accumulate in the cell simply by incubation with the AM esters since the resulting hydrophilic molecules can no longer pass through the cell membrane. This loading method, called the AM method, is an extremely simple way to load hydrophilic molecules into cells as compared to other known loading techniques such as microinjection, osmotic shock, or electroporation. Highly purified AM esters of various intracellular ion probes are now available from Dojindo.

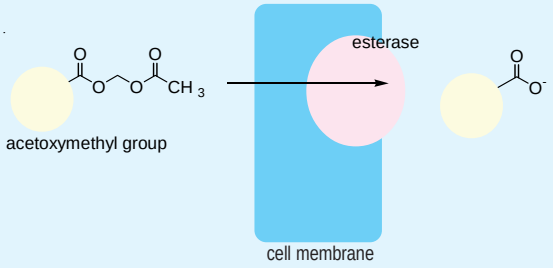


Fig. 15-2 Acetoxymethyl ester cleavage

Directions for AM Ester Use

AM ester is usually dispersed in dimethylsulfoxide (DMSO), and diluted with a buffer solution or a culture medium for incubation with living cells. However, because of the high hydrophobicity of AM ester, it is difficult to prepare more than 1 mM aqueous solution, even when DMSO is used. For the preparation of a high concentration AM solution to improve the loading efficiency into living cells, surfactants or BSA are required. Such agents help the AM ester disperse into an aqueous phase. Pluronic F127 or Cremophore EL are suitable for this purpose. Although such surfactants have low cytotoxicity, they should be used at concentrations of 0.2% or less. Sometimes, sonication is necessary to obtain a well-dispersed AM ester solution. The loading of a probe is usually accomplished by 10 to 60 minutes incubation at 37 °C in the CO_2 incubator. However, in some cases, incubation at a lower temperature may be more desirable to avoid the localization of the probes in cells. Please note that AM ester is sensitive to hydrolysis. Therefore, repeated freezing and thawing of the AM ester stock solution should be avoided because of the risk of humidity absorption by the DMSO solution. One approach to avoid spoiling is to divide 1 to 10 mM of AM ester in DMSO solution into individual use quantities, and keep them frozen. The incubation media should be amine and serum free. Serum contains some esterase activities, and amine tends to accelerate the hydrolysis of AM ester or reacts with AM ester to generate an amide form.

Autofluorescence

Living cells contain pyridine nucleotides and flavin nucleotides, which are fluorescent molecules. Muscle contractions or activation of metabolism in the cell can sometimes increase the fluorescence of these molecules. This autofluorescence often affects the estimation of intracellular ion concentrations when fluorescent probe techniques are used. The excitation and emission wavelength of pyridine nucleotides are 340 nm and 470 nm respectively. The fluorescent spectrum of flavin nucleotides is between 300 nm and 580 nm. This artifact can be a serious problem when using Fura 2, which has a similar excitation wavelength. Fluorescent noise from extracellular space causes another artifact. The extracellular fluorescent noise may be estimated by adding Mn^{2+} to quench the fluorescence after the experiment. There are two ways to avoid these artifacts altogether: one is to use a probe with a longer fluorescent wavelength, the other is a ratiometric measurement.

Ratiometry

If the excitation or emission maximum of a probe is shifted by the interaction with its target ion, it is possible to estimate near absolute intracellular ion concentration by measuring the ratio of two separate wavelength fluorescence intensities. The measurement can be independent of the sample thickness, concentration of probes loaded, leakage of probes from cells, photobleaching and so on. For Fura 2 and Indo 1, ratiometry may be used to measure the calcium concentration changes, while BCECF may be used for pH determination. For Fura 2 and BCECF, the excitation spectra shift. For Indo 1, the emission spectrum changes. In the case of digital imaging microscopy, it is more convenient to measure the emission wavelength after measuring the excitation wavelength. Fura 2 is thus the most popular calcium probe. On the other hand, a flow cytometer has a single excitatory light source; therefore, Indo 1 is more convenient. It should be noted that if the shape of cells changes during an experiment, ratiometry may not estimate the exact ion concentration.

Calibration

When pH indicator is loaded into cells, its pKa value is often changed because of differences in the ionic strengths, viscosities, and other factors. Thus, calibration of the fluorescent signal is needed after the experiment. Take nigericin for example. This ionophore of monovalent cations (10 mM) and potassium in high concentration (100-150 mM) are added to the sample to keep the pH value the same as that of the plasma membrane. The extracellular pH is then changed, and the ratio of the two fluorescent signals is measured. Dojindo also offers a probe, BCECF, to monitor the intracellular pH gradient. The probe is useful in monitoring acidic organelles, biogenesis of lysosomes, development of autophagic vacuoles and so on.

Fluorescent Chloride Indicator

Fluorescent chloride probes such as MQAE allow the monitoring of chloride ion in living cells. The fluorescent intensities of these chloride probes, which are based on quinoline structures, are influenced by counteranions. The chloride anion strongly suppresses fluorescent emission by quinoline. Therefore, a decreasing ratio of total fluorescence indicates an increase in the concentration of chloride ions. MQAE is cell permeable.

Inositols

Inositol phosphates and diacylglycerols are generated from phosphatidyl inositols by phospholipase C. They are involved in the movement of cellular calcium ion as second messengers. Inositol-1,4,5-triphosphate is an extremely important messenger that triggers calcium ion release from endoplasmic and sarcoplasmic reticulum. It is thought to increase calcium sensitivity of the inositol induced calcium release (IICR) channel. Thus, the IICR channel can be opened by a low level calcium ion concentration, causing a dramatic burst of calcium into the cytosol. The released calcium ions and diacylglycerol then activate protein kinase C, a key enzyme of signal transduction. Dojindo's inositol triphosphate is chemically synthesized, and is fairly stable at room temperature in aqueous solution.

XV-1. Ion Detection: Intracellular Fluorescent Probes

Coelenterazine-WS

Coelenterazine, β-cyclodextrin complex

Application: Luminescent calcium probe

Appearance: pale yellowish-brown powder
Absorbance: 0.07-0.25 at 430 nm

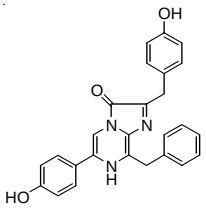
Storage Condition	Shipping Condition
-20 ºC	with blue ice or dry ice

Ordering Information

Product code	Unit
C397-10	1 mg*

* Coelenterazine content: 2%

Chemical structure



β-cyclodextrin complex

Product Description

Aequorin is a luminescent protein that contains coelenterazine as a luminescent compound. Since aequorin emits luminescence by calcium conjugation, it is used for intracellular calcium ion detection. However, the water solubility of coelenterazine is poor under physiological conditions, and coelenterazine is adsorbed to cell membranes. Dojindo's Coelenterazine-WS is a β-cyclodextrin complex of coelenterazine and its water solubility at neutral pH is drastically improved.

Reference

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Fluo 3

1-[2-Amino-5-(2,7-dichloro-6-hydroxy-3-oxo-9-xanthenyl)phenoxy]-2-(2-amino-5-methylphenoxy)ethane-*N*,*N*,*N*',*N*'-tetraacetic acid [CAS: 123632-39-3]

Application: Fluorescent calcium probe

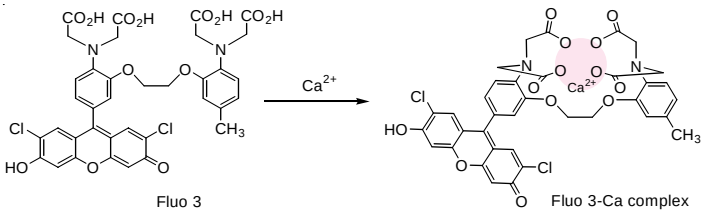
Appearance: reddish or dark reddish-brown powder
Purity: >70.0 %(HPLC)
MW: 769.53, $C_{36}H_{30}Cl_2N_2O_{13}$

Storage Condition	Shipping Condition
-20 ºC	ambient temperature

Ordering Information

Product code	Unit
F019-10	1 mg

Calcium Chelation



Fluo 3 → Fluo 3-Ca complex

Fluo 3-AM

1-[2-Amino-5-(2,7-dichloro-6-hydroxy-3-oxo-9-xanthenyl)phenoxy]-2-(2-amino-5-methylphenoxy)ethane-*N*,*N*,*N*',*N*'-tetraacetic acid, pentaacetoxymethyl ester [CAS: 121714-22-5]

Application: Fluorescent calcium probe

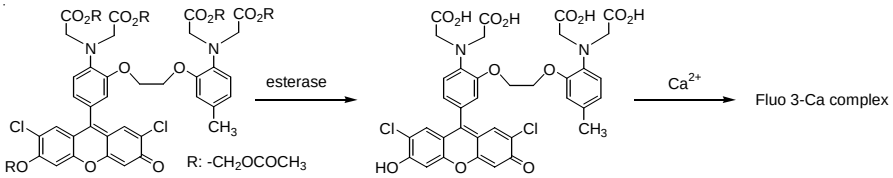
Appearance: red solid
Purity: >85.0 % (HPLC)
MW: 1129.85, $C_{51}H_{50}Cl_2N_2O_{23}$

Storage Condition	Shipping Condition
-20 ºC	ambient temperature

Ordering Information

Product code	Unit
F023-10	1 mg

Hydrolysis of AM ester



Fluo 3-AM → Fluo 3

XV-1. Ion Detection: Intracellular Fluorescent Probes

Product Description

Fluo 3 is a long wavelength calcium probe. Fluo 3 is practically non-fluorescent in its free ligand form, but its fluorescence increases 60-80 times when it forms complexes with calcium. Thus, it has been widely used with confocal laser fluorescent microscopy because the microscope has an argon laser. The long wavelength of the fluorescent signal is also convenient for minimizing photodamage to sample cells. Fluo 3 is also useful for caged calcium and others that are cleaved by the photo-irradiation in the UV region. Fluo 3-AM is an acetoxymethyl ester derivative of Fluo 3 that can be easily loaded into cells by incubation.

General Protocol (for Human T cells)*

Reagents:

2 mM Fluo 3-AM/DMSO (1 mg Fluo 3-AM in 442 µl DMSO)
Pluronic F127
Hanks' balanced salt solution (HBSS)
HEPES buffer saline (10 mM HEPES, 1 mM Na_2HPO_4, 137 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM glucose, 0.1% BSA, pH 7.4)

Protocol:

1. Add 16.5 mg Pluronic F127 to Fluo 3-AM/DMSO solution. Pluronic F127 prevents aggregation of Fluo 3-AM in HBSS and helps uptake with cells.
2. Dilute the Fluo 3-AM solution with HBSS to prepare 4 µM Fluo 3-AM working solution.
3. Add the Fluo 3-AM working solution to the cells and incubate at 37 °C for 20 min.
4. Add HBSS containing 1% fetal Calf serum equivalent to 5 times the volume of Fluo 3 -AM working solution (step 3).
5. Wash the cells 3 times with HEPES buffer saline. Then resuspend the cells to prepare $1x10^5$ cells per ml solution using HEPES buffer saline.
6. Incubate at 37 °C for 10 min. Then use the cells for fluorescent calcium ion detection.
7. Monitor the fluorescence at 528 nm (excitation: 490-500 nm).

*Cell staining conditions differ by cell types, so it is necessary to optimize the conditions for each experiment.

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XV-1. Ion Detection: Intracellular Fluorescent Probes

Fura 2

1-[6-Amino-2-(5-carboxy-2-oxazolyl)-5-benzofuranyloxy]-2-(2-amino-5-methylphenoxy)ethane-*N,N,N',N'*-tetraacetic acid, pentapotassium salt [CAS: 96314-98-6]

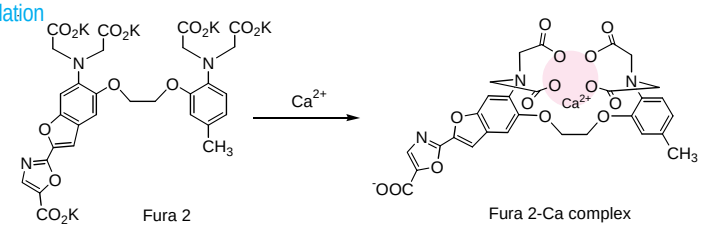
Application: Fluorescent calcium probe

Appearance: yellow or yellowish-orange powder
Purity: >98.0% (HPLC)
MW: 831.99, $C_{29}H_{22}K_5N_3O_{14}$

Ordering Information	
Product code	Unit
F014-10	1 mg

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Ca Chelation



Fura 2-AM

1-[6-Amino-2-(5-carboxy-2-oxazolyl)-5-benzofuranyloxy]-2-(2-amino-5-methylphenoxy)ethane-*N,N,N',N'*-tetraacetic acid, pentaacetoxymethyl ester [CAS: 108964-32-5]

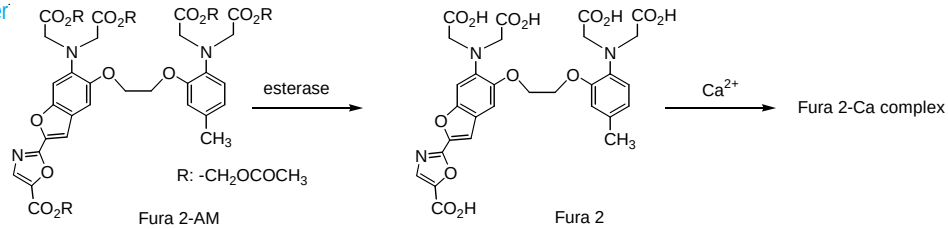
Application: Fluorescent calcium probe

Appearance: yellow solid
Purity: >98.0% (HPLC)
MW: 1001.85, $C_{44}H_{47}N_3O_{24}$

Ordering Information	
Product code	Unit
F015-10	1 mg

Storage Condition: -20 °C
Shipping Condition: ambient temperature

Hydrolysis of AM ester



Product Description

Fura 2 was developed to improve the fluorescent properties of Quin 2. The signal intensity in 1 mM of loaded Fura 2 corresponds to that of 30 mM of loaded Quin 2. This allows an experiment at a lower concentration of indicator using Fura 2 as compared to Quin 2. Fura 2 is one of the most widely used calcium indicators for ratiometric measurement. Many types of instrumentation are now available for experiments using Fura 2. Fura 2 is especially suitable for digital imaging microscopy. It is less susceptible to photobleaching than Indo 1. Changes in the cell shape can sometimes affect the fluorescent ratio at 340 nm and 380 nm. For example, fluorescent signal intensities at these wavelengths sometimes decrease simultaneously with smooth muscle contraction. For blood vessels, however, the increase of the signal intensity at 340 nm tends to be smaller on contraction, while the decrease of the signal intensity at 380 nm tends to be larger with its contraction. Fura 2-AM is an acetoxymethyl ester derivative of Fura 2 that can be easily loaded into cells by incubation.

General Protocol (for NG 108-15/ Neuronal Cell Line)*

Reagents:

1 mM Fura 2-AM/DMSO (1 mg Fura 2-AM in 1 ml DMSO)
Hanks' balanced salt solution (HBSS)
HEPES buffer saline (20 mM HEPES, 115 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 13.8 mM glucose, pH 7.4)

Protocol:

1. Culture cells on a glass-bottom dish using DMEM containing 5% fetal calf serum.
2. Change the medium to 1 mM dibutyl cAMP/DMEM, and culture the cells for 3-4 days to induce dendrites.
3. Dilute 1 mM Fura 2-AM DMSO solution with HEPES buffer saline to prepare 1 mM Fura 2-AM working solution.
4. Remove the culture medium, and add 0.5 ml of the Fura 2-AM working solution to the cells.
5. Incubate for 20 min. Then remove the Fura 2-AM working solution.
6. Wash the cells once with HEPES buffer saline. Then incubate the cells for 1 hour in the HEPES buffer saline.
7. Use the cells for fluorescent calcium ion detection.
8. Monitor the excitation spectra at 380 nm (calcium free) and 340 nm (calcium complex) with fixed emission at 510 nm.

*Cell staining conditions differ by cell types, so it is necessary to optimize the conditions for each experiment.

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Indo 1

1-[2-Amino-5-(6-carboxy-2-indolyl)phenoxy]-2-(2-amino-5-methylphenoxy)ethane-*N,N,N′,N′*-tetraacetic acid, pentapotassium salt [CAS: 96314-96-4 (free acid)]

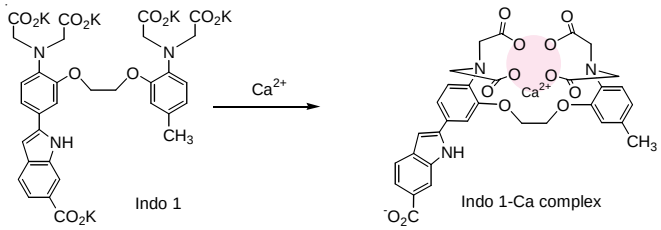
Application: Fluorescent calcium probe

Appearance: slightly yellowish-white powder
Purity: >95.0% (HPLC)
MW: 840.05, $C_{32}H_{26}K_5N_3O_{12}$

Ordering Information	
Product code	Unit
I004-10	1 mg

Storage Condition	Shipping Condition
-20 °C, protect from light	ambient temperature

Calcium Chelation



XV-1. Ion Detection: Intracellular Fluorescent Probes

Indo 1-AM

1-[2-Amino-5-(6-carboxy-2-indolyl)phenoxy]-2-(2-amino-5-methylphenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid, pentaacetoxymethyl ester [CAS: 112926-2-0]

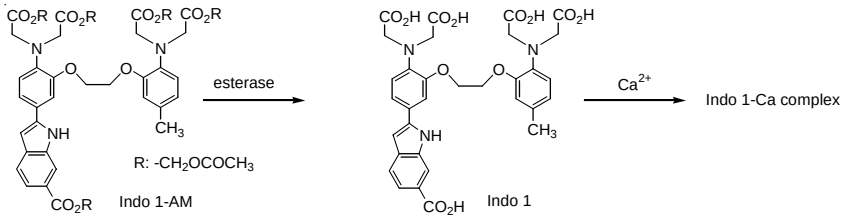
Application: Fluorescent calcium probe

Appearance: dark yellow-green reddish solid
Purity: >97.0% (HPLC)
MW: 1009.91, $C_{47}H_{51}N_3O_{22}$

Ordering Information	
Product code	Unit
I005-10	1 mg

Storage Condition	Shipping Condition
-20 °C, protect from light	ambient temperature

Hydrolysis of AM ester



Product Description

Indo 1 is another type of improved calcium indicator that can be used in ratiometry. Two separate wavelengths in the emission spectrum, usually at 410 nm and 480 nm, are measured in the ratiometry of Indo 1. Indo 1 is suitable for ratiometry using a flow cytometer that can measure fluorescent signals at dual wavelengths. Indo 1 is reported to have better properties for localization than Fura 2 after being loaded into cells. Indo 1-AM is an acetoxymethyl ester derivative of Indo 1 that can be loaded into cells by incubation.

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Quin 2

8-Amino-2-[(2-amino-5-methylphenoxy)methyl]-6-methoxyquinoline-*N*,*N*,*N'*,*N'*-tetraacetic acid, tetrapotassium salt [CAS: 73630-23-6]

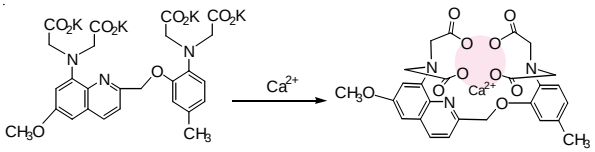
Application: Fluorescent calcium probe

Appearance: pale yellow powder
Purity: >95.0% (HPLC)
MW: 693.87, $C_{26}H_{23}K_4N_3O_{10}$

Ordering Information	
Product code	Unit
Q001-10	100 mg

Storage Condition	Shipping Condition
ambient temperature	ambient temperature

Calcium Chelation



Quin 2 Quin 2-Ca complex

Product Description

Quin 2 forms a stable fluorescent complex with calcium ($\log K_{CaY}$=7.1), but not with magnesium ($\log_{MgY}$=2.7). The complex has a high quantum yield (0.14) at emission wavelength 525 nm, excitation wavelength 339 nm, and emission wavelength 492 nm. Quin 2-AM is an acetoxymethyl ester derivative of Quin 2 that is readily permeable through cell membranes. Within the cell, the ester is hydrolyzed to Quin 2.

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XV-1. Ion Detection: Intracellular Fluorescent Probes

Rhod 2

1-[2-Amino-5-(3-dimethylamino-6-dimethylammonio-9-xanthenyl)phenoxy]-2-(2-amino-5-methylphenoxy)ethane-*N,N,N',N'*-tetraacetic acid, chlorid [CAS: 132523-91-2]

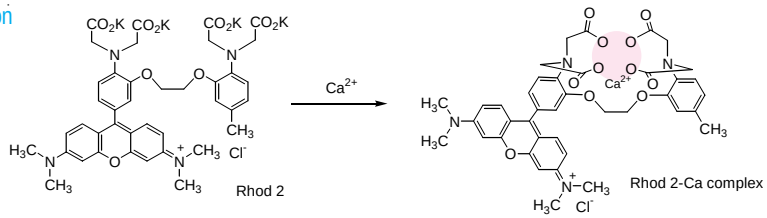
Application: Fluorescent calcium probe
Appearance: dark purple solid
Purity: >60.0% (HPLC)
MW: 791.24, $C_{40}H_{43}ClN_4O_{11}$

Ordering Information	
Product code	Unit
R001-10	1 mg

Storage Condition
-20 °C, protect from light

Shipping Condition
ambient temperature

Calcium Chelation



Rhod 2-AM

1-[2-Amino-5-(3-dimethylamino-6-dimethylammonio-9-xanthenyl)phenoxy]-2-(2-amino-5-methylphenoxy)ethane-*N,N,N',N'*-tetraacetic acid, tetraacetoxymethyl ester, chloride [CAS: 129787-64-0]

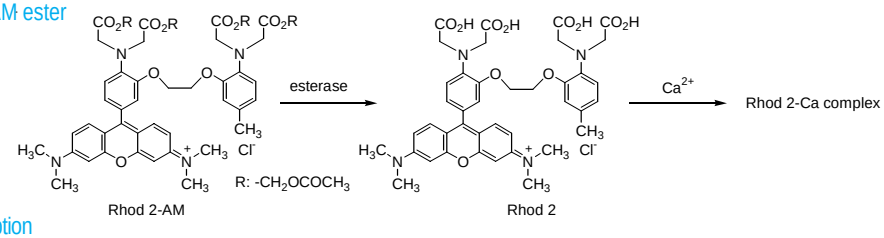
Application: Fluorescent calcium probe
Appearance: dark purple solid
MW: 1079.49, $C_{52}H_{59}ClN_4O_{19}$

Ordering Information	
Product code	Unit
R002-10	1 mg

Storage Condition
-20 °C, protect from light

Shipping Condition
ambient temperature

Hydrolysis of AM ester



Product Description

Rhod 2 has the longest fluorescent wavelength signal of all the calcium indicators. It has a rhodamine-like fluorophore whose excitation and emission maxima are 557 nm and 581 nm, respectively. This makes it a convenient excitation source for argon and krypton lasers. Although it was thought that the fluorescent signal of Rhod 2 only increases several times with the calcium complex, Dojindo's Rhod 2 increases its signal with calcium 80-100 times because of its high purity. Its signal intensity is the strongest of all the calcium probes. Rhod 2 is thus highly recommended as a probe for intracellular calcium monitoring using laser microscopes. Rhod 2 is reported to have a better loading profile at the point of localization, especially in neural slice cultures. The dissociation constant of Rhod 2 with calcium (K_d=1.0 mM) is the highest of all the fluorescent calcium probes, providing a wider range for monitoring calcium concentration. Rhod 2-AM is an acetoxymethyl ester derivative of Rhod 2 that can be easily loaded into cells using the AM method.

