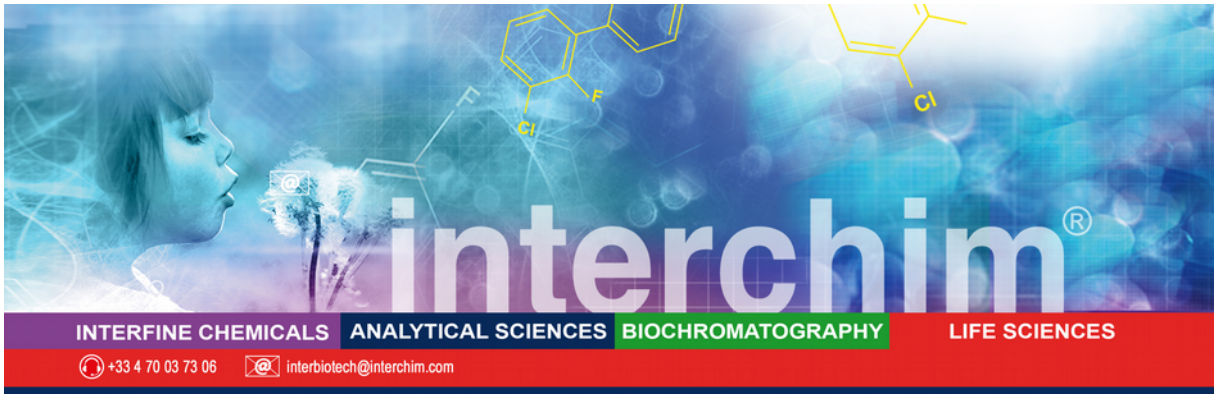
FT-44201A



Acidic-pH Sensors

Probes for pH in Lysosomes, endosomes, spermatozoa and acrosomes

Products Information

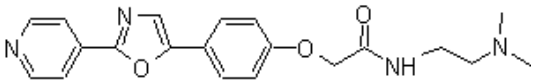
The existing pH probes are ill-adapted to study acidic organelles such as lysosomes, endosomes, spermatozoa and acrosomes because their fluorescence is significantly reduced at lower pH. In addition, most of the existing pH probes (such as BCECF and SNARF) are not selectively localized in acidic organelles. Following are efficient tracking dyes for these applications,

Cat #	Probe	Abs * (nm)	Em * (nm)	pKa
FP-44201A FP-022900 FP-024010	**Acidic pH Sensor 450/540Ratiometric**, Yellow/Blue PDMPO, DNP-160 (SE ester) (Dextran 10K)	384 to 329*	540 to 440*	3.9
FP-JQ7870 FP-JQ7880 FP-JQ7890	**Acidic pH Sensor 500, Green** (SE ester) (Dextran complex)	443	505	ND
FP-GCZ160 FP-GDA080 FP-GDA090	**Acidic pH Sensor 600, Red** (SE ester) (Latex beads)	575	597	ND
See also	Acidic-pH ***Tracking*** dyes FT-97578A (LysoTracker dyes: Blue DND-122, Green DND26, Yellow HCK123, Red DND99)			

Storage: –20°C >1 year or +4°C(M). Protect from light and moisture *ratiometric with pH 3 to 7