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BAPTA

O,O'-Bis(2-aminophenyl)ethyleneglycol-*N,N,N',N'*-tetraacetic acid, tetrapotassium salt, hydrate [CAS: 85233-19-8(free acid)]

Application: Calcium masking

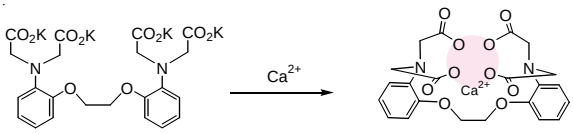
Appearance: white powder or crystalline powder
Purity: >95.0% (Titration)
MW: 628.79, $C_{22}H_{20}K_4N_2O_{10}$

Storage Condition	Shipping Condition
ambient temperature	ambient temperature

Ordering Information

Product code	Unit
B019-10	500 mg

Calcium Chelation



BAPTA BAPTA-Ca complex

BAPTA-AM

O,O'-Bis(2-aminophenyl)ethyleneglycol-*N,N,N',N'*-tetraacetic acid, tetraacetoxymethyl ester [CAS: 126150-97-8]

Application: Calcium masking

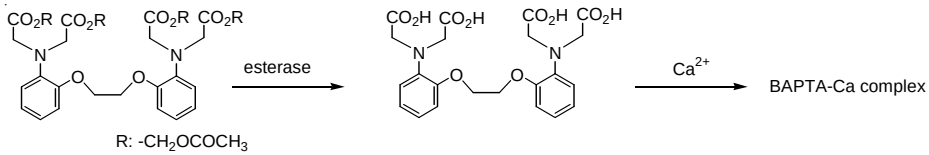
Appearance: white powder
Purity: >95.0% (Titration)
MW: 764.68, $C_{34}H_{40}N_2O_{18}$

Storage Condition	Shipping Condition
-20 °C	ambient temperature

Ordering Information

Product code	Unit
B018-10	25 mg

Hydrolysis of AM ester



BAPTA-AM BAPTA

Product Description

BAPTA is a calcium-selective chelator developed by Dr. Tsien. It has $\log K_{Ca}$=6.97 and $\log K_{Mg}$=1.77. The basic chelating unit resembles that of EGTA, but the two aliphatic nitrogen atoms are replaced by aromatic nitrogen. Thus, BAPTA is not protonated at physiological pH. BAPTA possesses pKa_3=5.47 and pKa_4=6.36. This property indicates that the deprotonation step is not included in its calcium complexation step, and it has a higher complexation rate than EGTA because it is not affected by proton interference. BAPTA-AM is an acetoxymethyl ester derivative of BAPTA that can be easily loaded into cells using the AM method. BAPTA-AM is useful for controlling the intracellular calcium concentration.

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XV-1. Ion Detection: Intracellular Fluorescent Probes

GEDTA (EGTA)

O,O'-Bis(2-aminoethyl)ethyleneglycol-*N,N,N',N'*-tetraacetic acid [CAS: 67-42-5]

Application: Calcium masking

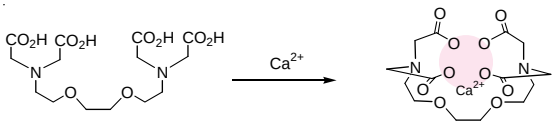
Appearance: white crystalline powder
Purity: >98.0% (Titration)
MW: 380.35, $C_{14}H_{24}N_2O_{10}$

Storage Condition	Shipping Condition
ambient temperature	ambient temperature

Ordering Information

Product code	Unit
G002-12	100 g

Calcium Chelation



GEDTA GEDTA-Ca complex

Product Description

EGTA is the most widely used calcium selective chelator. The calcium complex of EGTA is 100,000 times more stable than its Mg complex. It is utilized to prepare calcium buffers and control the calcium ion concentration.

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Dansylaminoethyl-cyclen

1-[2-[5-(Dimethylamino)-1-naphthalenesulfonamido]ethyl]-1,4,7,10,-tetraazacyclo-dodecane, tetrahydrochloride, dihydrate
[CAS: 184537-03-9]

Application: Fluorescent zinc probe, cell staining

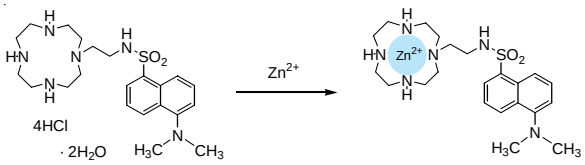
Appearance: slightly yellow crystalline powder
Purity: >95.0% (HPLC)
MW: 630.50, $C_{22}H_{40}Cl_4N_6O_2S \cdot 2H_2O$

Storage Condition	Shipping Condition
-20 °C, protect from light	ambient temperature

Ordering Information

Product code	Unit
D480-10	5 mg

Zinc Chelation



Dansylaminoethyl-cyclen Dansylaminoethyl-cyclen-Zn complex

Product Description

Dansylaminoethyl-cyclen is a water-soluble and cell membrane permeable fluorescent zinc indicator developed by Dr. E. Kimura. The aqueous solution of this reagent allows intracellular zinc ion to be monitored. The fluorescence intensity of the complex is 5 times that of the free ligand. Most other cations do not form fluorescent complexes with this reagent. Although cadmium ion forms a fluorescent complex, the stability constant of this complex is 10 times less than that of the zinc complex. Thus, cadmium ion does not affect zinc detection in most cases. Copper ion (Cu(II)), which forms a non-fluorescent complex, also does not affect zinc detection because it is extremely rare as a free ion in normal living cells. Recently, the zinc ion has gained a lot of attention; it is thought to play an important role in ischemia and subsequent selective neuronal cell death. Reactive oxygen species might damage cystein-residues in a zinc-finger motif to produce free zinc ions.

References

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Zinquin ethyl ester

Ethyl [[2-methyl-8-[[(4-methylphenyl)sulfonyl]amino]-6-quinolinyl]oxy]acetate
[CAS: 151606-29-0]

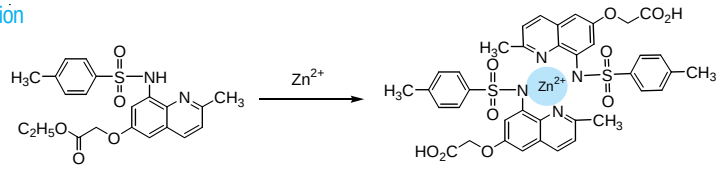
Application: Fluorescent zinc probe, cell staining

Appearance: white or slightly blue crystalline powder
Purity: >98.0% (HPLC)
MW: 414.48, $C_{21}H_{22}N_2O_5S$

Ordering Information	
Product code	Unit
Z215-10	1 mg

Storage Condition	Shipping Condition
-20 °C	ambient temperature

Zinc Chelation



Zinquin ethyl ester — Zinquin-Zn complex

Product Description

Zinquin ethyl ester is an analog of the widely used indicator TSQ. Though Zinquin ethyl ester itself is fluorescent, its fluorescence intensity is negligibly weak (1/30). Zinquin ethyl ester is membrane permeable, as are acetoxymethyl esters of calcium probes such as Fura 2-AM and Fluo 3-AM. Zinquin ethyl ester is thus useful to detect intracellular zinc ions. It forms a complex with a zinc ion with nitrogen atoms in the structure. This compound also forms a fluorescent complex with cadmium ion; however, detectable amounts of cadmium ions are not contained in normal living cells. Because the water solubility of Zinquin ethyl ester is poor, dimethylsulfoxide (DMSO) or ethanol is required as a solvent for preparing the stock solution.

General Protocol for Lymphoblastoid Cells*

Reagents:

2.4 mM Zinquin ethyl ester stock solution (1 mg Zinquin ethyl ester in 1 ml DMSO)
Zinquin ethyl ester stock solution is stable for 1 month at -20 °C.

Protocol:

1. Suspend cells in Hanks' balanced salt solution (HBSS) to prepare 5-10 x 10^6 cells per ml medium.
2. Add Zinquin ethyl ester stock solution to the cell suspension to prepare 2.4 μM Zinquin ethyl ester (1/1000 vol of the cell suspension) as the final concentration.
3. Incubate the cell suspension at 37 °C for 30 min.
4. Wash the cells 3 times with HBSS and then prepare 2-5 x 10^6 cells per ml of cell suspension.
5. Determine the fluorescence intensity of each cell using a fluorescence microscope or a confocal laser microscope coupled with an image analyzer.

* Cell staining conditions differ by cell type, so it is necessary to optimize the conditions for each experiment.

References

P. D. Zalewski, *et al.*, *Biochem. J.*, **296**, 403 (1993); P. D. Zalewski, *et al.*, *J. Histochem. Cytochem.*, **42**, 877 (1994); P. Coyle, *et al.*, *Biochem. J.*, **303**, 781 (1994); P. D. Zalewski, *et al.*, *Chem. Biol.*, **1**, 153 (1994); P. D. Zalewski, *et al.*, *Reprod. Fertil. Dev.*, **8**, 1097 (1996); I. A. Brand, *et al.*, *J. Biol. Chem.*, **271**, 1941 (1996); R. D. Palmiter, *et al.*, *EMBO J.*, **15**, 1784 (1996); N. Wellinghausen, *et al.*, *Cell Immunol.*, **171**, 255 (1996); D. Berendji, *et al.*, *FEBS Lett.*, **405**, 37 (1997); M. Tsuda, *et al.*, *J. Neurosci.*, **17**, 6678 (1997); J. W. Kleineke, *et al.*, *J. Pharmacol. Toxicol. Methods*, **38**, 181 (1997); N. Wellinghausen, *et al.*, *Eur. J. Immunol.*, **27**, 2529 (1997); H. Haase, *et al.*, *Biometals*, **12**, 247 (1999).

XV-1. Ion Detection: Intracellular Fluorescent Probes

TPEN

N,N,N',N'-Tetrakis(2-pyridylmethyl)ethylenediamine [CAS: 16858-02-9]

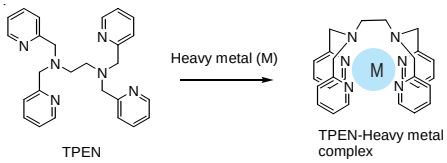
Application: Heavy metal ion masking

Appearance: white or slightly yellow crystals
Purity: >98.0% (Titration)
MW: 424.54, $C_{26}H_{28}N_6$

Storage Condition	Shipping Condition
ambient temperature	ambient temperature

Ordering Information	
Product code	Unit
T040-10	100 mg

Heavy Metal Chelate



Product Description

TPEN is a highly selective chelator of transition metal cations because the pyridine groups act as soft electron donators. TPEN is thus utilized for masking transition metal cations in fluorescent calcium monitoring. The masking function of the zinc cation is important for canceling the fluorescent artifact. TPEN is water-soluble and cell membrane permeable. Maximum wavelength is 260 nm.The physical properties of TPEN are as follows: pKa_1=10.27, pKa_2=3.32, pKa_3=4.85, and pKa_4=7.19; $LogK_{Mn}$=10.27, $logK_{Fe}$=14.61, $logK_{Zn}$=15.58, $logK_{Mg}$=1.7, and $logK_{Ca}$=4.4.

References

G. Anderegg, *et al.*, *Helv. Chim. Acta*, **50**, 2330 (1967); H. Toftlund, *et al.*, *Acta Chem. Scand. Ser.* **A35**, 575 (1981); P. Arslan, *et al.*, *J. Biol. Chem.*, **260**, 2719 (1985); H. Komulainen, *et al.*, *Neurochem. Int.*, **10**, 55 (1987); D. D. Maenz, *et al.*, *Biochim. Biophys. Acta*, **1069**, 250 (1991); B. Musicki, *et al.*, *Mol. Cell. Endocrinol.*, **92**, 215 (1993); M. Michikawa, *et al.*, *J. Neurosci. Res.*, **37**, 62 (1994); M. Ishido, *et al.*, *Jpn. J. Toxicol. Environ. Health*, **41**, 23 (1995); R. E. Sheridan, *et al.*, *Toxicon*, **33**, 539 (1995); M. Vignes, *et al.*, *Neurochem. Int.*, **29**, 371 (1996); V. A. Snitsarev, *et al.*, *Biophys. J.*, **71**, 1048 (1996); C. Usai, *et al.*, *Eur. J. Histochem.*, **41** Suppl 2, 189 (1997); M. Aoyama, *et al.*, *Biochem. J.*, **336**, 727 (1998); Y. H. Ahn, *et al.*, *Exp. Neurol.*, **154**, 47 (1998); C. H. Kim, *et al.*, *J. Neurochem.*, **72**, 1586 (1999); T. J. McNulty, *et al.*, *Biochem. J.*, **339**, 555 (1999); P. Meerarani, *et al.*, *Am. J. Clin. Nutr.*, **71**, 81 (2000).

BCECF

2',7'-Bis(carboxyethyl)-4 or 5-carboxyfluorescein [CAS: 85138-49-4]

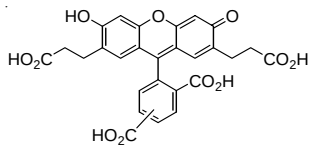
Application: Fluorescent pH probe

Appearance: reddish-brown or red cyrstalline powder
Purity: >85.0% (HPLC)
MW: 520.44, $C_{27}H_{20}O_{11}$

Ordering Information	
Product code	Unit
B031-10	5 mg

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



BCECF-AM

3'-*O*-Acetyl-2',7'-bis(carboxyethyl)-4 or 5-carboxyfluorescein, diacetoxymethyl ester [CAS:117464-70-7]

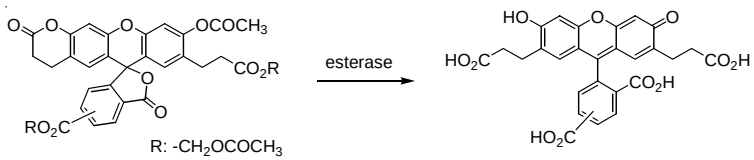
Application: Fluorescent pH probe

Appearance: orange or orange-brown crystals
Purity: >90.0% (HPLC)
MW: 688.59, $C_{35}H_{28}O_{15}$

Ordering Information	
Product code	Unit
B262-10	1 mg

Storage Condition: -20 °C, protect from light
Shipping Condition: ambient temperature

Hydrolysis of AM ester



BCECF-AM BCECF

Product Description of BCECFs

BCECF is the most widely used intracellular pH probe. Dr. Tsien and others improved this carboxyfluorescein by introducing two extra carboxylates that allow it to be retained better by the cell. BCECF is highly water-soluble because it has 4 to 5 negative charges at neutral pH; it becomes difficult to pass through the cell membrane after loading. Its pKa value, 6.97, is higher than that of carboxyfluorescein. BCECF has an isosbestic point at 439 nm in the excitation spectra, so it can be used in ratiometry, similar to Fura 2. Wavelengths of 505 nm and 439 nm are usually used for the ratiometric assay, and 490 nm and 450 nm filters are set in front of the excitation light source. The 530 nm filter is used for its fluorescent signal. Please note that the excitation spectrum is slightly different from the absorption spectra. BCECF-AM is an acetoxymethyl ester of BCECF. It enables the easy loading of BCECF into cells. BCECF-AM accumulates in a cell only by incubation as do the other acetoxymethyl esters. BCECF-AM is very sensitive to moisture; it should be carefully handled. The color of the DMSO solution changes from pale yellow to dark orange with decomposition of the AM form. Therefore, hydrolysis of the AM ester can be monitored by changes in color.

General Protocol (for Human Neutrophil)*

Reagents:

1 mM BCECF-AM/DMSO solution (1 mg BCECF in 1.45 ml DMSO)
HEPES buffer saline (20 mM HEPES, 153 mM NaCl, 5 mM KCl, 5 mM glucose, pH 7.4)

Protocol:

1. Suspend cells in HEPES buffer solution to prepare $4x10^7$ cells per ml.
2. Add 1 mM BCECF-AM/DMSO solution to the cell suspension to prepare 3 μM BCECF-AM (1/300 vol of cell suspension) as the final concentration.
3. Incubate the cell suspension at 37 °C for 30 min.
4. Wash the cells 3 times with HEPES buffer saline and then prepare $3x10^6$ cells per ml of the cell suspension.
5. Determine the fluorescence intensity using a fluorescence microscope or a confocal laser microscope coupled with an image analyzer.

* Cell staining conditions differ by cell type, so it is necessary to optimize the conditions for each experiment.

XV-1. Ion Detection: Intracellular Fluorescent Probes

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MQAE

N-Ethoxycarbonylmethyl-6-methoxyquinolinium bromide [CAS: 124505-60-8]

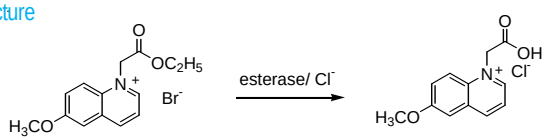
Application: Fluorescent chloride ion probe

Appearance: yellow powder
Purity: >95.0% (HPLC)
MW: 326.19, $C_{14}H_{16}BrNO_3$

Ordering Information	
Product code	Unit
M024-10	50 mg

Storage Condition	Shipping Condition
0-5 °C	ambient temperature

Chemical Structure



MQAE MQAE-Cl complex

Product Description

MQAE with bromide ion as a counteranion has fluorescence excitation at 355 nm and emission at 460 nm. The fluorescent intensity of MQAE decreases in proportion to the chloride increase in cells. Chloride ion concentrations less than 50 mM may be monitored without being affected by pH changes. MQAE has high water solubility and membrane permeability.

General Protocol*

Reagents:

5 mM MQAE/Krebs-HEPES buffer

Krebs-HEPES buffer (20 mM HEPES, 128 mM NaCl, 2.5 mM KCl, 2.7 mM $CaCl_2$, 1 mM $MgCl_2$, 16 mM glucose, pH 7.4)

Protocol:

1. Wash the cells with Krebs-HEPES buffer 3 times.
2. Add 5 mM Cl probe solution to the cells and incubate at 37 °C for 1 hour.
3. Wash the cells with Krebs-HEPES buffer 5 times.
4. Determine the fluorescence intensity using a fluorescence microscope coupled with an image analyzer.

* Cell staining conditions differ by cell type, so it is necessary to optimize the conditions for each experiment

References
G. A. Banker, *et al., Brain Res.*, **126**, 397 (1977); K. Ishibashi, *et al., Am. J. Physiol.*, **255**, F49 (1988); B. A. Stanton, *Am. J. Physiol.*, **254**, F80 (1988); A. S. Verkman, *et al., Anal. Biochem.*, **178**, 35 (1989); A. C. Chao, *et al., Biophys. J.*, **56**, 1071 (1989). P. L. McNeil, *Methods in Cell Biology, edited by Y-L. Wang and D. L. Taylor, San Diego CA: Academic*, **Vol. 29**, 153 (1989); A. S. Verkman, *Am. J. Physiol.*, **259**, C3 (1990); A. C. Chao, *et al., J. Membr. Biol.*, **113**, 193 (1990); M. Inoue, *et al., Neurosci. Lett.*, **134**, 75 (1991); A. C. Engblom, *et al., Biochim. Biophys. Acta*, **1153**, 262 (1993); C. Koncz, *et al., Am. J Physiol.*, **267**, H2114 (1994); W. E. Crowe, *et al., J. Membr. Biol.*, **144**, 81 (1995); E. Brochiero, *et al., Pflugers. Arch.*, **431**, 32 (1995); E. Woll, *et al., Pflugers. Arch.*, **432**, 486 (1996); G. P. Amorino, *et al., J. Membr. Biol.*, **152**, 217 (1996); J. Sahi, *et al., J. Cell. Physiol.*, **168**, 276 (1996); M. R. West, *et al., Anal. Biochem.*, **241**, 51 (1996); H. S. White, *et al., Epilepsy. Res.*, **28**, 167 (1997); T. Nakamura, *et al., Neurosci. Lett.*, **237**, 5 (1997); S. Paula, *et al., Biophys. J.*, **74**, 319 (1998); L. M. Maglova, *et al., Am. J. Physiol.*, **275**, C1330 (1998); U. Banderali, *et al., J. Physiol. (Lond.)*, **519**, 737 (1999).

DiBAC4(3) Bis(1,3-dibutylbarbituric acid)trimethine oxonol, sodium salt

Application: Membrane potential sensitive dye

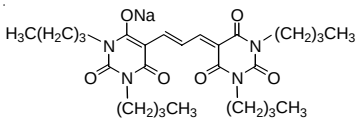
Appearance: red or reddish-orange powder
Purity: >98.0% (HPLC)
MW: 538.61, $C_{27}H_{39}N_4NaO_6$

Storage Condition	Shipping Condition
0-5 °C	ambient temperature

Ordering Information

Product code	Unit
D545-10	25 mg

Chemical Structure



Product Description

$DiBAC_4(3)$ is a Bis-oxonol type membrane potential sensitive dye. According to membrane depolarization detection, DiBAC distribution in cytosol is increased and the fluorescence intensitiy is increased. Since an argon laser (488 nm) can be used for $DiBAC_4(3)$ excitation, it is applicable for flow cytometry and confocal microscopy.

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Ins(1,4,5)P3 (synthetic) D-*myo*-Inositol-1,4,5-triphosphate, tripotassium salt [CAS: 85166-31-0]

Application: Cell signal transduction research

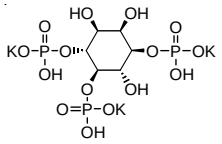
Appearance: colorless pellet
Purity: pass test (TLC)
MW: 534.37, $C_6H_{12}K_3O_{15}P_3$

Storage Condition	Shipping Condition
-20 °C, protect from light	ambient temperature

Ordering Information

Product code	Unit
I007-10	100 µg
I007-12	1 mg

Chemical Structure



Product Description

Inositol phosphates and diacylglycerols are generated from phosphatidyl inositols by phospholipase C. They are involved in cellular calcium ion movements as second messengers. Inositol-1,4,5-triphosphate ($Ins(1,4,5)P_3$) is an extremely important messenger that triggers calcium ion release from endoplasmic and sarcoplasmic reticulum. It is thought to increase the calcium sensitivity of the inositol induced calcium release (IICR) channel in the intracellular calcium source. Thus, the IICR channel can be opened with a low level of calcium ion concentration, causing a dramatic calcium burst into the cytosol. The released calcium ions and diacylglycerol then activate protein kinase C, which is one of the key enzymes in signal transduction.

References

Y. Nishizuka, *Nature*, **308**, 693 (1984); M. J. Berridge, *et al.*, *Nature*, **312**, 315 (1984); I. R. Batty, *et al.*, *Biochem. J.*, **232**, 211 (1985); D. J. Gawler, *et al.*, *Biochem. J.*, **276**, 163 (1991); M. Hirata, *et al.*, *Biochem. J.*, **276**, 333 (1991); S. DeLisle, *et al.*, *Am. J. Physiol.*, **266**, C429 (1994); J. Yang, *et al.*, *Biochem. Biophys. Res. Commun.*, **210**, 21 (1995); A. Meyer-Alber, *et al.*, *Scand. J. Gastroenterol.*, **30**, 384 (1995); S. Fukuhara, *et al.*, *Pept. Chem.*, **32nd**, 385 (1995); C. W. Taylor, *et al.*, *J. Membr. Biol.* , **145**, 109 (1995); P. D'Andrea, *et al.*, *Cell Calcium*, **17**, 367 (1995); J. F. Wootton, *et al.*, *Biophys. J.*, **68**, 2601 (1995); M. Hirata, *et al.*, *Biochim. Biophys.*, **1244**, 404 (1995); S. G. Ward, *et al.*, *J. Biol. Chem.*, **270**, 12075 (1995); F. B. Davis, *et al.*, *Metab., Clin. Exp.*, **44**, 865 (1995); E. Pasyk, *et al.*, *Am. J. Physiol.*, **268**, H138 (1995); K. Maiese, *et al.*, *Neurosci. Lett.*, **194**, 173 (1995); R. A. Wilcox, *et al.*, *Mol. Pharmacol.*, **47**, 1204 (1995); A. Bouron, *et al.*, *Neurosci. Lett.*, **195**, 37 (1995); A. M. Parsons, *et al.*, *Neuroscience*, **68**, 855 (1995). T. Michikawa, *et al.*, *Crit. Rev. Neurobiol.*, **10**, 39 (1996). D. Malchow, *et al.*, *Int. J. Dev. Biol.*, **40**, 135 (1996); D. L. Gill, *et al.*, *Biosci. Rep.*, **16**, 139 (1996); B. Minke, *et al.*, *Mol. Neurobiol.*, **12**, 163 (1996); D. Restrepo, *et al.*, *J. Neurobiol.*, **30**, 37 (1996); I. Parker, *et al.*, *Cell Calcium*, **20**, 105 (1996); T. J. Shuttleworth, *et al.*, *J. Exp. Biol.*, **200**, 303 (1997); S. R. James, *et al.*, *Cell Signal*, **9**, 329 (1997); C. W. Taylor, *et al.*, *Biochim. Biophys. Acta*, **1436**, 19 (1998); P. Dal Santo, *et al.*, *Cell*, **98**, 757 (1999); N J. Haughey, *et al.*, *J. Neurochem.*, **73**, 1363 (1999); T. Michikawa, *et al.*, *Neuron*, **23**, 799 (1999); J. P. Camina, *et al.*, *J. Biol. Chem.*, **274**, 28134 (1999). M. Fujieda, *et al.*, *J. Cell. Biochem.*, **75**, 215 (1999). S. Komatsu, *et al.*, *J. Pharmacol. Toxicol. Methods*, **41**, 33 (1999); M. M. Rodriguez, *et al.*, *Biochemistry*, **38**, 13787 (1999); 37. I. A. Graef, *et al.*, *Nature*, **401**, 703 (1999).

XV-2. Ion Detection: Metals and Anions

Introduction

Metal indicators, colorimetric metal chelators, and fluorometric metal chelators are chromogenic chelating agents. They can be used to determine the metal ion concentration in solutions. There are several methods for measuring a particular metal ion in solution. These methods include chelate titration, colorimetric detection, fluorometric detection, colorimetric detection coupled with solvent extraction, and precipitation titration. In general, metal indicators are utilized for chelate titration, and colorimetric and fluorometric chelating agents are utilized for the determination of the metal concentration by spectrophotometry.

Table 15-1. Application of Metal Indicators

Indicators	Metals	pH	Color Change
BT	alkaline earth metals	10	reddish purple
	Zn, Cd, Hg, Pb	7-10	-> blue
XO	In, Th, Bi	1-3	pink -> yellow
	Zn, Cd, rare earth metals, Hg, Pb	5-6	
NN	Ca	12-13	pink -> blue
PAN	Cu	3-10	pink -> yellow
Cu-PAN	Al	3-3.5	pink -> yellow
	Zn, Cd, Co, Hg, Fe, Pb, Ni	4-5	
PC	alkaline earth metals	11	pink -> colorless
MX	Ca	8	yellow -> purple
	Ni	10	
Calcein	Ca	12-13	green fluorescence -> no fluorescence
Calcein Blue	Ca	12-13	blue fluorescence -> no fluorescence

Chelate Titration

Metal indicators and chelating agents are used to determine the concentration of specific metal ions in solution by chelate titration. The endpoint of the titration can be determined by the color of the solution. The color of the metal indicator varies sharply with the association and dissociation of metal ions, so the total amount a metal ion can be estimated by the amount of titration reagent used. EDTA-metal complexes are very stable and have high dissociation constants. EDTA forms a 1:1 complex with most metal ions that are divalent or more. For these reasons, EDTA is a widely used titration reagent. Fluorescent metal indicators are useful for determining the endpoint of titration of metal ions in stained samples.

Spectrophotometry

Colorimetric chelating reagents form colored complexes with metal ions in pH-controlled solutions. Their selectivity depends on the dissociation constants of metal ions and their sensitivity depends on the molar absorptivity of the complex. However, few colorimetric chelating reagents are highly selective. To increase selectivity, the choice of masking reagents or solvents for the extraction procedures is important. The maximum wavelength of the complex is also an important factor for selectivity and sensitivity. For example, Nitroso-PSAP forms complexes with several heavy metal ions, but the maximum wavelength of the Nitroso-PSAP-Fe complex is considerably longer than that of the other metal complexes. Thus, iron can be determined without interference from other metal ions. Water-soluble colorimetric chelating agents enable the determination of metal ions in aqueous solution without solvent extraction. Therefore, these reagents are useful for automatic detection systems. Calcium is one of the most important metal ions for signal transduction in living cells. Several unique reagents are available for monitoring the calcium concentration in living cells (see Section XV-1).

Masking Reagent

EDTA forms stable complexes with various metal ions in chelate titration. The total consumption of EDTA solution indicates the total amount of mixed metal ions contained in the sample solution. To determine the amount of one specific metal ion in the sample solution, masking reagents should be added to remove other metal ions. Table 15-2 shows popular masking reagents for chelatometry and colorimetry.

Table 15-2. Application of Masking Reagents

Masking Reagent	Metal	pH
Acetylacetone	Al, Fe(III), Pd, Be, UO_2	acidic pH
Citric acid	Zr, Th	5-6
KCN	Co, Hg, Fe, Cu, Ni	>7
	Zu, Cd	
Diethyldithiocarbamate	Cu, Mn	alkaline pH
Dimercaptopropanol	Zn, Sb, Cd, Hg. Sn	alkaline pH/NH_4OH
	Pb, Bi	
Dimercaptopropane sulfonate	Zn, Cd, Hg, Sn	weak acidic pH
Oxalic acid Dithiooxialic acid	Sn	weak acidic pH
Tartaric acid	Ti	alkaline pH
	Sb, UO_2	5-6
Tiron	Al, Ti, Fe	acidic pH
Thiocarbazide	Cu	acidic pH
Thioglycolic acid	Zn, Cd, Hg, Cu, Pb	alkaline pH
Thiourea	Cu	2.5-6
	Pb, Pt	5-6
Thiosulfate	Cu, Bi	acidic pH
Triethanolamine	Al, Fe(III), Mn(III)	10-12/KOH, NaOH
o-Phenanthroline 2,2'-Dipyridine	Zn, Cd, Co, Hg, Cu, Ni	acidic pH
NH_4F	Al, Sn, Ti, Fe	acidic pH
	Ca, Sr, Ba, Mg	alkaline pH
KI	Hg(II)	>7
H_2S	Zn, Cd, Co, Hg	acidic pH
Na_2S	Cu, Ni	alkaline pH

Reagents for Ion Selective Electrodes

Real-time monitoring of electrolytes is increasing in importance for clinical use. For example, monitoring sodium and potassium ion concentrations in the blood flow is indispensable during cardiac surgery. Although lithium ion is used in the treatment of manic symptoms, its serum level must be strictly monitored due to its toxicity. For the simple and quick determination of these alkaline and alkaline earth metal ions, polyvinyl chloride (PVC) membrane electrodes have been widely used. The concentration of neutral carriers, plasticizers, and counteranions used to prepare the PVC electrode determines its ion selectivity. A large number of crown ether compounds have been developed. Some of them are superior to naturally existing neutral carriers such as valinomycin, which is highly selective for potassium ions.

XV-2. Ion Detection: Metals and Anions

Table 15-3 Reagent Index

Ion	Reagent
Ag	3,5-DiBr-PAESA
	o-Phenanthroline
Al	Calcein
	Cu-PAN
	PR (Pyrogallol Red)
	XO (Xylenol Orange)
As	Arsemate
	Bismuthiol-II
B	Azomethine H
Ba	Calcein
	Calcein Blue
	HNB
	PC (Phthalein Complexone)
Bi	Bismuthiol-II
	PAR
	XO (Xylenol Orange)
Ca	calcium ion monitoring: page 186
	BT
	Calcein
	Calcein Blue
	Chlorophosphonazo-III
	Cu-PAN
	HDOPP-Ca
	t-HDOPP-Ca
	HNB
	Murexide
	MX
	NN
	PC (Phthalein Complexone)
	Zincon
Cd	BT
	Zincon
Cl^-	chloride ion monitoring: page 187
	Bisthiourea-1
CN^-	Cyanoline Blue
Co	BT
	Calcein Blue
	Cu-PAN
	5-Br-PSAA
	PAN
	5-Br-PAPS
	Nitro-PAPS
	PAR
	PR (Pyrogallol Red)
	TPPS
Cr	Calcein Blue
Cs	Cesibor
Cu	Bathocuproine
	Bathocuproindisulfonic acid
	Calcein
	Calcein Blue
	Calcichrome
	Cu-PAN
	5-Br-PSAA
	PAN
	PR (Pyrogallol Red)
	SATP
Cu	Sodium bicinchoninate
	5-Br-PAPS
	Nitro-PAPS
	3,5-DiBr-PAESA
	TMPyP
	TPPS
	Zincon

Ion	Reagent
F^-	ALC
	Calcein Blue
Fe	Bathophenanthroline
	Bathophenanthrolinedisulfonic acid
	Calcein Blue
	N-PSAP
	5-Br-PAPS
	Nitro-PAPS
	Cu-PAN
	Diantipyrylmethane
	PAN
Ga	Cu-PAN
	PAR
Ge	PR (Pyrogallol Red)
H^+	Intracellular pH monitoring: page 186
Hg	BT
	Cu-PAN
	PC (Phthalein Complexone)
	Zincon
In	Cu-PAN
K	Bis(benzo-15-crown-5)
	Kalibor
La	Chlorophosphonazo-III
	PC (Phthalein Complexone)
Li	Dibenzyl-14-crown-4
	TTD-14-crown-4
Mg	BT
	C14-K22B5
	Calcein
	XB-I
	Chlorophosphonazo-III
	Cu-PAN
	PC (Phthalein Complexone)
Mo	Diantipyrylmethane
Na	Bis(12-crown-4)
Nb	XO (Xylenol Orange)
Nd	Chlorophosphonazo-III
NH_4^+	Cyanoline Blue
	TD19C6
Ni	Calcein Blue
	Calcichrome
	Cu-PAN
	NPSAP
	5-Br-PSAA
	PAN
	PAR
	SATP
	Nitro-PAPS
NO_2^-	DAN
Np	Chlorophosphonazo-III
OCN^-	Cyanoline Blue
Os	Bismuthiol-II
	PAN
	PDTS

Ion	Reagent
Pa	Chlorophosphonazo-III
Pb	BT
	TPPS
	Zincon
Pd	5-Br-PAPS
Pt	5-Br-PAPS
Pu	Chlorophosphonazo-III
Re	Bismuthiol-II
Rare	Chlorophosphonazo-III
earth	HNB
metals	Murexide
	MX
	PAN
Rh	5-Br-PAPS
Ru	5-Br-PAPS
Sb	Arsemate
Sc	Chlorophosphonazo-III
SCN^-	Cyanoline Blue
Se	DAB
	DAN
Sn	SATP
Sr	Calcein Blue
	Chlorophosphonazo-III
	PC (Phthalein Complexone)
Te	Bismuthiol-II
Th	Chlorophosphonazo-III
Ti	Chlorophosphonazo-III
	Diantipyrylmethane
	PR (Pyrogallol Red)
U	Chlorophosphonazo-III
	Diantipyrylmethane
UO_2	HNB
	NN
V	Cu-PAN
	DAB
	PAR
Zn	BT
	Calcein
	Cu-PAN
	5-Br-PAPS
	Nitro-PAPS
	PAN
	PAR
	XO (Xylenol Orange)
	Zincon
Zr	Calcein Blue
	Chlorophosphonazo-III

XV-2. Ion Detection: Metals and Anions

ALC

3-[*N*,*N*-Bis(carboxymethyl)aminomethyl]-1,2-dihydroxyanthraquinone [CAS: 3952-78-1]

Application: F^- detection, colorimetric

Appearance: orange-yellow or yellowish-brown powder
MW: 385.32, $C_{19}H_{15}NO_8$

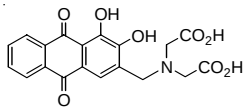
Storage Condition
ambient temperature, protect from light

Shipping Condition
ambient temperature

Ordering Information

Product code	Unit
A006-10	100 mg
A006-12	1 g

Chemical Structure



Product Description

ALC is utilized for colorimetric detection of fluoride ion. A red solution of the La(III) or Ce(III) complex turns blue in the presence of fluoride ions. The maximum wavelength of the fluoride complex is 620 nm at pH 4-5. As little as 0.1 to 1 ppm of fluoride ion can be determined using this method.The structure of this complex has been reported as $La_2L_2F_2$, $(La_5L_4F_2)_n$ or $Ce_5L_4F_4$ (L: ALC). ALC is insoluble in alcohol and ether, slightly soluble in water, and easily soluble in alkaline water. The fluoride complex can be extracted with *iso*-amylalcohol. Since ALC is less toxic than Alizarin red, it is used to mark and trace young fish. The aqueous solution of ALC is yellow at pH < 6, red at pH 6 - 10, and blue violet at pH > 11. ALC can also be used to detect aluminum ions. $pKa_1(COOH)=2.40, pKa_2(OH)=5.54, pKa_3(NH^+)=10.07$, $pKa_4(OH)=11.98$ (m=0.1).

References

R.Greenhalgh, *et al.*, *Anal. Chim. Acta*, **25**, 179 (1961); M. A. Leonard, *et al.*, *International Symposium on Analytical Chemistry*, **1969** (1969); D. Negoiu, *et al.*, *Au. Univ. Bucuresti, Chim.*, **18**, 27 (1969); K. Al-ami, *et al.*, *Analyst*, **95**, 1039 (1970); C. K. Laird, *et al.*, *Talanta*, **17**,173 (1970); B. A. Rahr, *et al.*, *Experientia*, **28**, 180 (1972); F. Ingma, *et al.*, *Talanta*, **20**, 135 (1973); F. Ingman, *Talanta*, **20**, 999 (1973); K. Tsukamoto, *Bull. Jpn. Soc. Sci. Fish.*, **51**, 903 (1985).

Arsemate

Diethyldithiocarbamic acid, silver salt [CAS: 1470-61-7]

Application: As, Sb detection, colorimetric

Appearance: yellow crystalline powder
Purity: >98.0% (Titration)
MW: 256.14, $C_5H_{10}AgNS_2$

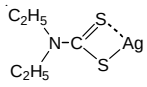
Storage Condition
ambient temperature, protect from light

Shipping Condition
ambient temperature

Ordering Information

Product code	Unit
A012-10	5 g
A012-12	25 g

Chemical Structure



Product Description

Arsemate is soluble in pyridine and chloroform, but insoluble in water and other organic solvents. For arsenic detection, a 0.5% Arsemate/pyridine solution is used. The sample should be treated with sulfuric acid, 15% potassium iodide, and 40% $SnCl_2$/HCl solution to convert arsenic to AsH_3. Arsemate reacts with AsH_3 in pyridine and forms red colloidal silver with a maximum wavelength at 520-540 nm. The detection range of arsenic using the Arsemate/pyridine solution is 4-12 ppm. Alternatively, 0.5% Arsemate/chloroform solution with 30 mM triethylamine can be used for the assay. For the colorimetric detection of arsenic, the AsH_3 gas generator is required.

References

B. W. Budesinsky, *Microchem. J.*, **24**, 80 (1979); A. G. Howard, *et al.*, *Analyst*, **105**, 338 (1980).

Azomethine H

8-Hydroxy-1-(salicylideneamino)-3,6-naphthalenedisulfonic acid, disodium salt [CAS: 32266-60-7]

Application: B detection, colorimetric

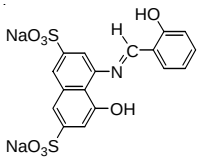
Appearance: yellowish-orange or yellowish-brown crystalline powder
Absorbance: >0.9 at 413 nm (6 ppm boron solution)
MW: 467.38, $C_{17}H_{11}NNa_2O_8S_2$

Ordering Information	
Product code	Unit
A015-10	5 g
A015-12	25 g

Storage Condition
ambient temperature, protect from light

Shipping Condition
ambient temperature

Chemical Structure



Product Description

Azomethine H is a colorimetric reagent for boron detection; it forms an orange complex with boron in aqueous solution. The molar absorptivity of this complex is 1.01×10^4 at 415 nm. The detection range of boron in sample solutions is 1-6 µg per ml. To detect boron in plant samples, EDTA is used to mask copper, iron, and aluminum ions. Azomethine H is used to detect microgram levels of boron in glass and steel samples.

Working Solution

1. Mix 9 g Azomethine H and 2 g ascorbic acid with 700 ml deionized or double-distilled water.
2. Heat the mixture at 70 °C or lower to dissolve Azomethine H.
3. Add water to prepare 1,000 ml assay solution.

References

R. Capelle, *Anal. Chim. Acta*, **24**, 555 (1961); T. M. Shanina, *et al., Z. Anal. Khim.*, **22**, 782 (1967); W. D. Basson, *et al., Analyst*, **94**, 1135 (1969); R. A. Edwards, *Analyst*, **105**, 139 (1980).

Bathocuproine

2,9-Dimethyl-4,7-diphenyl-1,10-phenanthroline [CAS: 4733-39-5]

Application: Cu(I) detection with solvent extraction, colorimetric

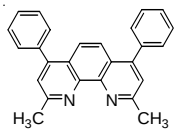
Appearance: white or yellow crystalline powder
Absorbance: >0.81 at 280 nm
>0.58 at 475 nm (Cu complex)
MW: 360.45, $C_{26}H_{20}N_2$

Ordering Information	
Product code	Unit
B001-10	100 mg

Storage Condition
ambient temperature

Shipping Condition
ambient temperature

Chemical Structure



Product Description

Bathocuproine is a Cu(I)-specific colorimetric reagent. An orange Bathocuproine-Cu(I) complex can be extracted with n-hexylalcohol or *iso*-amylalcohol. The maximum wavelength of the complex extract is 479 nm. The Cu(I) detection range is 0.1-10 ppm. Bathocuproine has been used to determine trace amounts of Cu(I) in wine and purified tellurium samples. Bathocuproine is soluble in organic solvents such as nitrobenzene and insoluble in water.

References

L. G. Borchardt, *et al., Anal. Chem.*, **29**, 414 (1957); W. M. Banick, *et al., Anal.Chim. Acta*, **16**, 464 (1957); B. Zak, *et al., Microchem. J.*, **6**, 67 (1962); A. A. Schilt, *Analytical applications of 1,10-Phenanthroline and related compounds, Pergamon Press, London, New York, Paris* (1969); P. Hulthe, *Analyst*, **95**, 351 (1970); K. L. Cheng *et al., Hand book of Orgamic Analytical Reagents, CRC Press Inc. Florida* (1982); M. Matsushita, *et al., Clin. Chim. Acta*, **216**, 103 (1993); L. M. Sayre, *et al., Science*, **274**, 1933 (1996).

XV-2. Ion Detection: Metals and Anions

Bathocuproinedisulfonic acid, disodium salt

2,9-Dimethyl-4,7-diphenyl-1,10-phenanthrolinedisulfonic acid, disodium salt [CAS:52698-84-7]

Application: Cu(I) detection, colorimetric

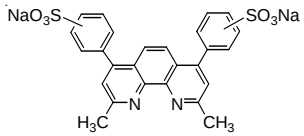
Appearance: white or pale yellowish-brown powder
Absorbance: >0.74 at 285 nm
>0.21 at 480 nm (Cu complex, 1 mM $CuSO_4$)
MW: 564.54, $C_{26}H_{18}N_2Na_2O_6S_2$

Storage Condition: ambient temperature, protect from light
Shipping Condition: ambient temperature

Ordering Information

Product code	Unit
B002-10	1 g

Chemical Structure



Product Description

Bathocuproinedisulfonic acid is a water-soluble analog of bathocuproine that emits fluorescence under UV light. Since the Bathocuproine-Cu(I) complex formed by this analog is water-soluble, Cu(I) can be determined by direct measurement of the assay solution at 483-522 nm without solvent extraction. The molar absorptivity of the bathocuproinedisulfonic acid-Cu(I) complex is 1.2×10^4.

Dr. Faizullah developed a system to quickly determine Cu(I) at around 30 ppm by using Bathocuproine-disulfonic acid in flow injection analysis. Dr. Sawada developed a highly sensitive Cu(I) detection system using the long flow cell. Dr. Sawada's system can detect as little as 0-15 ppb Cu(I) in water samples without pretreatment.

References

T. Yanetani, *Biochem. Biophys. Research Comm*., **3**, 549 (1960); D. Blair, *et al., Talanta*, **7**,163 (1961); D. E. Griffiths, *et al., J. Biol. Chem*., **236**, 1857 (1961); A. Mohindru, *et al., Nature*, **303**, 64 (1983); A.T. Faizullah, *et al., Anal. Chim. Acta*, **172**, 291 (1985); M. B. Kadiiska, *et al., Mol. Pharmacol*., **42**, 723 (1992); Y. Li, *et al., Arch. Biochem. Biophys*., **300**, 346 (1993); Y. Li, *et al., Cancer Res*., **54 (7 Suppl)**, 1895s (1994); F. Nagai, *et al., Carcinogenesis*, **16**, 837 (1995); F. Nagai, *et al., Toxicol Lett*., **89**, 163 (1996); A. M. Seacat, *et al., Arch. Biochem. Biophys*., **347**, 45 (1997); N. Yamashita, *et al., Chem. Res. Toxicol*., **11**, 855 (1998); F. Chen, *et al., Free Radic. Res*., **29**, 197 (1998); M. Murata, *et al., Biochemistry*, **38**, 7624 (1999); Y. Ohkuma, *et al., Arch. Biochem. Biophys*., **372**, 97 (1999).