● Sensor 450/540-ratiom. Yellow/Blue for Acidic pH – PDMPO, DNP-160 #44201

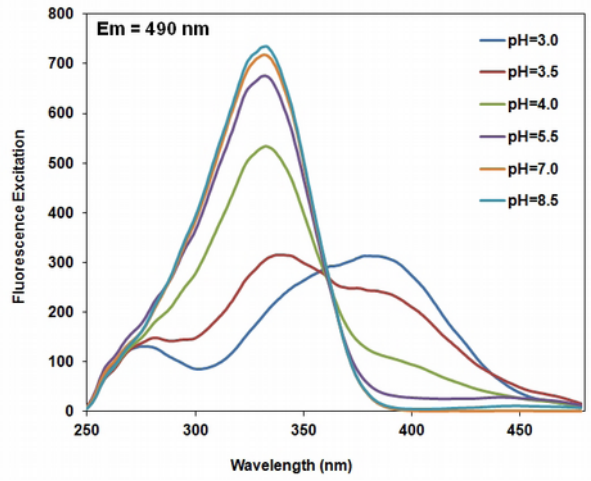
Names :	2-(4-pyridyl)-5-((4-(2-dimethylaminoethylaminocarbamoyl) methoxy) phenyl)oxazole; CAS: - LysoSensor ™Yellow/Blue DNP160, RatioWorks™ PDMPO
Catalog Number :	FP-44201A, 1 mg (M)
Molecular Weight :	MW= 366.42
Soluble in:	DMSO
Absorption / Emission :	$\lambda_{exc}\backslash\lambda_{em}$ (MeOH) = 405 / 550 nm Use ratio: Ex= 360 nm, and Em= 450 and 540 nm



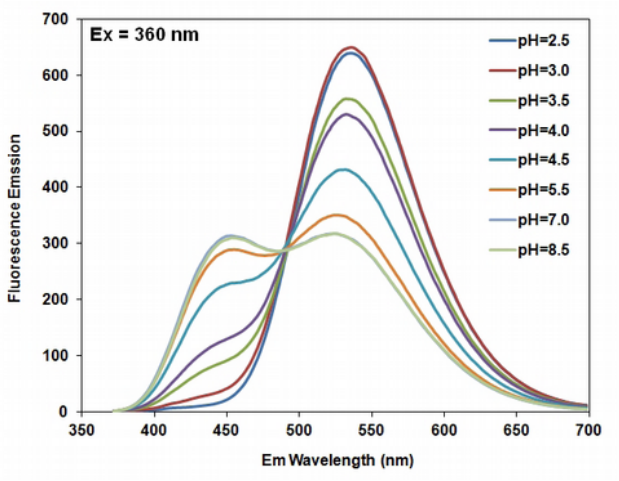
Also available as:
-Succinimidyl ester (SE, FP-022900): amine reactive
-Dextran conjugate (FP-024010: ~10K, soluble/water)

FT-44201A

Excitation spectrum



Emission spectrum (with exc.@360nm)



The PDMPO sensor with Yellow/Blue fluorescence, is characterized as acidotropic dual-excitation and dual-emission pH probe. It emits intense yellow fluorescence at lower pH and gives intense blue fluorescence at higher pH. This unique pH-dependent fluorescence makes PDMPO an ideal pH probe for acidic organelles with pKa = 4.2-4.5. PDMPO selectively labels acidic organelles (such as lysosomes) of live cells and the two distinct emission peaks can be used to monitor the pH fluctuations of live cells in ratio measurements.
The short emission band is ~450 nm while the longer emission is ~550 nm, making the common filter sets of Pacific Blue and Pacific Orange. Additionally, the very large Stokes shift and excellent photostability of PDMPO make PDMPO an excellent fluorescent acidotropic reagent for fluorescence imaging.
PDMPO can be well excited by the violet laser at 405 nm for flow cytometric applications.
PDMPO is available as a dextran conjugate, and other conjugates can be prepared using the SE ester derivate. Bioconjugates allow for specific detection of phagocytosis and endocytosis with reduced signal variability and improved accuracy, and for multiplexing cell functional analysis with green dyes such as GFP, Fluo-8, calcein, or FITC-labeled antibodies.

Assay Protocol (using the Dextran-PDMPO)

This is a guideline, and should be modified according to each specific needs.
Treat cells as desired before making the working solution.

1. **Prepare cells in growth medium as desired**

For example, plate adherent cells overnight in growth medium at 40 000 to 80 000 cells/well/100µL for 96-well or 10 000 to 20 000 cells/well/25µL for 384-well plates.

Note: Each cell line should be evaluated on an individual basis to determine the optimal cell density

2. **Prepare PDMPO Dextran loading solution**:

2.1 Prepare a 1mg/mL stock solution of PDMPO Dextran in 1 mL of sterile water or Hanks and 20 mM Hepes buffer (HHBS). The stock solution should be used promptly. Any unused solution need to be aliquoted and refrozen at < -20°C.