Bathophenanthroline

4,7-Diphenyl-1,10-phenanthroline [CAS:1662-01-7]

Application: Fe(II), Cu(I) and Ru(II) detection with solvent extraction, colorimetric

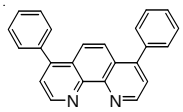
Appearance: white or pale yellow powder
Molar absorptivity: >21,000 at 535 nm (Fe complex)
MW: 332.40, $C_{24}H_{16}N_2$

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Ordering Information

Product code	Unit
B003-10	1 g
B003-12	5 g

Chemical Structure



Product Description

Bathophenanthroline is a highly selective colorimetric reagent for Fe(II) detection. Bathophenanthroline is soluble in organic solvents and slightly soluble in acidic aqueous solutions, but insoluble in neutral or alkaline solutions. It chelates with Fe(II) at pH 2-9, and the red color complex can be extracted with *iso*-amylalcohol ($\log\beta_3$=21.8, λmax=533 nm, ε=22,400). The detection range of Fe(II) by Bathophenanthroline is 10 ppb to 4 ppm. The pKa_2 of Bathophenanthroline is reported to be 4.30 in a 50% dioxane solution (m=0.3) at 25 °C, and the redox potential of FeL_3^{2+}-FeL_3^{3+} in the 1 M H_2SO_4 solution is 1.24 V. Cu(I) and Ru(II) can also be detected using Bathophenanthroline. The maximum wavelength of the Cu(I) and Ru(II) complexes are 457 nm and 460 nm, respectively.

References

G. Firedericksmith, *et al., Analyst*, **77**, 418 (1952); D. Blair, *et al., Talanta*, **7**, 163 (1961); F. R. Short, *et al., Anal. Chem*., **39**, 251 (1967); N. Majkic-Singh, *et al., Clin. Chem.,* **26**, 1360 (1980); C. Carlsson, *et al., Biochim. Biophys. Acta*, **638**, 345 (1981); K. L. Cheng, *et al., Handbook of Organic Analytical Reagents*, *CRC Press Inc., Florida* (1982); N. Liappis, *et al., Klin. Padiatr*., **194**, 60 (1982); E. Glaser, *et al., Acta. Chem. Scand. B*, **42**, 175 (1988); D. P. Derman, *et al., Ann. Clin. Biochem*., **26**, 144 (1989); C. Charlier, *et al., Ann. Biol. Clin*., **50**, 191 (1992).

Bathophenanthrolinedisulfonic acid, disodium salt

4,7-Diphenyl-1,10-phenanthrolinedisulfonic acid, disodium salt [CAS:52746-49-3]

Application: Fe(II) and Cu(I) detection, colorimetric

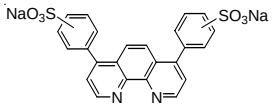
Appearance: white, pale pink, or pale brown powder
Absorptivity: < 0.01 at 535 nm (Fe^{2+} free)
>0.50 at 535 nm (Fe^{2+} complex)
MW: 536.49, $C_{24}H_{14}N_2Na_2O_6S_2$

Storage Condition	Shipping Condition
ambient temperature	ambient temperature

Ordering Information

Product code	Unit
B004-10	1 g

Chemical Structure



Product Description

Bathophenanthrolinedisulfonic acid, disodium salt is a water-soluble derivative of Bathophenanthroline. It is insoluble in organic solvents. Its pKa_1 and pKa_2 are 2.83 and 5.20, respectively. Due to its high water solubility, Bathophenanthrolinedisulfonic acid can be used to detect Fe(II) in aqueous samples such as blood or biological fluids. The detection range of Fe^{2+} using Bathophenanthrolinedisulfonic acid is 0.25-4 ppm at 535 nm. The molar absorptivity of the complex is 2.24×10^4. The reduction and oxidation potential of FeL_3^{2+}-FeL_3^{3+} in 1 M H_2SO_4 solution is reported to be 1.09 V. Cu^+ can also be detected using Bathophenanthrolinedisulfonic acid. The maximum wavelength and molar absorptivity of the Cu^+ complex is 483 nm and 1.23×10^4, respectively.

References

G. Plomteux, *et al., Ann. Biol. Clin.*, **45**, 52 (1987); P. F. Bell, *et al., Biol. Trace Elem. Res.*, **30**, 125 (1991); R. E. Cowart, *et al., Anal. Biochem.*, **211**, 151 (1993); R. P. Glahn, *et al., J. Nutr.*, **125**, 1833 (1995).

Bis(benzo-15-crown-5)

Bis[(benzo-15-crown-5)-4-methyl]pimelate [CAS:69271-98-3]

Application: Ionophore for K^+ selective electrode

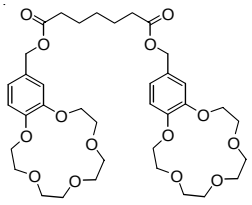
Appearance: white powder
Purity: >98.0% (HPLC)
MW: 720.80, $C_{37}H_{52}O_{14}$

Storage Condition	Shipping Condition
ambient temperature	ambient temperature

Ordering Information

Product code	Unit
B020-10	100 mg

Chemical Structure



Product Description

Bis(benzo-15-crown-5) is a potassium selective ionophore. Its potassium ion selectivity is almost equal to that of valinomycin, which is a well-known neutral carrier for the potassium ion in nature. This reagent, combined with NPOE as a plasticizer, is the most recommended ionophore for potassium-selective PVC membrane electrodes. This ionophore also forms a thallium complex, so it can be used for thallium sensors.

Ion selectivity: $LogK^{pot}(K/Na)$:-3.6, $LogK^{pot}(K/Rb)$:-0.7, $LogK^{pot}(K/NH_4)$:-2.0, $LogK^{pot}(K/Cs)$:-2.0
Formulation: Ionophore: 2.7%, NPOE: 70%, TFPB: 50 mol% of ionophore/PVC, THF

References

K. Kimura, *et al., J. ElectroAnal. Chem.*, **95**, 91 (1979); H. Tamura, *et al., Bull. Chem. Soc. Jpn.*, **53**, 547 (1980); H. Tamura, *et al., J. ElectroAnal. Chem.*, **115**, 115 (1980); H. Tamura, *et al., Anal. Chem.*, **54**, 1224 (1982); H. Tamura, *et al., Mikrochim. Acta*, **1983(II)**, 287 (1983); Y. Umezawa, *Handbook of Ion-selective electrodes: Selectivity coefficients, CRC Press, Inc., N. W., Boca Raton, Florida* (1990), 635-641; Y. Yamashoji, *et al., Anal. Sci.*, **7**, 485 (1991).

XV-2. Ion Detection: Metals and Anions

Bis(12-crown-4)

Bis[(12-crown-4)methyl]-2-dodecyl-2-methylmalonate [CAS: 80403-59-4]

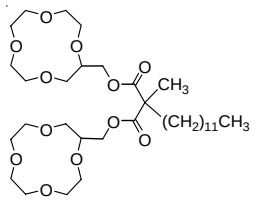
Application: Ionophore for Na^+ selective electrode

Appearance: pale yellow liquid
Purity: >95.0% (HPLC)
MW: 662.85, $C_{34}H_{62}O_{12}$

Ordering Information	
Product code	Unit
B021-10	100 mg
B021-12	500 mg

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



Product Description

The $NAS_{11\text{-}8}$ glass electrode is utilized as a selective sodium ion sensor. However, it is easily spoiled by biomaterials in sample solutions. Bis(12-crown-4), a sodium ionophore, is 100 times more selective for sodium than potassium in the PVC electrode using NPOE as a plasticizer. The same selectivity for sodium over potassium is reported when Bis(12-crown-4) is used on a coated wire electrode. It can be used in either a liquid membrane or a PVC membrane. TFPB, a lipophilic anion, can help improve detection sensitivity.

Ion selectivity: $LogK^{pot}(Na/Li)$:-3.0, $LogK^{pot}(Na/K)$:-2.0, $LogK^{pot}(Na/Cs)$:-2.0, $LogK^{pot}(Na/Rb)$:-2.4, $LogK^{pot}(Na/NH_4)$:-3.0, $LogK^{pot}(Na/Mg)$:-4.0, $LogK^{pot}(Na/Ca)$:-4.0, $LogK^{pot}(Na/Ba)$:-3.7, $LogK^{pot}(Na/Sr)$:-3.7
Formulation: Ionophore: 6%, NPOE: 70%, TFPB: 50 mol% of ionophore

References

H. Tamura, *et al., Anal. Chem.*, **54**, 1224 (1982); T. Shono, *et al., J. electoral. Chem.*, **132**, 99 (1982); H. Tamura, *et al., Mikrochim. Acta [Wien]*, **1983(II)**, 287 (1983); T. Maruizumi, *et al., Mikrochim. Acta*, **1986(I)**, 331 (1986); G. J. Moody, *et al., Analyst*, **114**, 15 (1989); G. J. Moody, *et al., Anal. Proc.*, **26**, 8 (1989); Y. Umezawa, *Handbook of Ion-selective electrodes: Selectivity coefficients, CRC Press, Inc., N. W., Boca Raton, Florida*, 717, (1990)

Bisthiourea-1

2,7-Di-*tert*-butyl-9,9-dimethyl-4,5-bis(*N'*-*n*-butyl-thioureido)xanthene [CAS:187404-67-7]

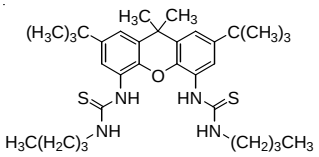
Application: Ionophore for Cl^- selective electrode

Appearance: white or pale yellow powder
Purity: >98.0% (HPLC)
MW: 582.91, $C_{33}H_{50}N_4OS_2$

Ordering Information	
Product code	Unit
B432-10	25 mg

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



Product Description

Bisthiourea-1 is a chloride-selective neutral ionophore, and it is utilized for preparation of chloride electrodes. Bisthiourea-1 forms a 1:1 complex with chloride ion. The detection limit of chloride ion using Bisthiourea-1 is $(6.5^{\pm}3.0) \times 10^{-6}$ M.

References

K. P. Xiao, *et al., Anal. Chem.*, **69**, 1038 (1997); P. Buhlmann, *et al., Tetrahedron*, **53**, 1647 (1997).

5-Br-PAPS

2-(5-Bromo-2-pyridiylazo)-5-[*N*-*n*-propyl-*N*-(3-sulfopropyl)amino]phenol, disodium salt, dihydrate [CAS:91599-24-5]

Application: Zn^{2+} detection, colorimetric

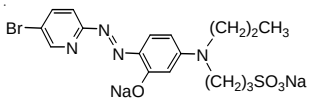
Appearance: orange or reddish-brown cyrstalline powder
Absorptivity: >0.42 at 449 nm
MW: 537.34, $C_{17}H_{19}BrN_4Na_2O_4S,2H_2O$

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Ordering Information

Product code	Unit
B026-10	100 mg

Chemical Structure



Product Description

5-Br-PAPS, a water-soluble derivative of 5-Br-PADAP, is a highly sensitive colorimetric reagent for zinc detection. This reagent forms a red chelate with zinc at pH 7.5-9.5 (λmax=552 nm, ε=13.3 x 10^4). 5-Br-PAPS is also utilized for the determination of Cu(II), Fe(II), Ni(II), Co(II), Pt(II), Ru(II), and Rh(II). 5-Br-PAPS is utilized to determine serum levels of Zn, Cu, Fe, Ni, and Co. For serum Zn levels, Fe ions can be masked with citric acid or metaphosphoric acid, and fluoride and Cu ions can be masked with 2-mercaptobenzothiazole or dithiocarboxysarcosine. 5-Br-PAPS-Ti complex can be used for hydrogen peroxide detection.

Table 15-4 Spectral Data of 5-Br-PAPS-Metal Complex

Metal	λmax	Molar Absorptivity	Metal	λmax	Molar Absorptivity
Co(II)	558 nm	93,000	U(VI)	578 nm	73,000
Cu(II)	560 nm	116,000	V(V)	600 nm	60,000
Ni(II)	559 nm	117,000	Zn(II)	553 nm	133,000

References

T. Makino, *et al.*, *Clin. Chim. Acta*, **120**, 127 (1982); C. Matsubara, *et al.*, *Anal. Chem.*, **57**, 1107 (1985); D. Horiguchi, *et al.*, *Anal. Sci.*, **1**, 461 (1985); R. Homsher, *et al.*, *Clin. Chem.*, **31**, 1310 (1985); Y. Shijo, *et al.*, *Analyst*, **113**, 519 (1988); Y. Shijo, *et al.*, *Analyst*, **113**, 1201 (1988); N. Uehara, *et al.*, *Anal. Sci.*, **5**, 111 (1989); I. Kasahara, *et al.*, *Anal.Chim. Acta*, **219**, 239 (1989); E. Ohyoshi, *et al.*, *J. Inorg. Biochem.*, **52**, 157 (1993); J. Shum-Cheong-Sing, *et al.*, *Ann. Biol. Clin.*, **52**, 765 (1994); E. Ohyoshi, *et al.*, *J. Inorg. Biochem.*, **75**, 213 (1999).

5-Br-PSAA

2-(5-Bromo-2-pyridylazo)-5-[*N*-*n*-propyl-*N*-(3-sulfopropyl)amino]aniline, sodium salt [CAS:86035-60-7]

Application: Fe(II) and Co(III) detection, colorimetric

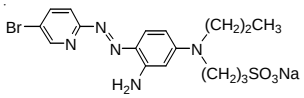
Appearance: reddish-orange or orange powder
Molar absorptivity: >86,000 at 558 nm
MW: 478.34, $C_{17}H_{21}BrN_5NaO_3S$

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Ordering Information

Product code	Unit
B027-10	100 mg

Chemical Structure



Product Description

5-Br-PSAA is a water-soluble colorimetric reagent for Fe(II) and Co(III) detection. This reagent forms colored complexes with Fe, Ni, Cu, and Co, but not with Zn. The Fe(II) and Co(III) complexes are very stable, and even EDTA cannot remove metals from these complexes. However, Cu(II) and Ni(II) complexes are decomposed by EDTA, so 5-Br-PSAA can be used to determine Fe(II) in the presence of Cu(II) or Ni(II). The molar absorptivity of Fe(II) is reported to be 8.9x10^4 (λmax=558 nm). The 5-Br-PSAA assay is 4 times as sensitive as Bathophenanthroline assay. Dr. Sakai and others determined ppb levels of Pb (ε=98,400) using 5-Br-PSAA by flow injection analysis. Dr. Makino and others determined Cu(II) in 0.2 ml of serum by direct colorimetric detection (λmax=580 nm, ε=64,000).

References

D. Horiguchi, *et al.*, *Anal. Chim. Acta*, **151**, 457 (1983); T. Sakai, *et al.*, *Anal. Chim. Acta*, **214**, 271 (1988); T. Makino, *Clin. Chim. Acta*, **185**, 7 (1989).

XV-2. Ion Detection: Metals and Anions

BT

2-Hydroxy-1-(1-hydroxy-2-naphthylazo)-6-nitro-4-naphthalenesulfonic acid, sodium salt [CAS:1787-61-7]

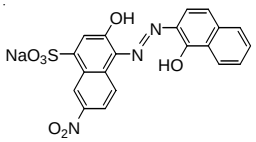
Application: Metal indicator, Ca^{2+} and Mg^{2+} detection, colorimetric

Appearance: black or dark brown powder
MW: 461.38, $C_{20}H_{12}N_3NaO_7S$

Ordering Information	
Product code	Unit
B015-10	25 g

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



Product Description

BT in aqueous solution changes color according to pH: red at pH<6, blue at pH 7-11, and orange at pH>12. Its proton dissociation constants are reported to be pKa_2=6.3 and pKa_3=11.55 (m=0.08, 20 °C). BT is one of the most commonly used indicators of Ca, Mg, Zn, Cd, Pb, Hg, and rare earth metals for EDTA chelate titration. BT solution turns red in the presence of the metals at pH 10, and then changes to blue at the endpoint of the titration. Cu and Fe(III), as well as Al, Co, and Mn(III), should be masked with KCN because they interfere with the color change. Triethanolamine is also a suitable masking reagent for Al, Fe(III), and Mn(III). Since metal ions such as Mn(III) and Fe(III) oxidize BT, hydroxylamine and hydrochloride should be added to prevent oxidation.

Table 15-5 Metal Detection Conditions

Metal	Conditions	Wavelength	Molar Absorptivity	Detection Range	Interference
Cd, Zn	pH 6, $CHCl_3$ extraction with phenanthroline	522 nm	22,000	0.22-5.6 ppm	-
Co(II)	pH 5.5-8.2, benzene extraction with capriquat	587 nm	66,000	0.1-0.8 ppm	Mn, Ni, VO_3^-
Mg	pH 9.5-11.2, butanol extraction	545 nm	20,000	0.5-5 ppm	Ca, Cd, Fe, Zn
rare earth metals	pH 6.35-6.8, isoamyl alcohol extraction with diphenylguanidine	500-600 nm	26,000-30,000	0.1-15 ppm	-

References

H. V. Connerty, *et al.*, *Am. J. Clin Pathol.*, **50**, 499 (1968); N. I. Shestidesyatnaya, *et al.*, *Zhur. Anal. Khim.*, **33**, 303 (1978); J. L. Seltzer, *et al.*, *Coll. Relat. Res.*, **7**, 399 (1987); M. Obuchowski, *et al.*, *Acta Biochim. Pol.*, **38**, 177 (1991); T. Kvernmo, *et al.*, *Int. J. Biochem. Cell. Biol.*, **28**, 303 (1996); D. W. Jung, *et al.*, *Anal. Biochem.*, **263**, 118 (1998); R. T. Gettar, *et al.*, *J. Chromatogr. A*, **855**, 111 (1999).

C14-K22B5

4,13-Bis[*N*-(1-adamantyl)carbamoylacetyl]-8-tetradecyl-1,7,10,16-tetraoxa-4,13-diazacyclooctadecane

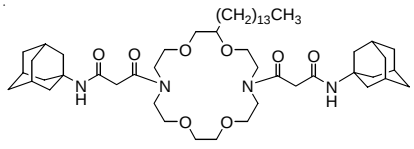
Application: Ionophore for Mg selective electrode

Appearance: white powder
Purity: >95.0% (HPLC)
MW: 897.28, $C_{52}H_{88}N_4O_8$

Ordering Information	
Product code	Unit
C391-10	10 mg

Storage Condition: -20 °C
Shipping Condition: ambient temperature

Chemical Structure



Product Description

C14-K22B5 is the best magnesium selective ionophore available. The PVC membrane stability is improved by this ionophore's high lipophilicity due to tetraoctadecane and adamantyl groups. C14-K22B5 has a linear response between $1x10^{-5}$ M and 0.1 M magnesium ion.

Reference

K. Suzuki, *et al.*, *Anal. Chem.*, **67**, 324 (1995).

Calcein

Bis[*N*,*N*-bis(carboxymethyl)aminomethyl]fluorescein [CAS:1461-15-0]

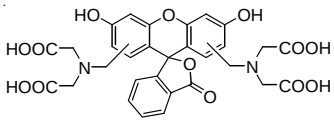
Application: Metal indicator for Ca^{2+}, fluorometric

Appearance: orange-yellow powder
Free fluorescein: pass test
MW: 622.53, $C_{30}H_{26}N_2O_{13}$

Ordering Information	
Product code	Unit
C001-10	1 g
C001-12	5 g

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



Product Description

Calcein is a fluorescent metal indicator for calcium detection with EDTA titration. It is slightly soluble in water. The proton dissociation constants are reported to be pKa_1=2.1, pKa_2=2.9, pKa_3=4.2, pKa_4=5.5, pKa_5=10.8 and pKa_6=11.7. Calcein solution emits a yellowish-green fluorescence in acidic conditions, and no fluorescence in basic conditions. However, Calcein will emit fluorescence in the presence of metal ions such as Al, Ba, Ca, Cu, Mg, and Zn in basic conditions. Therefore, Calcein is also used for direct fluorometric EDTA titration of these metal ions. Since Calcein has a clear endpoint even if the sample has color or contains phosphate, it is suitable for calcium detection in biological samples or fertilizers. TPC, murexide, or PPC are added to get a clear endpoint. For example, by mixing equal amounts of TPC with Calcein, intense green fluorescence will turn reddish-purple with no fluorescence at the endpoint.

References

J. Korbl, *et al.*, *Collection*, **23**, 622 (1958); D. F. H. Wallach, *et al.*, *Anal. Chem.*, **31**, 456 (1959); R. G. Yalman, *et al.*, *Anal. Chem.*, **31**, 1230 (1959); D. H. Wilkins, *et al.*, *Talanta*, **2**, 201 (1959); J. H. Austin, *et al.*, *Chemist-Analyst*, **55**, 11 (1966); H. K. Suzuki, *et al.*, *Stain Technolgy*, **41**, 50 (1966); K. Sato, *et al.*, *Nippon Yakurigaku Zasshi*, **110 (Suppl 1)**, 199P (1997).

Calcein Blue

8-[*N*,*N*-Bis(carboxymethyl)aminomethyl]-4-methylunbelliferone [CAS: 54375-47-2]

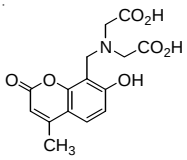
Application: Metal indicator, fluorometric

Appearance: white or pale yellow powder
MW: 321.28, $C_{15}H_{15}NO_7$

Ordering Information	
Product code	Unit
C002-08	1 g

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



Product Description

Calcein Blue is a fluorescent metal indicator in EDTA titration. It is highly soluble in alkaline solutions, but not so soluble in water. Its proton dissociation constants are reported to be pKa_1=2.45, pKa_2=7.24 and pKa_3=11.3. Calcein Blue solution emits bright blue fluorescence (λem=445 nm) at pH 4-11, but no fluorescence at pH 12 or higher. The blue fluorescence of the Calcein Blue solution at pH 4-11 is quenched by metal ions such as Co, Cu, Mn, Ni, and Pb. However, Calcein Blue forms a highly fluorescent complex with alkaline earth metals at pH 12 or higher. Since the endpoint of the chelate titration is clear by UV irradiation at 200-370 nm in a dark room, highly stained samples can be used. Calcein Blue is also suitable to determine Fe and Zr ions. Calcein Blue-Zn chelate can detect 10 ppb of F^-. Calcein Blue aqueous solution emits laser at 449-490 nm.

References

J. H. Eggers, Talanta, **4**, 38 (1960); D. H. Willkins, *Talanta*, **4**, 182 (1960); H. N. Elsheimer, *Talanta*, **14**, 97 (1967); M. A. Salam-Khan, *et al.*, *Anal. Chim, Acta*, **43**, 453 (1968); R. V. Hems, *et al.*, *Anal. Chem.*, **42**, 784 (1970); T. L. Har, *et al.*, *Anal. Chem.*, **43**, 136 (1971); T. Imasaka, *et al.*, *Bull. Chem. Soc. Jpn.*, **49**, 2687 (1976); K. A. Matsoukas, *et al.*, *Anal. Chim. Acta*, **227**, 211 (1989).

XV-2. Ion Detection: Metals and Anions

Cesibor

Tetrakis(4-fluorophenyl)borate, sodium salt, dihydrate [CAS: 25776-12-9]

Application: Cs^+ detection, precipitation titration

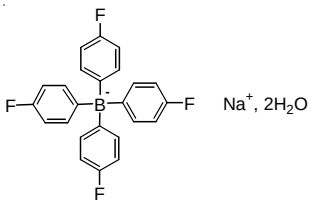
Appearance: white powder
Purity: >98.0% (Precipitation titration)
MW: 450.21, $C_{24}H_{16}BF_4Na$, $2H_2O$

Storage Condition	Shipping Condition
ambient temperature	ambient temperature

Ordering Information

Product code	Unit
C007-10	1 g

Chemical Structure



Product Description

Cesibor is a cesium ion selective tetraphenylborate compound, and is utilized as a precipitation reagent for cesium ion. Cesibor can precipitate only cesium ion from a mixture of ammonium, potassium, and cesium ions.

References

C. E. Moore, *et al., Anal. Chim. Acta*, **35**, 1 (1966); C. M. Tsai, *et al., Radiochem. Radioanal. Lett.*, **20**, 167 (1975); M. Tsubouchi, *et al., Anal. Chem.*, **57**, 781 (1985).

Chlorophosphonazo-III

2,7-Bis(4-chloro-2-phosphonophenylazo)-1,8-dihydroxy-3,6-naphthalenedisulfonic acid, disodium salt [CAS: 1914-99-4]

Application: Alkaline earth metals detection, colorimetric

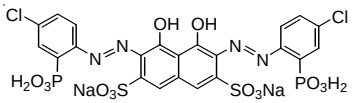
Appearance: dark purple powder
MW: 801.33, $C_{22}H_{14}Cl_2N_4Na_2O_{14}P_2S_2$

Storage Condition	Shipping Condition
ambient temperature	ambient temperature

Ordering Information

Product code	Unit
C010-10	100 mg
C010-12	1 g

Chemical Structure



Product Description

Chlorophosphonazo-III is a colorimetric reagent for thorium, uranium, lanthanoids, actinoids, and alkaline earth metals. This reagent is readily soluble in water and is violet at pH 4 or lower, blue in NaOH solution, and brilliant green in acids such as H_2SO_4, HCl and diluted HNO_3 (it decomposes in concentrated HNO_3). Its proton dissociation constants are reported to be -1.1, -1.1, 0.6, 0.8, 1.5, 2.5, 5.47, 7.20, 12.15 and 15.13 (μ=0.1, 20 °C). Chlorophosphonazo-III is used as a selective colorimetric reagent for calcium in a mixed Ca/Mg sample in the pH range of 2-3. It is also a useful indicator for the precipitation titration of SO_4^{2-} using Ba^{2+} as a standard solution.

Table 15-6 Metal Detection Conditions Using Chlorophosphonazo-III

Metal	λmax	Molar Absorptivity	pH	Metal	λmax	Molar Absorptivity	pH
Ba	665 nm	-	5.9	Pu	-	138,000	3-5 M HCl
Ca	668 nm	14,600	2.2	Sr	670 nm	122,000	2 M HCl
Gd	668 nm	190,000	11.2 / Butanol	Sr	660 nm	-	2.5-5.5
Mg	669 nm	14,500	7.0	Th	670 nm	122,000	2 M HCl
Nd	680 nm	40,000	-	Ti	690 nm	10,800	1-2
Np	672 nm	62,200	2-5	U	670 nm	78,600	4-5
Pa	630 nm	30,800	4-5 M HNO_3	Zr	690 nm	33,000	2 M HCl

References

J. W. Ferguson, *et al., Anal.Chem.*, **36**, 796 (1964). B. Budensinsky, *Talanta*, **16**, 1277 (1969). E. B. Sandell, *et al., Photometric Determination of Traces of Metals, John Wiley & Sons, Inc. New York* (1978).

Co(III)-5-Cl-PADAP

Bis[2-(5-chloro-2-pyridylazo)-5-diethylaminophenolato]cobalt(III) chloride [CAS: 81342-98-5]

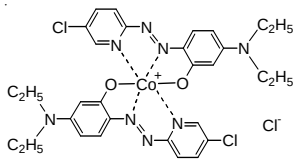
Application: Anionic detergent detection with solvent extraction, colorimetric

Appearance: dark purple crystals
Molar absorptivity: >84,000 at 585 nm
MW: 701.92, $C_{30}H_{32}Cl_3CoN_8O_2$

Ordering Information	
Product code	Unit
C021-10	100 mg

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



Product Description

Since Co(III)-5-Cl-PADAP is not influenced by chloride or nitrate ions, it is suitable for the colorimetric determination of anionic detergents in seawater. Anionic surfactant can be determined at the ppb level.

Protocol

1. Add 1 ml HCl (5 M), 1 ml Co(III)-5-Cl-PADAP (0.1% w/v), and 5 ml toluene to 50 ml of sample solution.
2. Shake the mixture for 5 minutes.
3. Discard the aqueous phase and wash the toluene solution with 25 ml Co(III)-5-Cl-PADAP (0.002% w/v, pH 1 HCl).
4. Measure the absorbance of the toluene phase at 560 nm.

References

S. Taguchi, *et al.*, *Talanta*, **27**, 289 (1980); S. Taguchi, *et al.*, *Talanta*, **28**, 616 (1981); M. Taga, *et al.*, *Anal. Sci*., **4**, 181 (1988); M. Taga, *et al.*, *Anal. Sci*., **5**, 219 (1989); M. Taga, *et al.*, *Bull. Chem. Soc. Jpn*., **62**, 1482 (1989).

Cu-PAN

Mixture of Cu-EDTA and PAN (11.1:1)

Application: Metal indicator

Appearance: gray reddish-brown or yellowish-brown powder

Ordering Information	
Product code	Unit
C016-10	10 g

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Product Description

Cu-PAN, a mixture of Cu-EDTA and pyridylazo-naphthol PAN, is a very popular metal indicator for chelatometry. Usually, 20-50% aqueous dioxane (or *i*sopropylalcohol) or 50-70% aqueous methanol (or ethanol) is used as a solvent (1 g Cu-PAN per 100 ml solvent). The solution should be heated during the chelating titration. However, a clear endpoint can be seen without heating by adding an equal volume of organic solvents such as isopropylalcohol or dioxane.

Applications

Chelate titration (acidic condition): Al, C, Co, Fe(III), Ga, Hg, In, Ni, V, Zn, rare earth metal ions
Chelate titration (alkaline condition): Ca, Cu, Mg, Mn(II)

References

H. Flashka, *et al.*, *Chemist-Analyst*, **45**, 58 (1956); H. Flashka, *et al.*, *Z. Anal. Chim*., **152**, 77 (1956).

XV-2. Ion Detection: Metals and Anions

Cyanoline Blue

Mixture of monopyrazolone and bisbyrazolone (12.5:1)

Application: CN^- detection, colorimetric

Appearance: white or pale yellow powder

Storage Condition	Shipping Condition
ambient temperature	ambient temperature

Ordering Information

Product code	Unit
C017-10	25 g

Product Description

Cyanoline Blue is a mixture of monopyrazolone and bispyrazolone. It is used as a colorimetric reagent for CN^- detection. The detection range of CN^- by this method is 5 ppb to 10 ppm. The maximum wavelength is 620 nm. A ready-to-use solution can be prepared by dissolving 0.27 g Cyanoline Blue in 20 ml pyridine, followed by dilution with 100 ml of water. The solution is stable for one week at 4 °C. Cyanoline Blue is also used to detect SCN^-, OCN^-, NH_3, and vitamin B_{12}.

References

J. M. Kruse, *et al.*, *Anal. Chem.*, **25**, 446 (1953); L. Prochazkova, *Anal. Chem.*, **36**, 865 (1964); S. Baar, *Analyst*, **91**, 268 (1966).

DAN

2,3-Diaminonaphthalene [CAS 771-97-1]

Application: Se detection, colorimetric and fluorometric
Nitrite detection, fluorometric

Appearance: white or pale yellowish-brown powder

Melting Point: 185-200°C

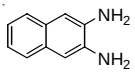
MW: 158.20, $C_{10}H_{10}N_2$

Storage Condition	Shipping Condition
-20 °C, protect from light	ambient temperature

Ordering Information

Product code	Unit
D027-10	1 g

Chemical Structure



Product Description

DAN is a highly selective colorimetric and fluorometric reagent for Se detection. This reagent is almost insoluble in cold water and alcohol, but it is higly soluble in water at 50 °C. DAN forms 4,5-benzopiaselenol by the complex with Se, and this complex is extracted with chloroform, toluene, or cyclohexane. Since the Se complex is volatile, Se can be determined by gas chromatography. HPLC can detect Se in tap and rain water at the picogram level. Further, DAN generates fluorescent naphthalenetriazole by the reaction with nitrite ion, so it is also used for the fluorometric determination of nitrite.

Applications

Solvent extraction, colorimetric: Se(IV) (in toluene at pH 1.2-2.2, λmax=380 nm, ε=11,800, detection range 0-4 ppm)
Solvent extraction, fluorometric: Se(IV) (λem=520 nm in toluene, λem=538 nm in cyclohexane, detection range 1-100 ppb)

References

J. H. Wiersma, *Anal. Lett.*, **3**, 123 (1970); G. L. Wheeler, *et al.*, *Microchem. J.*, **19**, 390 (1974); Y. Shibata, *et al.*, *Analyst*, **110**, 1269 (1985); E. M. Rodrigues, *et al.*, *Talanta*, **41**, 2025 (1994); A. M. Miles, *et al.*, *Methods in Enzymology*, **7**, 40 (1995).

Diantipyrylmethane

Di(4-antipyryl)methane, monohydrate [CAS 1251-85-0]

Application: Ti detection, colorimetric

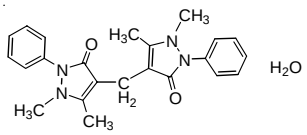
Appearance: white crystalline powder
Purity: >99.0% (HPLC)
MW: 406.48, $C_{23}H_{24}N_4O_2$, H_2O

Ordering Information	
Product code	Unit
D008-10	25 g

Storage Condition
ambient temperature

Shipping Condition
ambient temperature

Chemical Structure



Product Description

Diantipyrylmethane is a colorimetric reagent for Ti detection, and is also used as a gravimetric and solvent extraction reagent for various kinds of metal ions. This reagent is readily soluble in mineral acids and organic solvents, and slightly soluble in benzene (1.7%) and carbon tetrachloride (0.28%). Diantipyrylmethane forms a yellow complex with Ti in 0.5-4.0 M HCl solution (λmax=385-390 nm, ε=15,000). The resulting Ti complex can be extracted by organic solvents in the presence of I^-, SCN^-, tartaric acid, or pyrocatechol. The detection range is 0.2-3.0 ppm. Diantipyrylmethane also makes a brown water-soluble complex with Fe in a weak acidic solution.

Application

Solvent extraction, colorimetric detection: Fe (lmax=450 nm, e=5,400), Mo, Ti, U
Gravimetric detection: Be, Ca, Cd, Co, Cu, Hg, Ir, Os, Pb, Sr, Tl, Zn

References

V. P. Zhivopistsev, *Zabodsk. Lab.*, **31**, 1043 (1965); K. L. Cheng, *et al.*, *Handbook of Organic Analytical Reagents*, *CRC press, Fiorida*, p. 23 (1982); C. H. Chung, *Anal. Chim. Acta*, **154**, 259 (1983); C. H. Chung, *Anal. Chim. Acta*, **154**, 259 (1983); N. Uehara, *et al.*, *Analyst*, **116**, 27 (1991).

Dibenzyl-14-crown-4

6,6-Dibenzyl-1,4,8,11-tetraoxacyclotetradecane [CAS: 106868-21-7]

Application: Li^+ selective electrode

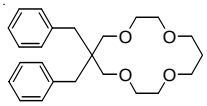
Appearance: white crystalline powder
Purity: >98.0% (HPLC)
MW: 384.51, $C_{24}H_{32}O_4$

Ordering Information	
Product code	Unit
D043-10	50 mg

Storage Condition
ambient temperature

Shipping Condition
ambient temperature

Chemical Structure



Product Description

Dibenzyl-14-crown-4 is 200 times more selective for lithium than for sodium or potassium. Though Dibutyl-phenanthroline is 1,000 times more selective for lithium than sodium at a controlled pH, Dibenzyl-14-crown-4 is less pH-sensitive.

Selectivity: $LogK^{pot}(Li/Na)$: -2.5, $LogK^{pot}(Li/K)$: -2.3, $LogK^{pot}(Li/Cs)$: -1.6, $LogK^{pot}(Li/Rb)$: -2.2, $LogK^{pot}(Li/NH_4)$: -3.1, $LogK^{pot}(Li/H)$: -3.2, $LogK^{pot}(Li/Mg)$: -4.3, $LogK^{pot}(Li/Ca)$: -4.9, $LogK^{pot}(Li/Ba)$: -4.7, $LogK^{pot}(Li/Sr)$: -4.3
Formulation: Ionophore: 1%, NPOE: 70%, K-TCPB: 50 mol% of ionophore/PVC, THF

References

S. Kitazawa, *et al.*, *J. Am. Chem. Soc.*, **106**, 6978 (1984); S. Kitazawa, *et al.*, *Analyst*, **110**, 295(1985); K. Kimura, *et al.*, *J. Chem. Soc. Perkin Trans II*, 1945 (1986); K. Kimura, *et al.*, *Anal. Chem.*, **59**, 2331 (1987); R. A. Bartsch, *et al.*, *Anal. Chim. Acta*, **272**, 285 (1993); R. A. F. Linton, *et al.*, *Br. J. Anaesth.*, **71**, 262 (1993).

XV-2. Ion Detection: Metals and Anions

HDOPP-Ca

Bis(4-*n*-octylphenyl)phosphate, calcium salt

Application: Ion exchanger for Ca^{2+} selective electrode

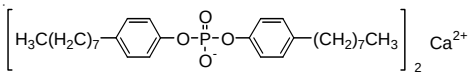
Appearance: white powder
Molar absorptivity: >3,600 at 270 nm
MW: 987.29, $C_{56}H_{84}CaO_8P_2$

Ordering Information

Product code	Unit
H003-10	1 g

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



Product Description

HDOPP-Ca is a liquid cation exchanger for use in the calcium ion selective electrode. HDOPP is the most suitable compound among the dialkyl phosphate derivatives, and is used in either a liquid membrane or a PVC membrane using DOPP (dioctylphenylphosphate calcium salt) as a plasticizer.

References

J. W. Ross, *Science*, **156**, 1378 (1967); J. Ruzicka, *et al., Anal. Chim. Acta*, **67**, 155 (1973); H. M. Brown, *et al., Anal. Chim. Acta.*, **85**, 261 (1976); B. J. Birch, *et al., Detection limits of calcium ion-selective electrodes in relation to ligand-containing systems, Pungor, E. (Editor), Ion-selective Electrodes, Akademiai Kiado, Budapest and Elsevier, Amsterdam*, p.335 (1977); J. D. R. Thomas, *Lab. Pract.*, **27**, 857 (1978).

HFPB

Tetrakis[3,5-bis(1,1,1,3,3,3-hexafluoro-2-methoxy-2-propyl)phenyl]borate, sodium salt, trihydrate [CAS: 120945-63-3]

Application: Lipophilic anion, counter anion for ion selective electrode

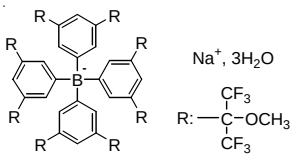
Appearance: white crystalline powder
Purity: >95.0% (Titration)
MW: 1836.65, $C_{56}H_{36}BF_{48}NaO_8$, $3H_2O$

Ordering Information

Product code	Unit
H209-10	100 mg

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



Product Description

Highly lipophilic tetraphenylborate (HFPB) is a very stable anion in a wide pH range. It is used as a cation phase transfer reagent. Vitamin B_{12} electrode in which HFPB was used as an ion site detects 10^{-7} M level of vitamin B_{12} with linear response.

References

G. H. Zhang, *et al., Anal. Sci.*, **4**, 527 (1988); G. H. Zhang, *et al., Anal. Chem.*, **62**, 1644 (1990); K. Fujiki, *et al., J. Fluorine. Chem.*, **1992**, 1; T. Katsu, *et al., Anal. Lett.*, **29**, 1281 (1996); T. Katsu, *et al., Electroanalysis*, **8**, 1101 (1996).

HNB

2-Hydroxy-1-(2-hydroxy-4-sulfo-1-naphthylazo)-3,6-naphthalendisulfonic acid, trisodium salt [CAS: 63451-35-4]

Application: Alkaline earth metal ions, rare earth metal ions detection

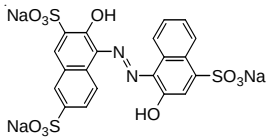
Appearance: dark purple powder
Sensitivity: pass test
MW: 620.48, $C_{20}H_{11}N_2Na_3O_{11}S_3$

Storage Condition	Shipping Condition
ambient temperature	ambient temperature

Ordering Information

Product code	Unit
H007-10	1 g

Chemical Structure



Product Description

HNB is a metal indicator for calcium, and a colorimetric reagent for alkaline earth metal ions, rare earth metal ions, and uranium ions. HNB is readily soluble in water and alcohol. Aqueous solution of HNB is blue at pH 7-12, red above pH 13, and pink in the presence of calcium at pH 10. The detection range of alkaline earth metal ions and rare earth metal ions are 1-600 ppm and 1-300 ppm, respectively. The maximum wavelength of the complex is 650 nm. HNB is also used for UO_2(II) detection. The proton dissociation constants of HNB are reported to be pKa_4=6.44 and pKa_5=12.91(μ=0.1, 24 °C).

References

G. Catledge, *et al., Clin. Chem.*, **11**, 521 (1965); E. F. Steagall, *J. A.O. A. C.*, **48(4)**, 723 (1965); K. Ueno, *et al., Analyst*, **95**, 583 (1970); A. Itoh, *et al., Analyst*, **95**, 583 (1970); M. Sugawara, *et al., Bull. Chem. Soc. Jpn.*, **50**, 3206 (1977); M. M. Ferris, *et al., Analyst*, **111**, 351 (1986).

Kalibor

Tetraphenylborate, sodium salt [CAS: 143-66-8]

Application: Lipophilic anion, counter anion for ion selective electrode

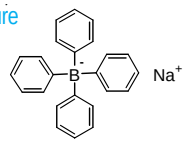
Appearance: white crystalline powder
Purity: >99.5% (Titration)
MW: 342.22, $C_{24}H_{20}BNa$

Storage Condition	Shipping Condition
ambient temperature	ambient temperature

Ordering Information

Product code	Unit
K003-12	25 g
K003-14	100 g

Chemical Structure



Product Description

Kalibor is utilized for gravimetric analyses of K^+, Rb^+, Cs^+, NH_4^+, amine compounds, alkaloids, onium compounds, and other monovalent ions, such as Ag(I), Cu(I) and Tl(I).

References

A. M. Amin, *et al., Chem. Anal.*, **43**, 13 (1954); R. F. Muraca, *et al., Chem. Anal.*, **43**, 102 (1954); H. Flaschka, *Chem. Anal.*, **44**, 60 (1955); R. E. Jensen, *et al., Anal. Chem.*, **44**, 846 (1972); N. M. Hanken, *J. Paleontology*, **53**, 738 (1979).

XV-2. Ion Detection: Metals and Anions

Murexide

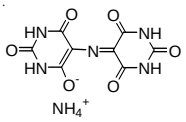
Purpuric acid, ammonium salt [CAS 3051-09-0]

Application: Ca^{2+} and rare earth metal ion detection, colorimetric

Appearance: reddish-purple powder
Sensitivity: pass test
MW: 284.19, $C_8H_8N_6O_6$

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
M011-10	5 g
M011-12	25 g

MX

Mixture of ammonium purpurate and potassium sulfate (1:250)

Application: Ca^{2+} and rare earth metal ions detection, colorimetric

Appearance: pink or reddish-purple powder
Sensitivity: pass test

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Ordering Information

Product code	Unit
M012-08	100 g

Product Description of Murexide and MX

MX is a 1:250 mixture of Murexide and potassium sulfate. Murexide is a metal indicator for Ca, Co, Cu, Ni, Th, and rare earth metals; it is also a colorimetric reagent for calcium and rare earth metals. Murexide is slightly soluble in water, alcohol, and ether. Murexide is unstable in aqueous solution. Its proton dissociation constants are reported to be pKa_1=0, pKa_2=9.2 and pKa_3=10.5. The solution is yellow in strong acidic conditions, reddish-purple in weak acidic conditions, and blue-purple in alkaline conditions. Detection conditions for calcium are pH 11.3, maximum wavelength 506 nm and detection range 0.2-1.2 ppm.

Reference

K. S. Balaji, *et al., Anal. Chem.*, **50**, 1972 (1978).

Nitro-PAPS

2-(5-Nitro-2-pyridylazo)-5-[*N*-n-propyl-*N*-(3-sulfopropyl)amino]phenol, disodium salt, dihydrate [CAS: 143205-66-7]

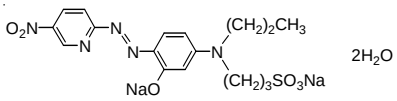
Application: Heavy metal ions detection, colorimetric

Appearance: dark green or dark greenish-brown powder
Purity: >90.0% (Absorbance)
MW: 503.45, $C_{17}H_{19}N_5Na_2O_6S$, $2H_2O$

Ordering Information	
Product code	Unit
N031-10	100 mg

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



Product Description

Nitro-PAPS is a highly sensitive colorimetric reagent for Fe(II) detection that forms a water-soluble complex at pH 3.0-8.0 (λmax=582 nm, ε=107,000). This reagent is suitable for the determination of Fe(II) in serum, and can also be used to determine micromolar levels of Cu, Zn, Ni, and Co. Cu and Zn in serum are masked by a mixture of thioglycolic acid and SDS. Zn in serum can be determined by Nitro-PAPS using CN^- as a masking reagent of Fe and Cu.

Table 15-7 Spectral Data of Nitro-PAPS-Metal Complex

Metal	λmax	Molar Absorptivity	Metal	λmax	Molar Absorptivity
Co(II)	590 nm	113,000	Ni(II)	568 nm	150,000
Cu(II)	566 nm	71,000	Zn(V)	566 nm	150,000
Fe(II)	582 nm	107,000			

References

T. Makino, *et al.*, *Clin. Chim. Acta*, **171**, 19 (1988); T. Makino, *Clin. Chim. Acta*, **197**, 209 (1991); S. Yamashita, *et al.*, *Clin. Chem.*, **38**, 1373 (1992); J. D. Artiss, *et al.*, *Am. J. Clin. Pathol.*, **108**, 269 (1997).

Nitroso-PSAP

2-Nitroso-5-[*N*-*n*-propyl-*N*-(3-sulfopropyl)amino]phenol [CAS 80459-15-0]

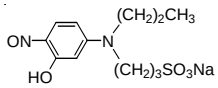
Application: Fe(II) detection, colorimetric

Appearance: yellow or yellowish-brown powder
Purity: >97.0% (Absorbance)
Molar absorptivity: >44,000 at 756 nm (Fe complex)
MW: 302.35 $C_{12}H_{18}N_2O_5S$

Ordering Information	
Product code	Unit
N010-10	100 mg
N010-12	1 g

Storage Condition: 0-5 °C
Shipping Condition: ambient temperature

Chemical Structure



Product Description

Nitroso-PSAP is a highly sensitive water-soluble colorimetric reagent for Fe(II) detection that forms a green complex with Fe(II) (λmax=756 nm, $\varepsilon=4.5\times10^4$). This reagent is suitable for the determination of blood serum levels of Fe, Co, Ni, and Cu. The detection range is from 5 ppb to 5 ppm under the same detection condition. Nitroso-PSAP is useful in flow injection analyses. The workable pH range of the nitroso compounds is 5.6-10.1. The molar absorptivities and maximum wavelengths of the Co, Cu, and Ni complexes are 4.0×10^4 (490 nm), 2.8×10^4 (429 nm), and 2.6×10^4 (394 nm), respectively.

Table 15-8 Spectral Data of Nitroso-PSAP-Metal Complex

Metal	λmax	Molar Absorptivity	Metal	λmax	Molar Absorptivity
Co(II)	490 nm	40,000	Fe(II)	756 nm	45,000
Cu(II)	429 nm	28,000	Ni(II)	394 nm	26,000

References

N. Ohno, *et al.*, *Analyst*, **112**, 1127 (1987); I. Yoshida, *et al.*, *Anal. Sci.*, **4**, 69 (1988); M. Yamada, *et al.*, *Jpn. J. Lab. Auto.*, **13**, 659 (1988).

XV-2. Ion Detection: Metals and Anions

NN

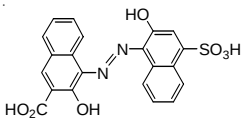
2-Hydroxy-1-(2-hydroxy-4-sulfo-1-naphthylazo)-3-naphthoic acid [CAS: 3737-95-9]

Application: Ca detection, colorimetric

Appearance: dark purple powder
MW: 438.41, $C_{21}H_{14}N_2O_7S$

Storage Condition	Shipping Condition
ambient temperature	ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
N013-08	1 g

Product Description

NN is a colorimetric and chelatometric reagent for calcium (pH 12, λmax=470 nm) and UO_2^{2+} (λmax=570 nm, ε=1.36x10^5). Although NN is soluble in water, the solution is unstable and decomposes in a few minutes, especially in the presence of oxidative ions. Its proton dissociation constants are reported to be pKa_2=9.26 and pKa_3=13.67 (μ=0.1, 24 °C). An NN solution of 0.1% w/v in 50% aqueous methanol is stable for one week if stored in a cool and dark place. Ascorbic acid is used as a stabilizer of the NN stock solution. Al, Cu, Fe(III), and Ni interfere with the determination of calcium by NN and should be masked by KCN or triethanolamine.

Reference

Patton, *et al.*, *Anal. Chem.*, **28**, 1026 (1956).

NN diluted with potassium sulfate

Mixture of NN and potassium sulfate (1:200)

Application: Ca detection, colorimetric

Appearance: purple powder
Sensitivity: pass test

Storage Condition	Shipping Condition
ambient temperature	ambient temperature

Ordering Information

Product code	Unit
N012-10	25 g

PAN

1-(2-Pyridylazo)-2-naphthol [CAS: 85-85-8]

Application: Metal indicator, heavy metal ion detection

Appearance: orange-yellow or orange-red crystalline powder
MW: 249.27, $C_{15}H_{11}N_3O$

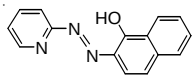
Storage Condition
ambient temperature

Shipping Condition
ambient temperature

Ordering Information

Product code	Unit
P002-10	1 g
P002-12	10 g

Chemical Structure



Product Description

PAN is a metal indicator and colorimetric reagent for heavy metal and rare earth metal ions. This reagent is slightly soluble in acidic solutions and soluble in alkaline solutions. Its proton dissociation constants are reported to be pKa_1=2.9 and pKa_2=11.6 (μ=0.1, $NaClO_4$, at room temperature). The aqueous solution is yellow at pH<12 and red at pH>12. The distribution constants, $logK_D$, of chloroform/water and carbon tetrachloride/water are reported to be 5.4 and 4.0, respectively.

Applications

Chelate titration: Bi, Cd, Ce, Cu, Ga, In, Tl, UO_2(II), Zn
Solvent extraction colorimetry: Ag, Bi, Cd, Co, Cr, Cu, Fe, Ga, Hf, Hg, In, Ir, Mn, Nb, Ni, Os, Pd, Pt, Rh, Ru, Sb, Ti, Tl, UO_2(II), VO(II), Zn, Zr, rare earth metals

Table 15-9 Characteristics of PAN Metal Complex

Metal	$LogK_{ML}$	$LogK_{ML2}$	Condition	Metal	$LogK_{ML}$	$LogK_{ML2}$	Conditions
Co(II)	>12 -	50%	dioxane	Al	12.9	-	50% ethanol
Cu(II)	15.6	8.4	50% dioxane	Ga	15.1	-	50% ethanol
Mn(II)	8.5	7.9	50% dioxane	In	13.1	-	50% ethanol
Ni	12.7	12.6	50% dioxane	Eu	12.4	11.4	μ=0.05 ClO_4^-
Zn	11.210.2	50%	dioxane				

* M=metal ion, L=PAN

Table 15-10 Photometric Detection Conditions Using PAN

Metal	Conditions	λmax (nm)	ε ($x10^{-4}$)	Extraction Solvent	Range (ppm)
Cd	pH 8.7-19	555	4.9	Chloroform	0-2.5
Hf	pH 4, 40% methanol	545	3.9	Water	0.2-3.6
Mn(II)	pH 8.8-9.6, NH_3, KCN	562	4.8	Chloroform	0.2-1.2
Ni	pH 4-10, NH_3, KCN	570	5.0	Benzene	0-1.5
Os(VIII)	pH 3.5-5.2	550	2.8	Chloroform	0-9.2
Zn	pH 8.0-9.5, Triton X100	555	5.6	Water	0.2-2
rare earth metals	pH 8-9.5	530	6-7	Chloroform	0-2

Reference

S. Shibata, *2-Pyridylazo Compounds in Analytical Chemistry, Ed. by H. A. Flaschka and A. J. Barnard, Jr., "Chelates in Analytical Chemistry", IV, Marcel Dekker, Inc., NY* (1972).

XV-2. Ion Detection: Metals and Anions

PAR

4-(2-Pyridylazo)resorcinol [CAS: 1141-59-9]

Application: Heavy metal ions detection, colorimetric

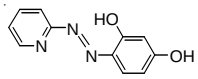
Appearance: yellowish-orange or orange-red powder
Sensitivity: pass test
MW: 215.21, $C_{11}H_9N_3O_2$

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Ordering Information

Product code	Unit
P003-10	1 g
P003-12	5 g

Chemical Structure



Product Description

PAR is a metal indicator and colorimetric reagent for heavy metal ions. The solubility of this reagent in water (5 mg per 100 ml at 10 °C) is higher than that of PAN. PAR is slightly soluble in alcohol. The aqueous solution is red at pH 5.5 or less, orange at pH 6-12.5, and red at pH 13 or higher. Its proton dissociation constants are reported to be $pKa_1(NH^+)$=3.1, pKa_2(*p*-OH)=5.6 and pKa_3(*o*-OH)=11.9 (m=0.2). PAR forms complexes with various metal ions that can be extracted with chloroform.

Application

Chelate titration: Al, Bi, Cd, Cu, Ga, Hg, In, Mn, Ni, Pb, Zn
Precipitation titration: MoO_4(II), WO_4(II)
Colorimetry: Al, Au, Bi, Co, Cr, Cu, Fe, Hf, Ga, In, Nb, Ni, Pb, Pd, Sb, Sn, Ti, Tl, U, V, Zn

Table 15-11 Photometric Detection Conditions Using PAR

Metal	Conditions	Complex	λmax (nm)	ε (x10^{-4})	Solvent
Bi	pH 3.0-6.0, ClO_4^-, antipyrine	$M(HL)_2X_2ClO_4$	520	2.9	chloroform-isobutanol (1:1)
Co(II)	pH 4-10, TPAC	ML_2X	518	6.0	chloroform
V(V)	pH 4.6-5.1, Crystal violet	MLX	585	11.0	benzene-MIBK (3:2) + 15% ethanol
Zn	pH 6-7, Diphenylguanidine	ML_2X_3	515	6.7	chloroform

M=metal ion, L=PAR, X=antipyrine, TPAC, Crystalviolet or Diphenylguanidine

PC

3,3'-Bis[*N*,*N*-bis(carboxymethyl)aminomethyl]-*o*-cresolphthalein [CAS: 2411-89-4]

Application: Alkaline earth metal detection, colorimetric

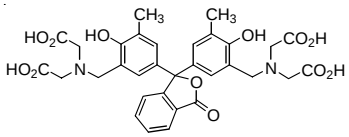
Appearance: white or slightly yellowish-pink powder
MW: 636.60, $C_{32}H_{32}N_2O_{12}$

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Ordering Information

Product code	Unit
P004-10	1 g
P004-12	5 g

Chemical Structure



Product Description

PC is a colorimetric reagent and metal indicator for alkaline earth metal ions. This reagent is slightly soluble in water and readily soluble in aqueous ammonia or organic solvents. The aqueous solution is colorless at pH 11, but turns a strong pink in the presence of alkaline earth metals. PC is widely utilized for the determination of serum calcium by an autoanalyzer.

Applications

Chelate titration: Ca, Ba, Mg, Sr, CN^-, SO_4^{2-}
Colorimetry: Ca, Ba, Hg(II), La, Mg, Sr

Conditions

Ba: pH 11.3, 575 nm, detection range 0-5 ppm Ca: pH 10.5, 575 nm, detection range 0-1 ppm
Hg: pH 10, 585 nm, detection range 0.1-4 ppm La: cetylpyridiniumbromate, 617 nm, detection range 0-2 ppm
Mg: pH 10, 570 nm, detection range 5-30 ppm Sr: pH 11.2, 575 nm, detection range 0-3 ppm

Reference

K. L. Cheng, *et al., Handbook of Organic Analytical Reagents, CRC Press, Inc., Florida* (1982).

o-Phenanthroline

1,10-Phenanthroline, monohydrate [CAS: 5144-89-8]

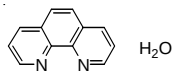
Application: Ag and Fe detection, colorimetric

Appearance: white crystalline powder
Purity: 99.0-101.0% (Titration)
Sensitivity: pass test
MW: 198.22, $C_{12}H_8N_2$, H_2O

Ordering Information	
Product code	Unit
P007-10	5 g
P007-12	25 g

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



Product Description

o-Phenanthroline is a colorimetric reagent for iron detection. It is also used as a reagent for the solvent extraction of anions. This reagent is slightly soluble in water (3.3 g/l at room temperature) and benzene (14 g/l at room temperature), and fairly soluble in alcohol (540 g/l), acetone, and diluted mineral acids. Its proton dissociation constants are reported to be pKa_1=0.70 and pKa_2=4.98 (μ=0.1, 25 °C). *o*-Phenanthroline forms a red chelate with Fe(II) [$\log\beta_3$=21.1(μ=0.1, 20 °C), λmax=510 nm, ε=1.1x10^5]. This complex is not easily extracted by organic solvents. The redox potential of FeL_3^{3+}-FeL_3^{2+} (L: *o*-phenanthroline) in 1 M H_2SO_4 is reported to be 1.06 V, and the color of the solution changes from red to pale blue. This reagent also forms colored complexes with Cu, Ni, and Co, and colorless complexes with Cd and Zn. Therefore, *o*-Phenanthroline can be used as a colorimetric reagent for Cu, Ni, and Co, and as a masking reagent of Cd and Zn. FeL_3^{2+} is also utilized for precipitation detection and ion-pair extraction.

Applications

Masking: Cd, Zn
Reduction and oxidation indicator: Ce
Colorimetry: Ag, Fe(II) :510 nm, detection limit 0.8 ppm
Fluorometry: Ru(II), Eu
Extraction colorimetry: halogen, ClO_4^-, $PtCl_6^{2-}$, ReO_4^-, $HCrO_4^-$, SCN^-, $AuCl_4$, $Ag(CN)_2^-$, $Sn(C_2O_4)_3^{2-}$, phosphomolybdate and organic compounds (Trichloroacetic acid, dehydroacetic acid, syclamic acid, saccharin, salicylic acid, alkylbenzenesulfonic acid, maleic acid, pentachlorophenol, tetraphenylborate, and chloranilic acid)

References

W. W. Brandt, *et al.*, *Anal. Chem.*, **21**, 1313 (1949); C. V. Banks, J. W. O'Langlin, *Anal. Chem.*, **29**, 1412 (1957); R. Pribil, *et al.*, *Chemist-Analyst.*, **48**, 87 (1959); R. Pribil, *et al.*, *Collection*, **24**, 3103 (1959). H. Veening, *et al.*, *Anal. Chem.*, **32**, 1426 (1960); F. Vydra, *et al.*, *Chemist-Analyst*, **52**, 88 (1963); A. N. Sevchenko, *et al.*, *Izv. Akad. Nauk. SSSR, Ser. Fiz.*, **27**, 710 (1963); L. I. Kononenko, *et al.*, *Zavod. Lab.*, **30**, 779 (1964); B. W. Bailey, *et al.*, *Talanta*, **13**, 1661 (1966); R. M. Dagnall, *et al.*, *Talanta*, **11**, 1533 (1964); R. Pribil, *Talanta*, **13**, 1223 (1966); K. L. Cheng, K. Ueno, T. Imamura, *Handbook of Organic Analytical Reagents*, *CRC Press Inc.*, *Florida* (1982).

PR

Pyrogallol Red [CAS: 32638-88-3]

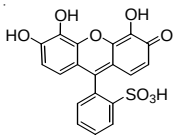
Application: Heavy metal ions and rare earth metal ions detection, colorimetric

Appearance: dark reddish-brown or dark purple powder
Sensitivity: pass test
MW: 400.36, $C_{19}H_{12}O_8S$

Ordering Information	
Product code	Unit
P012-10	1 g

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



XV-2. Ion Detection: Metals and Anions

Product Description

PR is a colorimetric reagent and indicator for heavy metal ions and rare earth metals. This reagent is hardly soluble in water and alcohol, and insoluble in organic solvents. The color of PR in aqueous solution is orange-red in weak acidic pH, yellow in neutral pH, and violet in alkaline pH. The aqueous PR solution turns red in the presence of Bi or Pb at acidic pH, and blue-violet in the presence of Co, Ni, and rare earth metal ions at neutral or alkaline pH. Its proton dissociation constants are reported to be pKa_1=2.56, pKa_2=6.28, pKa_3=9.75 and pKa_4=11.94.

Applications

Chelate titration: Bi, Co, Ni, Pb, rare earth metals
Precipitation titration: Br^-, Cl^-, I^-, SCN^-
Colorimetry: Ag, Al, Cu, Fe, Ge, In, Mo, Sb, Ti, U, V, W, rare earth metals

References

M. Malat, *et al.*, *Collection*, **24**, 2815 (1959); R. M.Dagnall, *et al.*, *Talanta*, **11**, 1533 (1964); K. L. Cheng, *et al.*, *Handbook of Organic Analytical Reagents*, *CRC Press, Inc., Florida* (1982).

SATP

Salicylideneamino-2-thiophenol [CAS: 3449-05-6]

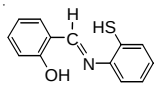
Application: Heavy metal detection with solvent extraction, colorimetric

Appearance: white crystalline powder
MW: 229.30, $C_{13}H_{11}NOS$

Ordering Information	
Product code	Unit
S003-10	1 g
S003-12	5 g

Storage Condition	Shipping Condition
ambient temperature	ambient temperature

Chemical Structure



Product Description

SATP is a colorimetric solvent extraction reagent for Cu, In, Ni, and Sn. It is insoluble in water, but soluble in organic solvents. SATP forms yellow or brown complexes with various metal ions. The Sn(II) complex can be extracted with benzene (maximum wavelength: 415 nm, molar absorptivity: 1.61×10^5), xylene and toluene.

Sodium bicinchoninate

4,4'-Dicarboxy-2,2'-biquinoline, disodium salt [CAS: 979-88-4]

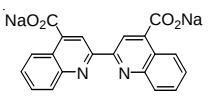
Application: Cu(I) determination, colorimetric

Appearance: slightly yellow or yellow powder
Molar absorptivity: >15,500 at 332 nm
MW: 388.28, $C_{20}H_{10}N_2Na_2O_4$

Ordering Information	
Product code	Unit
B037-10	5 g

Storage Condition	Shipping Condition
ambient temperature	ambient temperature

Chemical Structure



Product Description

Sodium bicinchoninate is a $Cu(I)^-$ specific colorimetric reagent that is more sensitive than neocuproine. Its absorption maximum is at 562 nm (molar absorptivity: 7,900). In alkaline solution, Cu(II) is reduced by proteins to Cu(I), which forms a purple complex with Sodium bicinchoninate. The protein detection range of this assay is 1-2,000 mg per ml.

References

O. H. Lowry, *et al.*, *J. Biol. Chem.*, **193**, 265 (1951); R. F. McFeeters, *Anal. Biochem.*, **103**, 302 (1980); P. K. Smith, *et al.*, *Anal. Biochem.*, **150**, 76 (1985); M. Belew, *et al.*, *Anal. Biochem.*, **151**, 438 (1985); M. G. Redinbaugh, *et al.*, *Anal. Biochem.*, **153**, 267 (1986).

TD19C6

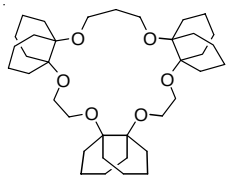
2,6,13,16,23,26-Hexaoxaheptacyclo[25.4.4.4^{7,12}.4^{17,22}.0^{1,17}.0^{7,12}.0^{17,22}]tritetracontane

Application: Ammonium selective electrode
Appearance: white powder
Purity: pass test (TLC)
MW: 602.88, $C_{37}H_{62}O_6$

Ordering Information	
Product code	Unit
T402-10	10 mg

Storage Condition	Shipping Condition
ambient temperature	ambient temperature

Chemical Structure



Product Description

TD19C6 is an ammonium ion selective ionophore. It is difficult to separate ammonium ion and potassium ion because they are close in ion size. The selectivity of TD19C6 for ammonium ion is about 10 times that of potassium ion, which is nearly the same selectivity of nonactin.

TFPB

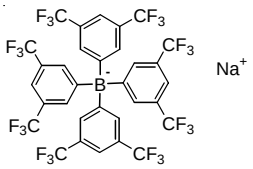
Tetrakis[3,5-bis(trifluoromethyl)phenyl]borate, sodium salt, dihydrate [CAS: 79060-88-1]

Application: Lipophilic anion, phase transfer reagent
Appearance: white crystalline powder
Purity: >99.0% (Titration with zephiramine)
MW: 886.20, $C_{32}H_{12}BF_{24}Na$

Ordering Information	
Product code	Unit
T037-10	100 mg

Storage Condition	Shipping Condition
ambient temperature	ambient temperature

Chemical Structure



Product Description

Tetraphenylborate is unstable in acidic conditions because protons attack the *ipso*-carbon of the phenyl group and benzene is released. However, the electron density of the *ipso*-carbon of TFPB is low due to a trifluoromethyl group. Thus TFPB is stable in acidic conditions (such as 50 mM sulfuric acid solution). TFPB is used in Friedel-Craft alkylation or diazo coupling reactions as a phase transfer reagent. It is also used for ion selective electrodes. TFPB is known as Kobayashi's reagent.

References

H. Kobayashi, *et al., Chem. Lett.*, **1981**, 579; H. Kobayashi, *et al., Chem. Lett.*, **1982**, 1185; Y. Takahashi, *et al., Chem. Lett.*, **1982**, 1187; H. Iwamoto, *et al., Bull. Chem. Soc. Jpn.*, **56**, 796 (1983); H. Iwamoto, *et al., Fluorine Chem.*, **24**, 535 (1984); H. Nishida, *et al., Bull. Chem. Soc. Jpn.*, **57**, 2600 (1984); Y. Shiraki, *et al., Bull. Chem. Soc. Jpn.*, **58**, 3041 (1985); N. Ichibashi, *et al., Anal. Sci.*, **4**, 527 (1988); G. H. Zhang, *et al., Anal. Chem.*, **62**, 1644 (1990); T. Nagamura, *et al., J. Chem. Soc. Chem. Commun.*, **1991**, 72; M. Kira, *et al., J. Am. Chem. Soc.*, **114**, 6697 (1992).

XV-2. Ion Detection: Metals and Anions

Tiron

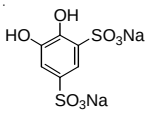
1,2-Dihydroxy-3,5-benzenedisulfonic acid, disodium salt, monohydrate [CAS: 149-45-1]

Application: Fe, Al, Ti detection, colorimetric

Appearance: white powder or crystals
Sensitivity: pass test
MW: 332.22, $C_6H_4Na_2O_8S_2$, H_2O

Storage Condition	Shipping Condition
ambient temperature	ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
T021-10	5 g

Product Description

Tiron is used as a metal indictor of Fe, and as a colorimetric reagent of Fe, Al, Ti, and other metal ions. Tiron is readily soluble in water as a colorless solution. The pKa_2 is reported to be 12.6 (μ=0.1, 20 °C). Tiron forms colored chelates with Fe, Ti, and other metal ions. The color of the Fe complex is blue at pH 1-4 (1:1, logK=20.4), purple at pH 5-7 (1:2, logK=15.1), and red at pH 7 or over (1:3, logK=10.8).

Applications

Chelate titration: Fe
Colorimetry: Al, B, Co, Cu, Fe, Ga, Mo, Nb, Os, Sr, Ti, UO_2(II), V
Fluorometry: rare earth metals
Masking reagent: Al, Cr, Fe, Ti

TMPyP

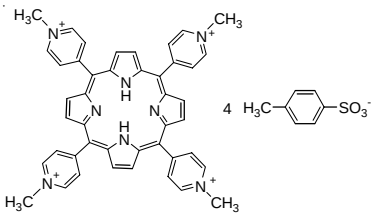
5,10,15,20-Tetrakis(*N*-methylpyridinium-4-yl)-*21H, 23H*-porphine, tetrakis(*p*-toluenesulfonate) [CAS: 36951-72-1]

Application: Cu detection, colorimetric

Appearance: purple powder
Molar absorptivity: >140,000 at 423 nm (Cu complex)
>280,000 at 445 nm (Acidity)
MW: 1363.61, $C_{72}H_{66}N_8O_{12}S_4$

Storage Condition	Shipping Condition
ambient temperature	ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
T001-12	1 g

Product Description

TMPyP is a highly sensitive colorimetric reagent for Cu. Its proton dissociation constants are reported to be pKa_1=0.8 and pKa_2=2.06. The maximum wavelength of the Soret band is 422 nm (molar absorptivity: 149,000) at pH 4-7. In 1 M HCl solution, the maximum wavelength of the Soret band is 446 nm (molar absorptivity: 193,000).

TOPO

Tri-*n*-octylphosphine oxide [CAS: 78-50-2]

Application: Solvent extraction

Appearance: white or pale yellow crystalline powder
Purity: >98.0% (GC)
MW: 386.63, $C_{24}H_{51}OP$

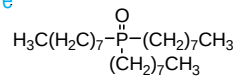
Ordering Information

Product code	Unit
T024-10	5 g
T024-12	25 g

Storage Condition
ambient temperature, protect from metals

Shipping Condition
ambient temperature

Chemical Structure



Product Description
TOPO dissolves in cyclohexane. TOPO forms adducts with metal chelates to enhance solvent extraction efficiency (Synergistic effect). It can be used for rare earth metals, actinoids, and other metal ions indicated below.

Applications
Solvent extraction: Al, Au, Co, Cr, Fe, Hf, Re, Ti, UO_2^{2+}, V, Zr, rare earth metals, actinoids

References
R. Y. Xie, *et al.*, *Analyst*, **112**, 61 (1980); S. Tsurubou, *et al.*, *Anal. Chim. Acta*, **248**, 501 (1991).

TPPS

5,10,15,20-Tetraphenyl-*21H, 23H*-porphinetetrasulfonic acid, disulfuric acid, tetrahydrate [CAS: 35218-75-8]

Application: Heavy metal ion detection, colorimetric

Appearance: deep green or greenish-blue powder
Molar absorptivity: >510,000 at 413 nm, pH 6.5
MW: 1203.21, $C_{44}H_{30}N_4O_{12}S_4$, $2H_2SO_4$, $4H_2O$

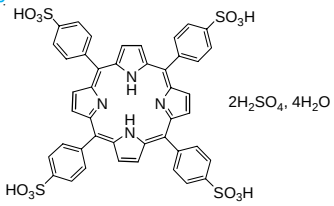
Ordering Information

Product code	Unit
T003-10	100 mg

Storage Condition
ambient temperature

Shipping Condition
ambient temperature

Chemical Structure



Product Description
TPPS is a water-soluble porphyrin that is reddish-purple in aqueous solution at pH 6.5 (maximum wavelength: 413 nm, molar absorptivity: 5.1×10^5) and green at pH 4 (maximum wavelength: 434 nm, molar absorptivity: 5.0×10^5). Its proton dissociation constants are reported to be pKa_1=4.86 and pKa_2=4.96. TPPS selectively forms a complex with Cu^{2+} in acidic conditions, and with Cd, Cu, Fe, Pb, and Pd in alkaline conditions.

Table 15-12 Colorimetric Detection Conditions Using TPPS

Metal	Conditions	λmax (nm)	ε ($\times 10^{-5}$)	Range (ppb)	Interference
Cu(II)	pH 4	434	4.8	6-60	Zn
Cd(II)	pH 12, bipyridine	432	4.5	(0)-100	-
Fe(II)	pH 3.9-4.2	395	1.4	20-180	Co, Cu, Mn, Pd, Sn, Zn
Pb(II)	pH 10.2, KCN	464	2.8	50-500	Cr, In, Mn, Sn
Pd(II)	pH 3	413	2.2	(0)-250	Cu, Hg

XV-2. Ion Detection: Metasl and Anions

XB-I

3-[3-(2,4-Dimethylphenylcarbamoyl)-2-hydroxynaphthalen-1-yl-azo]-4-hydroxybenzenesulfonic acid, sodium salt [CAS: 14936-97-1]

Application: Mg detection, colorimetric

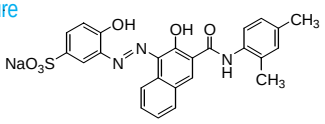
Appearance: dark red or dark reddish-brown powder
MW: 513.50, $C_{25}H_{20}N_3NaO_6S$

Storage Condition	Shipping Condition
ambient temperature	ambient temperature

Ordering Information

Product code	Unit
X001-10	1 g
X001-12	5 g

Chemical Structure



Product Description

XB-I is a colorimetric reagent for Mg detection. It is slightly soluble in water and alcohol, and readily soluble in aqueous alkaline solution. The aqueous solution of XB-1 is red, and turns reddish-violet in the presence of Mg at pH 9 (maximum wavelength: 510 nm, molar absorptivity: 49,000, detection range 0.02-0.4 ppm).

Reference

C. K. Mann, *et al., Anal. Chem.*, **28**, 202 (1956).

XO

3,3'-Bis[*N,N*-bis(carboxymethyl)aminomethyl]-*o*-cresolsulfonphthalein, disodium salt [CAS: 1611-35-4]

Application: Metal indicator

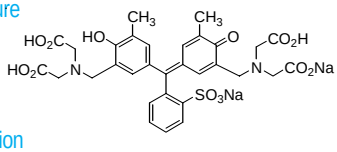
Appearance: orange-red or reddish-purple powder
Sensitivity: pass test
MW: 716.62, $C_{31}H_{30}N_2Na_2O_{13}S$

Storage Condition	Shipping Condition
ambient temperature	ambient temperature

Ordering Information

Product code	Unit
X003-10	1 g
X003-12	5 g

Chemical Structure



Product Description

XO is a colorimetric reagent and metal indicator of various metal ions. It is readily soluble in water. Its proton dissociation constants are reported to be -1.74, -1.09, 0.76, 1.15, 2.58, 3.23, 6.40, 10.5, and 12.6 (ion strength: 0.2). The color of the aqueous XO solution is yellow at pH < 6.3 and pink at pH > 6.5, and turns reddish-violet in the presence of metal ions. XO forms complexes with various metal ions in acidic conditions. Therefore, XO can be used to determine Ca, Cd, Mg, Pb, and Zn in the presence of Zephiramine in acidic conditions. XO is also a very versatile indicator for EDTA titration in acidic solutions.

Table 15-13 Colorimetric Determination of Metal Ions Using XO

Metal	Condition	λmax (nm)	Range (ppm)	Interference
Al	pH 3.4, 100 °C	536	0.2-1	Fe(III), Th
Bi	80-120 mM HNO_3, ascorbic acid	530	0.4-3.2	—
Fe(III)	60-100 mM $HClO_4$	535	0.12-1.8	Zr
Np(IV)	4 M HNO_3, trimethylbenzene	535	(0)-5.5	Cr(VI), Th
Pu(IV)	0.1 M HNO_3	560	0.5-4.0	>6000x UO_2^{2+}
Th	pH 3, diphenylguanidine, butanol	578	0.14-0.83	U, F^-
UO_2^{2+}	pH 3.5-3.7, ascorbic acid, 100 °C	568	0.8-4.0	Cu, Fe, Pd
Zr	acetic acid, TOA	550	0.03-2	Mo, Pd, Ti: + error; Cd, Cu, Hg: - error

Reference

B. Budesinsky, *Xylenol orange and methylthymol blue as chromogenic reagents, chelates in analytical chemistry, Ed. by H. A. Flaschka, and A. J. Barnard, Jr., Marcel Dekker, Inc., New York.*, **1**, 15 (1967).

Zephiramine

Benzyldimethyltradecylammonium chloride, dihydrate [CAS: 147228-81-7]

Application: K^+ detection, precipitation

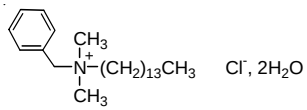
Appearance: white crystalline powder
Purity: >98.0% (Titration)
MW: 404.07, $C_{23}H_{42}ClN$, $2H_2O$

Storage Condition	Shipping Condition
ambient temperature	ambient temperature

Ordering Information

Product code	Unit
Z001-10	25 g

Chemical Structure



Product Description

Zephiramine is a cationic surfactant used as a sensitizer in photometric determination of metal ions. This reagent is highly soluble in water, acetone, and alcohol, and slightly soluble in benzene. The CMC value of Zephiramine is 0.37 mM. The distribution constants (Kd) of organic solvent/water of Zephiramine are 0.09 (benzene, toluene), 2.4 (dichloroethane), 11 (chloroform), 0.1 (tetrachlorocarbon) and 0.53 (MIBK). Zephiramine is also used for Kalibor titration, colloid titration, and as a surfactant for liquid scintillators.

Zincon

1-(2-Hydroxycarbonyl-phenyl)-5-(2-Hydroxy-5-sulfophenyl)-3-phenylformazan, sodium salt [CAS: 62625-22-3]

Application: Cu and Zn detection, colorimetric

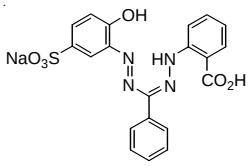
Appearance: dark reddish-purple powder
Sensitivity: pass test
MW: 462.41, $C_{20}H_{15}N_4NaO_6S$

Storage Condition	Shipping Condition
ambient temperature	ambient temperature

Ordering Information

Product code	Unit
Z002-10	1 g
Z002-12	5 g

Chemical Structure



Product Description

Zincon is a colorimetric reagent for Zn and Cu detection, and a metal indicator in EDTA titration. This reagent is insoluble in organic solvents, but slightly soluble in water and alcohol. Its proton dissociation constants are reported to be pKa_1=7.9-8.3, pKa_2=7.9-8.3 and pKa_3=13-14. The aqueous solution of Zincon is yellow or orange-yellow in weak alkaline conditions, and turns blue in the presence of Cu (maximum wavelength: 600 nm, molar absorptivity: 1.9×10^4) and Zn (pH 8.5-9.5, maximum wavelength: 620 nm, molar absorptivity: 23,000). Dr. Koupparis and others used Zincon for the automated flow injection spectrometric determination of 1-10 mg per ml levels of Zn at 80 samples per hour. The detection limit was reported to be 0.05 µg per ml.

Applications

Chelate titration: Ca, Cd, Hg, Pb, Zn
Colorimetry: Cu, Zn

References

D. W. Margerum, *et al.*, *Anal.Chem*, **32**, 1157 (1960); K.Yoshimura, *et al.*, *Talanta*, **25**, 579 (1978); G. Ackermann, *et al.*, *Talanta*, **26**, 693 (1979); K. Yoshimura, *et al.*, *Talanta*, **32**, 345 (1985); M. A. Koupparis, *et al.*, *Analyst*, **111**, 1311 (1986); C. M. Corns, *et al.*, *Ann. Clin. Biochem.*, **24**, 591 (1987); E. Hilario, *et al.*, *J. Biochem. Biophys. Methods*, **21**, 197 (1990); R. Liu, *et al.*, *Analyst*, **120**, 569 (1995); P. Richter, *et al.*, *Analyst*, **122**, 1045 (1997); M. P. Cuajungco, *et al.*, *Brain Res.*, **799**, 97 (1998).

XVI. Metal Chelates

Introduction

Organic compounds that coordinate metal ions into circular structures (chelate circles) are called chelating reagents. Most chelating reagents include oxygen, nitrogen, or sulfur atoms in their molecules. Chelate structures with five or six member rings form the most stable chelate circle. In chelating reactions of typical chelating reagents, such as ethylenediamine, acetylacetone, and oxine, several molecules are coordinated with one metal ion. Ethylenediamine tetraacetic acid (EDTA), which has many coordinated atoms, forms a very stable chelate between one molecule of EDTA and metal ion. Chelating reagents are utilized for chelate titration, isolation, and separation of metal ions. They are also used to mask certain ions, solubilize metals in organic solvents, and for gas chromatography of metal ions. Metal indicators are chelating reagents that can be used to colorimetrically or fluorometrically determine metal ion concentration in solutions. Acetylacetone (AA) and its analogs are utilized for solvent extractions of metal ions from aqueous solutions. These metal complexes are used as catalysts of polymerization, dryness, and combustion. Chelating reagents, including EDTA, EDTA analogs, metal salts, and others, are available in bulk quantities from Dojindo. The following table indicates the stability constants of chelators with various metal ions and chelate reagents.

Table 16-1 Stability Constants

Metal	EDTA	NTA	CyDTA	DTPA	EDTA-OH	GEDTA	TTHA*	DHEG	IDA	DTPA-OH	NTP	Me-EDTA	HIDA	EDDP	EDTPO	NTPO	BAPTA
Ag(I)	7.32	5.16	8.15	8.70	6.71	6.88	8.67 (13.89)	-	-	-	-	-	-	-	-	-	-
Al(III)	16.13	9.50	18.63	18.40	12.43	13.90	19.7 (28.90)	-	8.16	14.40	-	-	-	-	-	-	-
Am(III)	18.16	-	18.79	22.92	-	-	-	-	6.93	-	-	-	9.75	-	22.47	-	-
Ba(II)	7.76	4.83	8.64	8.63	5.54	8.41	8.22 (11.63)	-	1.67	4.91	-	8.10	3.42	-	11.14	-	5.77
Be(II)	9.27	7.11	10.81	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bi(III)	27.90	-	31.20	29.70	21.80	23.80	-	-	-	-	-	-	-	-	-	-	-
Bk(III)	-	-	19.60	22.79	-	-	-	-	-	-	-	-	-	-	-	-	-
Ca(II)	10.96	6.41	12.50	10.74	8.14	11.00	10.06 (14.16)	-	2.59	6.69	-	10.40	5.30	1.00	6.93	6.68	6.97
Cd(II)	16.46	9.54	19.23	19.31	13.60	16.70	18.65 (26.85)	6.30	5.73	12.10	3.40	16.00	7.41	5.60	16.53	-	12.20
Ce(III)	15.98	10.83	16.76	20.50	14.11	15.70	19.20	7.50	6.18	-	-	16.79	8.50	-	21.10	-	-
Ce(IV)	24.20	10.97	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cf(III)	19.09	-	19.42	22.57	-	-	-	-	-	-	-	-	9.61	-	-	-	-
Cm(III)	18.45	-	18.81	22.99	-	-	-	-	-	-	-	-	9.27	-	21.89	-	-
Co(II)	16.31	10.38	18.92	18.40	14.40	12.50	17.1 (28.80)	6.10	6.95	13.92	4.80	14.40	9.00	7.30	15.49	-	8.70
Co(III)	40.60	-	-	-	-	-	-	-	29.60	-	-	-	-	-	-	-	-
Cr(II)	13.61	-	-	-	-	-	-	-	-	-	-	-	7.73	-	-	-	-
Cr(III)	23.40	>10	-	-	-	2.54	-	-	-	-	-	-	-	-	-	-	-
Cs(I)	0.15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cu(II)	18.80	12.96	21.30	21.53	17.35	17.80	19.2 (32.60)	8.60	10.63	17.21	9.10	17.70	13.38	15.10	18.95	-	11.70
Dy(III)	18.30	11.74	19.69	22.82	15.30	17.42	23.29	7.60	6.88	-	-	19.09	8.88	-	-	-	-
Er(III)	18.38	12.03	20.20	22.74	15.42	17.40	13.19 (26.92)	7.70	7.09	-	-	19.61	9.24	-	-	-	-
Eu(III)	17.35	11.52	18.77	22.39	15.35	17.10	23.85	8.00	6.73	-	-	18.26	8.99	-	-	-	-
Fe(II)	14.33	8.84	16.27	16.55	12.20	11.92	17.10	4.31	5.80	12.90	-	14.30	6.78	6.30	-	-	-
Fe(III)	25.10	15.87	28.05	28.60	19.80	20.50	26.8 (40.50)	-	10.42	-	-	-	11.64	13.10	19.60	-	-
Fm(III)	-	-	-	22.70	-	-	-	-	-	-	-	-	-	-	-	-	-
Ga(III)	20.27	13.60	22.91	23.00	16.90	-	-	-	-	-	-	-	9.01	-	-	-	-
Gd(III)	17.00	11.54	18.80	22.46	15.22	16.94	23.83	7.70	6.68	-	-	18.21	-	-	21.80	-	-
Hf(IV)	29.50	20.34	-	35.40	-	-	19.08	-	-	-	-	-	14.63	-	-	-	-
Hg(II)	21.80	14.60	24.30	27.00	20.10	23.12	26.8 (39.10)	14.17	11.76	18.40	-	21.70	5.48	-	-	-	-
Ho(III)	18.05	11.90	19.89	22.78	15.32	17.38	23.59	7.60	6.97	-	-	19.30	9.14	-	21.85	-	-
In(III)	24.95	16.90	28.74	29.00	17.16	-	-	-	9.54	-	-	-	11.00	-	-	-	-
La(III)	15.50	10.36	16.75	19.48	13.46	15.79	22.22 (25.62)	7.30	5.88	-	-	16.42	8.00	5.80	21.15	-	-
Li(I)	2.79	2.51	4.13	-	-	1.17	-	-	-	-	-	-	-	-	-	-	-
Lu(III)	19.65	12.49	20.91	22.44	15.88	17.81	-	7.50	7.61	-	-	20.56	9.50	-	-	-	-
Mg(II)	8.69	5.46	10.32	9.30	7.00	5.21	8.10 (14.38)	1.15	2.94	5.30	<1	8.80	3.50	1.60	5.69	6.49	1.77
Mn(II)	14.04	7.44	16.78	15.60	10.70	12.30	14.68 (21.19)	3.90	-	9.06	-	14.50	6.40	3.40	12.70	-	8.73

* The data in parentheses are the stability constants of the 1:2 (metal:chelate) complexes

XVI. Metal Chelates

Table 16-1 Stability Constants (continued)

Metal	EDTA	NTA	CyDTA	DTPA	EDTA-OH	GEDTA	TTHA*	DHEG	IDA	DTPA-OH	NTP	Me-EDTA	HIDA	EDDP	EDTPO	NTPO	BAPTA
Mn(III)	24.80	-	28.90	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mo(V)	6.36	-	17.68	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mo(VI)	19.50	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Na(I)	1.66	2.15	2.70	-	-	1.38	-	-	-	-	-	-	-	-	-	-	-
Nb(V)	40.78	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Nd(III)	16.61	11.26	17.69	21.60	14.86	16.28	22.82 (26.75)	7.60	6.50	-	-	17.54	8.80	-	21.47	-	-
Ni(II)	18.62	11.54	19.40	20.32	17.00	13.60	18.1 (32.40)	7.70	8.90	16.63	5.80	14.20	10.20	9.30	15.30	-	-
NpO_2(II)	9.70	-	-	-	-	-	-	-	6.27	-	-	-	6.08	-	-	-	-
Pa(V)	8.19-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pb(II)	18.04	11.39	19.68	18.80	15.50	14.71	17.1 (28.10)	7.50	7.45	14.40	-	17.30	9.41	-	-	-	11.30
Pd(II)	26.40	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pm(III)	16.75	-	18.71	-	-	-	-	-	-	-	-	-	8.97	-	-	-	-
Pr(III)	16.40	11.07	17.23	21.07	14.61	16.50	23.45	7.70	6.44	-	-	17.17	8.64	-	-	-	-
Pu(III)	18.12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pu(IV)	17.66	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pu(VI)	16.39	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sb(III)	24.80	-	-	-	20.20	-	-	-	-	-	-	-	-	-	-	-	-
Sc(III)	23.10	12.70	25.40	-	-	25.40	-	8.00	-	-	-	-	-	-	-	-	-
Sm(III)	16.70	11.53	18.63	22.34	15.28	23.85	23.81	7.80	6.64	-	-	17.97	9.10	-	22.39	-	-
Sn(II)	18.30	-	-	-	-	23.85	-	-	-	15.20	-	15.10	-	-	-	-	-
Sr(II)	8.63	4.98	10.54	9.68	6.92	8.50	9.26 (12.70)	-	2.23	5.33	-	10.70	3.77	-	5.41	-	5.13
Tb(III)	17.81	11.59	19.30	22.71	15.32	17.27	23.61	8.00	6.78	-	-	18.64	9.08	-	-	-	-
Th(IV)	23.20	12.40	29.25	28.78	18.50	-	31.90	7.80	9.32	-	-	-	10.70	-	-	-	-
Ti(III)	17.30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TiO(II)	17.30	12.30	19.90	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tl(I)	6.53	4.75	5.33	5.97	5.45	-	-	-	1.32	4.40	-	-	-	-	-	-	-
Tl(III)	22.50	18.00	38.30	48.00	-	-	-	-	-	-	-	-	-	-	-	-	-
Tm(III)	18.62	12.20	20.46	22.72	15.59	17.48	-	-	7.22	-	-	20.08	9.35	-	21.41	-	-
U(IV)	25.80	-	26.90	-	-	-	-	-	-	-	-	-	-	-	-	-	-
UO_2(II)	10.40	9.56	-	-	-	-	-	-	8.93	-	-	-	8.32	-	-	-	-
V(II)	12.70	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VO(II)	18.77	-	19.40	-	-	-	-	-	9.01	-	-	-	-	-	-	-	-
V(V)	18.05	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y(III)	18.09	11.48	19.41	22.05	14.65	16.82	-	7.20	6.78	-	-	18.78	9.22	-	15.06	-	-
Yb(III)	18.88	12.40	20.80	22.62	15.88	17.78	23.58	7.70	7.42	-	-	20.25	9.38	-	-	-	-
Zn(II)	16.50	10.66	18.67	18.75	14.50	14.50	16.65 (28.70)	6.50	7.27	13.70	5.30	16.20	8.33	7.60	17.05	-	9.38
Zr(IV)	29.90	20.80	20.74	36.90	-	-	19.74	-	-	-	-	-	-	-	-	-	-

* The data in parentheses are the stability constants of the 1:2 (metal:chelate) complexes

Table 16-2 Acid Dissociation Constants

pKa	EDTA	NTA	CyDTA	DTPA	EDTA-OH	GEDTA	TTHA	DHEG	IDA	DTPA-OH	NTP	Me-EDTA	HIDA	EDDP	EDTPO	NTPO	BAPTA
a_1	1.99	1.89	2.43	2.14	2.51	2.00	2.42	8.14	2.58	1.60	3.62	2.60	2.25	6.87	1.46	0.30	-
a_2	2.67	2.49	3.52	2.38	5.31	2.68	2.95	-	9.33	2.60	4.24	3.03	8.75	9.60	2.72	1.50	-
a_3	6.16	9.73	6.12	4.26	9.86	8.85	4.16	-	-	6.96	9.30	6.20	-	-	5.05	4.64	5.47
a_4	10.26	-	11.70	8.60	-	1.38	6.16	-	-	9.49	-	10.84	-	-	6.18	5.86	6.36
a_5	-	-	-	10.53	-	9.46	9.40	-	-	-	-	-	-	-	6.63	7.30	-
a_6	-	-	-	-	-	-	10.19	-	-	-	-	-	-	-	7.43	12.10	-
a_7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	9.22	-	-
a_8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	10.60	-	-

XVI. Metal Chelates

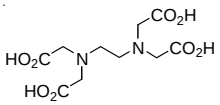
4H(EDTA·free acid)

Ethylenediamine-*N*,*N*,*N*',*N*'-tetraacetic acid [CAS: 60-00-4]

Appearance: white powder
Purity: >99.0% (Titration)
MW: 292.24, $C_{10}H_{16}N_2O_8$

Storage Condition	Shipping Condition
ambient temperature	ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
H001-10	500 g

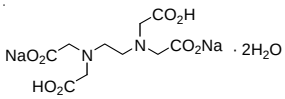
2NA(EDTA·2Na)

Ethylenediamine-*N*,*N*,*N*',*N*'-tetraacetic acid, disodium salt, dihydrate [CAS: 6381-92-6]

Appearance: white powder
Purity: >99.5% (as dihydrate,Titration)
MW: 372.24, $C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$

Storage Condition	Shipping Condition
ambient temperature	ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
N001-10	500 g

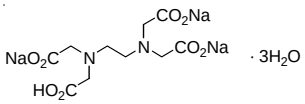
3NA(EDTA·3Na)

Ethylenediamine-*N*,*N*,*N*',*N*'-tetraacetic acid, trisodium salt, trihydrate [CAS: 150-38-9]

Appearance: white powder
Purity: >98.0% (as trihydrate,Titration)
MW: 412.23, $C_{10}H_{13}N_2Na_3O_8 \cdot 3H_2O$

Storage Condition	Shipping Condition
ambient temperature	ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
N002-10	500 g

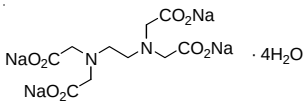
4NA(EDTA·4Na)

Ethylenediamine-*N*,*N*,*N*',*N*'-tetraacetic acid, tetrasodium salt, tetrahydrate [CAS: 67401-50-7(trihydrate)]

Appearance: white crystalline powder
Purity: >98.0% (as tetrahydrate,Titration)
MW: 452.23, $C_{10}H_{12}N_2Na_4O_8 \cdot 4H_2O$

Storage Condition	Shipping Condition
ambient temperature	ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
N003-10	500 g

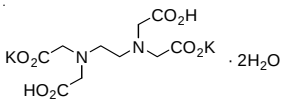
2K(EDTA·2K)

Ethylenediamine-*N*,*N*,*N*',*N*'-tetraacetic acid, dipotassium salt, dihydrate [CAS: 25102-12-9]

Appearance: white powder
Purity: >99.0% (as dihydrate,Titration)
MW: 404.45, $C_{10}H_{14}K_2N_2O_8 \cdot 2H_2O$

Storage Condition	Shipping Condition
ambient temperature	ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
K001-10	50 g
K001-12	500 g

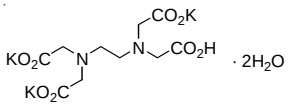
3K(EDTA·3K)

Ethylenediamine-*N,N,N',N'*-tetraacetic acid, tripotassium salt, dihydrate [CAS: 65501-24-8]

Appearance: white powder
Purity: >99.0% (as dihydrate,Titration)
MW: 442.54, $C_{10}H_{13}K_3N_2O_8$, $2H_2O$

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
K002-10	50 g
K002-12	500 g

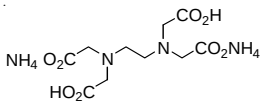
$2NH_4$(EDTA·$2NH_4$)

Ethylenediamine-*N,N,N',N'*-tetraacetic acid, diammonium salt [CAS: 20824-56-0]

Appearance: white powder
Purity: >99.0% (Titration)
MW: 326.30, $C_{10}H_{22}N_4O_8$

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
N008-10	500 g

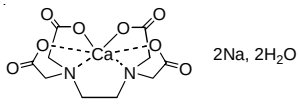
Ca(II)-EDTA

Ethylenediamine-*N,N,N',N'*-tetraacetic acid, calcium, disodium salt, dihydrate [CAS: 62-33-9]

Appearance: white powder
Purity: >99.0% (Titration)
MW: 410.30, $C_{10}H_{12}CaN_2Na_2O_8$, $2H_2O$

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
E008-10	50 g

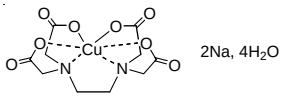
Cu(II)-EDTA

Ethylenediamine-*N,N,N',N'*-tetraacetic acid, copper, disodium salt, tetrahydrate [CAS: 39208-15-6]

Appearance: blue powder
Purity: >98.0% (Titration)
MW: 469.80, $C_{10}H_{12}CuN_2Na_2O_8$, $4H_2O$

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
E010-08	10 g

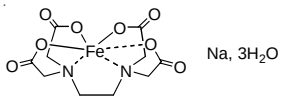
Fe(III)-EDTA

Ethylenediamine-*N,N,N',N'*-tetraacetic acid, iron, sodium salt, trihydrate [CAS: 15708-41-5]

Appearance: yellowish-brown powder
Purity: >98.0% (Titration)
MW: 421.09, $C_{10}H_{12}FeN_2NaO_8$, $3H_2O$

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
E011-10	50 g
E011-12	500 g

XVI. Metal Chelates

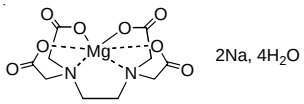
Mg(II)-EDTA

Ethylenediamine-*N,N,N',N'*-tetraacetic acid, magnesium, disodium salt, tetrahydrate [CAS: 14402-88-1]

Appearance: white powder
Purity: >99.0% (Titration)
MW: 430.56, $C_{10}H_{12}MgN_2Na_2O_8$, $4H_2O$

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
E013-10	25 g

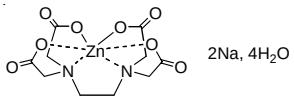
Zn(II)-EDTA

Ethylenediamine-*N,N,N',N'*-tetraacetic acid, zinc, disodium salt, tetrahydrate [CAS: 39208-16-7]

Appearance: white powder
Purity: >99.0% (Titration)
MW: 471.64 $C_{10}H_{12}N_2Na_2O_8Zn$, $4H_2O$

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
E017-10	25 g
E017-12	500 g

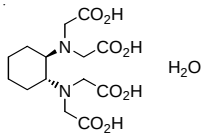
CyDTA

trans-1,2-Diaminocyclohexane-*N,N,N',N'*-tetraacetic acid, monohydrate [CAS: 125572-95-4]

Appearance: white powder
Purity: >99.0% (as monohydrate, Titration)
MW: 364.35, $C_{14}H_{22}N_2O_8$, H_2O

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
C018-10	25 g

Product Description

CyDTA is utilized for masking various metal ions. The two amino groups of CyDTA are in the *trans* formation, and its free acid is water-soluble. CyDTA chelates at a slower rate than EDTA with metal ions, especially Al, C, Ni, and Zr. However, the stability constants of CyDTA-metal complexes are higher than that of EDTA. Dojindo offers highly purified CyDTA for use as a masking reagent on automatic amino acid analyzers. It does not interfere with the ninhydrin reaction.

References

R. Pribil, *Collection Czechoslov. Chem. Commun.*, **20** 162 (1955); M. R. Verma, *et al.*, *Nature*, **179**, 1244 (1957); R. Pribil, *et al.*, *Talanta*, **10**, 1287 (1963); K. E. Burke *et al.*, *Anal. Chem.*, **36**, 172 (1964); R. Pribil, *et al.*, *Chemist-Analyst*, **55**, 38 (1966); W. H. Evans, *Analyst*, **92**, 685 (1967); D.E. Jordan, *et al.*, *Anal. Chim. Acta*, **37**, 42 (1967); D. L. Fuhrman, *et al.*, *Talanta*, **14**, 1199 (1967); L. Szekeres, *Microchem. J.*, **13**, 349 (1970); G. S. Yoneda, *et al.*, *Bioinorg. Chem.*, **8**, 369 (1978); E. J. Billo, *et al.*, *J. Inorg. Biochem.*, **10**, 331 (1979); T. Obe, *et al.*, *Radioisotopes*, **36**, 384 (1987); K. Saeki, *et al.*, *J. Biochem.*, **106**, 606 1989). N, Kurebayashi, *et al.*, *J. Muscle Res. Cell Motil.*, **12**, 355 (1991); T. Fujita, *et al.*, *Clin. Chem.*, **39**, 2130 (1993); M. C. Silvestrini, *et al.*, *Biochimie*, **77**, 531 (1995).

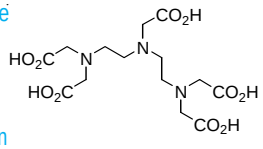
DTPA

Diethylenetriamine-*N,N,N′,N″,N″*-pentaacetic acid [CAS: 67-43-6]

Appearance: white crystalline powder
Purity: >99.0% (Titration)
MW: 393.35, $C_{14}H_{23}N_3O_{10}$

Storage Condition	Shipping Condition
ambient temperature	ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
D022-10	5 g
D022-12	25 g

Product Description

The stability constant of the DTPA-metal complex is the second highest, next only to the CyDTA-metal complex. DTPA is a free acid that has low solubility in water. DTPA is useful as a strong masking reagent. It is also used for the extraction of metal ions such as Al, Fe, Ma, Zn, Cu, Cd, and Ni from soil at pH 5.3.

References

R. Pribil, *et al.*, *Chemist-Analyst*, **56**, 23 (1967); W. A. Norvell, *Soil Sci. Soc., Am. J.*, **48**, 1285 (1984); R. Radi, *et al.*, *Arch. Biochem. Biophys.*, **288**, 481 (1991); X. Shi, *et al.*, *Chem. Res. Toxicol.*, **6**, 277 (1993); E. Kukielka, *et al.*, *Arch. Biochem. Biophys.*, **308**, 70 (1994); W. A. Prutz, *et al.*, *Arch. Biochem. Biophys.*, **332**, 110 (1996); J. H. Kang, *et al.*, *Mol. Cells*, **7**, 553 (1997); A. V. Kachur, *et al.*, *Radiat Res.*, **150**, 475 (1998); R. S. Prosser, *et al.*, *J. Magn. Reson.*, **141**, 256 (1999).

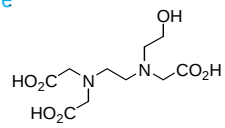
EDTA-OH

N-(2-Hydroxyethyl)ethylenediamine-*N,N′,N′*-triacetic acid [CAS: 150-39-0]

Appearance: white crystalline powder
Purity: >99.0% (Titration)
MW: 278.26, $C_{10}H_{18}N_2O_7$

Storage Condition	Shipping Condition
ambient temperature	ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
E005-10	5 g
E005-12	25 g

XVI. Metal Chelates

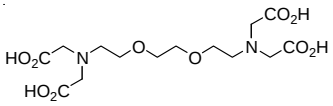
GEDTA (EGTA)

O,O'-Bis(2-aminoethyl)ethyleneglycol-*N,N,N',N'*-tetraacetic acid [CAS: 67-42-5]

Appearance: white crystalline powder
Purity: >97.0% (Titration)
MW: 380.35, $C_{14}H_{24}N_2O_{10}$

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
G002-12	100 g

References

R. A. Burg, *et al., Chemist-Analyst*, **49**, 100 (1960); J. H. Austin, *et al., Chemist-Analyst*, **55**, 11 (1966); N. Negrini, *et al., Plant Cell Environ.*, **18**, 159 (1995); J. A. H. Timmermans, *et al., J. Nutr.*, **125**, 1981S (1995); C. D. Lindsay, *et al., Toxicol. in Vitro*, **9**, 213 (1995); X. Boulenc, *et al., Int. J. Pharm.*, **123**, 13 (1995); J. Wu, *et al., Scand. J. Gastroenterol.*, **30**, 590 (1995); J. Jost, *et al., Gene*, **157**, 265 (1995); L. Graff, *et al., Res. Commum. Mol. Pathol. Pharmacol.*, **88**, 271 (1995); D. J. Sanchez, *et al., J. Appl. Toxicol.*, **15**, 285 (1995); V. Kolhe, *et al., Asian J. Chem.*, **7**, 568 (1995); M. Gliesing, *et al., Cell Mol. Biol.*, **41**, 867 (1995); T. L. Stewart, *et al., J. Neurochem.*, **66**, 131 (1996); L. Li, *et al., J. Invest. Dermatol.*, **106**, 254 (1996); R. Mondragon, *et al., J. Eukaryot. Microbiol.*, **43**, 120 (1996); M. J. MacDonald, *et al., Diabetes*, **46**, 1996 (1997); A. Clayton, *et al., J. Cell. Sci.*, **111**, 443 (1998); C. I.. Marin-Briggiler, *et al., Biol. Reprod.*, **61**, 673 (1999); L. J. Yang, *et al., Biochem. Pharmacol.*, **57**, 425 (1999).

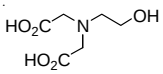
HIDA

N-(2-Hydroxyethyl)iminodiacetic acid [CAS: 93-62-9]

Appearance: white crystalline powder
Purity: >98.0% (Titration)
MW: 177.16, $C_6H_{11}NO_5$

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
H006-10	5 g

References

G. Anderegg, *et al., Helv. Chim. Acta*, **38**, 1940 (1955); V. Jovanovic, *et al., Eur. J. Nucl. Med.*, **6**, 375 (1981); J. H. Turner, *et al., Eur. J. Nucl. Med.*, **13**, 432 (1987); M. Chatterjee, *et al., Nucl. Med. Commun.*, **11**, 305 (1990).

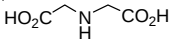
IDA

Iminodiacetic acid [CAS: 142-73-4]

Appearance: white crystalline powder
Purity: >99.0% (Titration)
MW: 133.10, $C_4H_7NO_4$

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
I001-10	25 g
I001-12	500 g

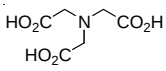
NTA

Nitrilotriacetic acid [CAS: 139-13-9]

Appearance: white crystalline powder
Purity: >99.0% (Titration)
MW: 191.14, $C_6H_9NO_6$

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
N016-10	500 g

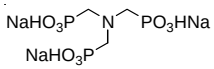
NTPO

Nitrilotris(methylenephosphonic acid), trisodium [CAS: 7611-50-9]

Appearance: white powder
Purity: >75.0% (Titration)
MW: 365.00, $C_3H_9NNa_3O_9P_3$

Storage Condition	Shipping Condition
ambient temperature	ambient temperature

Chemical Structure



Ordering Information	
Product code	Unit
N030-10	5 g

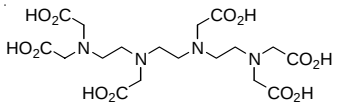
TTHA

Triethylenetetramine-*N*,*N*,*N'*,*N"*,*N"'*,*N"'*-hexaacetic acid [CAS: 869-52-3]

Appearance: white crystalline powder
Purity: >98.0% (Titration)
MW: 494.45, $C_{18}H_{30}N_4O_{12}$

Storage Condition	Shipping Condition
ambient temperature	ambient temperature

Chemical Structure



Ordering Information	
Product code	Unit
T031-10	5 g

References for Chelating Agents

A. E. Martell, *et al.*, "Chemistry of The Metal Chelate Compounds," *Prentice-Hall, Inc., New Jersey* (1952); G. Schwarzenbach, "Complexometric Titration," H. Irving, *Meteun & Co., Ltd., London; Interscinece Publishers, Inc., New York* (1957); F. J. Welcher, "The Analytical Uses of Ethylenediamine Tetraacetic Acid," *D. Van Nostrand Co., Inc., Prinston, Toronto, London,* (1958); H. Flaschka, "EDTA Titration", *Pergamon Press, London, New York, Paris, Los Angeles,* (1959); S. Chaberek, A. E. Martell, "Organic Sequestering Agents," *John Wiley & Sons Inc., New York* (1959); R. L. Smith, "The Sequestration of Metals," *Chapman & Hall Ltd., London* (1959); D. P. Graddon, "Introduction to Co-ordination Chemistry," *Pergamon Press Ltd.,* (1961); A. Ringbom, "Complexation in Analytical Chemistry," *John Wiley & Sons Inc., New York* (1963); T. S. West, "Complexometry with EDTA and Related Reagents," *BDH* (1969); R. Pribil, "Analytical Applications of EDTA and Related Compound," *Pergamon Press Oxford, New York, Toronto, Syndey, Braunschweig* (1972). E. Bishop, "Indicators," *Pergamon Press* (1973).

XVII. Specialty Chemicals

D-Luciferin K salt

(*S*)-4,5-Dihydro-2-(6-hydroxy-2-benzothiazolyl)-4-thiazolecarboxylic acid, potassium salt [CAS: 115144-35-9]

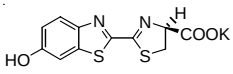
Application: Luciferase substrate, bioluminescence

Appearance: pale yellow or yellow powder
Purity: >98.0% (HPLC)
MW: 318.42, $C_{11}H_7N_2O_3S_2K$

Ordering Information	
Product code	Unit
L226-10	25 mg

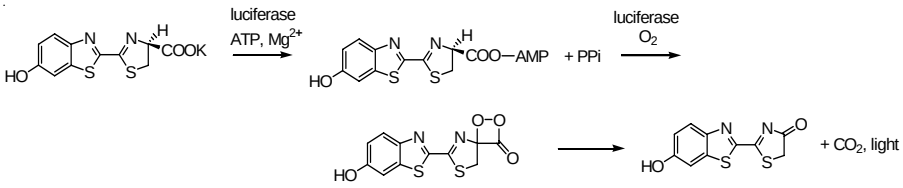
Storage Condition	Shipping Condition
-20 °C	ambient temperature

Chemical Structure



Product Description

D-Luciferin is a firefly luciferase substrate. Its quantum efficiency is 0.88, which is 20 times that of Luminol. The reaction mechanism is shown below. First, Luciferin reacts with ATP by luciferase in the presence of magnesium ion, then it is oxidized to form dioxetane structure and emits yellow-green light. Luciferin-luciferase luminescence is used for ATP monitoring to determine cell viability and bacteria counting. It is also used for reporter gene assays.



References

V. T. Nguyen, *et al.*, *Anal. Biochem.*, **171**, 404 (1988); S. P. M. Crouch, *et al.*, *J. Immunol. Methods*, **160**, 81 (1993); S. R. Ford, *et al.*, *Methods Mol. Biol.*, **102,** 3 (1998).

Ferrocenyl PEG

11-Ferrocenylundecyl polyoxyethylene ether [CAS: 126879-04-7]

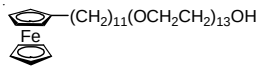
Application: Surfactant for organic thin layer preparation

Appearance: yellow or orange-brown wax
Purity: >98.0% (HPLC)
MW: 929.01, $C_{47}H_{84}FeO_{14}$

Ordering Information	
Product code	Unit
F017-12	1 g

Storage Condition	Shipping Condition
0-5 °C	ambient temperature

Chemical Structure



Ferrocenyl TMA

11-Ferrocenyltrimethylundecylammonium bromide

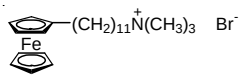
Application: Surfactant for organic thin layer preparation

Appearance: yellowish-orange powder
Purity: >95.0% (HPLC)
MW: 478.33, $C_{24}H_{40}BrFeN$

Ordering Information	
Product code	Unit
F018-12	1 g

Storage Condition	Shipping Condition
0-5 °C	ambient temperature

Chemical Structure



Product Description of Ferrocenyl Surfactants

Ferrocenyl PEG and Ferrocenyl TMA are used to prepare a thin layer of a hardly dissolved organic compound on a solid surface. For example, these surfactants can solubilize phtharocyanine in aqueous solution. After preparing a homogeneous phtharocyanine solution, a thin layer of phtharocyanine is formed on a solid surface by electrolysis of the ferrocenyl surfactant.

References
T. Saji, *et al.*, *J. Am. Chem. Soc.*, **107**, 6865 (1985); J. S. Facci, *et al.*, *Langmuir*, **2**, 732 (1986); K. Hoshino, *et al.*, *Chem. Lett.*, **29**, 979 (1986); K. Hoshino, *et al.*, *J. Am. Chem. Soc.*, **109**, 5881 (1987); T. Saji, *et al.*, *J. Electrochem. Soc.*, **136**, 2954 (1989); K. Saji, *et al.*, *J. Am. Chem. Soc.*, **113**, 452 (1991).

WSC

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide, hydrochloride [CAS: 25952-53-8]

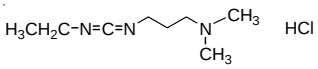
Application: Condensing agent

Appearance: white powder
Purity: >98.0% (Titration)
MW: 191.70, $C_8H_{18}ClN_3$

Ordering Information	
Product code	Unit
W001-10	5 g
W001-12	25 g

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



Product Description of Calixarenes
WSC is a water soluble carbodiimide. It is also soluble in alcohol, acetone, chloroform, dioxane, and DMF. WSC is used as a condensing agent for peptide syntheses. WSC and its urea form can be easily removed after the reaction due to their high water solubility. WSC, EDC, EDAC, EDCI, and ethyl-CDI are the same reagent.

References
J. H. Saunders, *et al.*, *Chem. Rev.*, **45**, 203 (1948); R. J. Slocombe, *et al.*, *J. Am. Chem. Soc.*, **72**, 1888 (1950); K. D. Kopple, *et al.*, *Org. Biol. Chem.*, **84**, 4457 (1962); H. Ozawa, *et al.*, *Biochemistry.*, **9**, 2158 (1970); T. Y. Lin, *et al.*, *Biochemistry*, **9**, 984 (1970); J. C. Sheehan, *et al.*, *J. Am. Chem. Soc.*, **95**, 875 (1973); M. E. Addy, *et al.*, *Biochem, Biophys. Res. Commun.*, **52**, 1034 (1973); A. Williams, *et al.*, *J. Am. Chem. Soc.*, **103**, 7090 (1981); H. Yamada, *et al.*, *Biochemistry*, **20**, 4836 (1981); J. W. Chase *et al.*, *Proc. Natl. Acad. Sci. USA*, **80**, 5480 (1983); J. V. Staros, *et al.*, *Anal Biochem.*, **156**, 220 (1986); M. Taniuchi, *et al.*, *Proc. Natl. Acad. Sci. USA*, **83**, 1950 (1986); M. Taniuchi, *et al.*, *Proc. Natl. Acad. Sci. USA*, **83**, 4094 (1986).

DPO

2,5-Diphenyloxazole [CAS: 92-71-7]

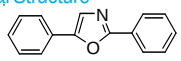
Application: Scintillator reagent

Appearance: white crystalline powder
Purity: >99.0% (HPLC)
MW: 221.25, $C_{15}H_{11}NO$

Ordering Information	
Product code	Unit
D018-10	25 g
D018-12	100 g

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



POPOP

1,4-Bis(5-phenyl-2-oxazolyl)benzene [CAS: 1806-34-4]

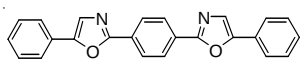
Application: Scintillation reagent

Appearance: pale yellow needles
Purity: >99.0% (HPLC)
MW: 364.40, $C_{24}H_{16}N_2O_2$

Ordering Information	
Product code	Unit
P009-10	5 g
P009-12	25 g

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



XVII. Speciality Chemicals

Alq3, sublimed

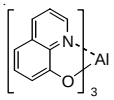
Tris(8-hydroxyquinolinato)aluminum(III) [CAS: 2085-33-8]

Application: Electron carrier

Appearance: yellow or yellowish-green powder
Purity: >98.5% (Titration)
MW: 459.43, $C_{27}H_{18}AlN_3O_3$

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
T203-10	1 g

Bathocuproine, sublimed

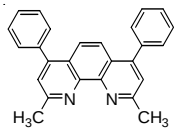
2,9-Dimethyl-4,7-diphenyl-1,10-phenanthroline [CAS: 4733-39-5]

Application: Electron carrier

Appearance: white or yellow crystalline powder
Purity: >98.0% (HPLC)
MW: 360.45, $C_{26}H_{20}N_2$

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
B446-10	1 g

TAZ-01

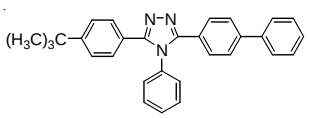
3-(4-Biphenylyl)-4-phenyl-5-(4-*tert*-butylphenyl)-1,2,4-triazole [CAS: 150405-69-9]

Application: Electron carrier

Appearance: white crystals
Purity: >98.0% (HPLC)
MW: 429.56, $C_{30}H_{27}N_3$

Storage Condition: ambient temperature
Shipping Condition: ambient temperature

Chemical Structure



Ordering Information

Product code	Unit
B363-10	1 g

XIX-1. Indexes: Product Code Index

XIX-1. Indexes: Product Code Index

XIX-1. Indexes: Product Code Index

XIX-2. Indexes: CAS Number Index

XIX-2. Indexes: CAS Number Index

XIX-2. Indexes: CAS Number Index

XIX-3. Indexes: Alphabetical Index

XIX-3. Indexes: Alphabetical Index

- D -

- E -

- F -

- G -

XIX-3. Indexes: Alphabetical Index

XIX-3. Indexes: Alphabetical Index