Note: Avoid repeated freeze-thaw cycles, and protect from light.

2.2 Prepare a 20-100ug/mL RatioWorks™ PDMPO Dextran loading solution in HHBS.

3. Run Endocytosis Assay:
- Replace the medium with PDMPO Dextran loading solution
- Incubate at 37°C for 5-20 minutes + Wash and replace with HHBS
- Read Fluorescence at Ex/Em= 360/540 and 360/450 nm

3.1 Remove the medium, and add 100 µL/well (96-well plate) or 25 µL/well (384-well plate) PDMPO Dextran loading solution into the cell plate (from Step 2.2).

Note1: It is important to replace the growth medium with HHBS buffer (100 µL/well for 96-well plate or 25 µL/well for 384-well plate before dye-loading) if your compounds interfere with the serum.

Note2: Rapid trafficking of RatioWorks™ PDMPO dextran from early endosomes to late endosomes and subsequent fusion with lysosomes can occur. To aid the visualization of RatioWorks™ PDMPO dextran within the endosomes, we recommend increasing the labeling concentration and decreasing the loading time, and imaging immediately.

3.2 Incubate the dye-loading plate at cell incubator for 5 to 20 minutes.

3.3 Wash and replace the dye-loading solution with HHBS or growth medium.

3.4 Run the endocytosis assay by monitoring the fluorescence at Ex = 360 nm, and Em = 450 and 540 nm for ratio measurements.

Note: The fluorescence signal from PDMPO dextran is stable for at least one hour after trafficking to lysosomes has occurred. Because lysosomes have a lower pH compared to endosomes, the signal from PDMPO dextran within the lysosomes is brighter than the signal from PDMPO dextran within the endosomes. The lysosomal PDMPO dextran concentration is directly dependent on endocytotic uptake; therefore, the modulation of endocytosis can be inferred from the intensity of PDMPO dextran signal from the lysosomes.

● Sensor 500 Green for Acidic pH (443/505nm)

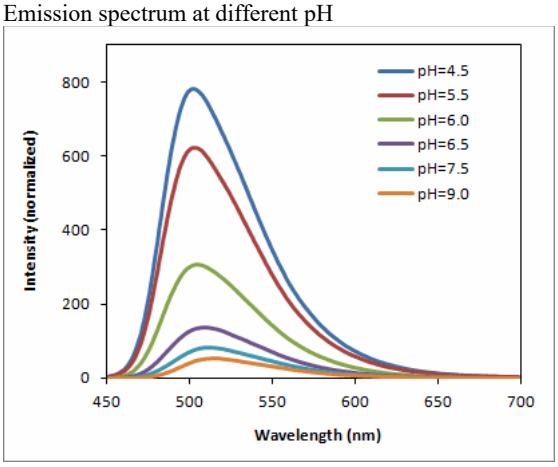
Name :	CAS: - LysoSensor ™, Protonex™
Catalog Number :	FP-JQ7870, 1 mg (M)
Molecular Weight :	MW= 398.46
Soluble in:	DMSO
Absorption / Emission :	$\lambda_{exc}\backslash\lambda_{em}$ (MeOH) = 443 / 505 nm

Also available as:

-Succinimidyl ester (SE) MW: 539.54
FP-JQ7880

-Dextran complex
FP-JQ7890

Emission spectrum at different pH



Acidic pH Sensor 500 (Green) dye has a demonstrated pH-dependent fluorescence which fluorescence, unlike most of the existing fluorescent dyes, dramatically increases as pH decreases from neutral to acid. The lack of fluorescence outside the cell eliminates the wash steps. This enables, together its brightly Green fluorescence in acidic compartments, the specific detection of cellular acidic compartments with reduced signal variability and improved accuracy for imaging or flow applications. This dye provides a powerful tool to monitor acidic cell compartments such as phagosomes, lysosomes and endosomes.The spectral properties are similar to those of Fluorescein, making his use suitable with the common filter set of FITC. It can be also used for multiplexing cellular functional analysis with red dyes such as RFP or TR/SR101-labeled antibodies.

● Sensor 600 Red for Acidic pH (575/597nm)

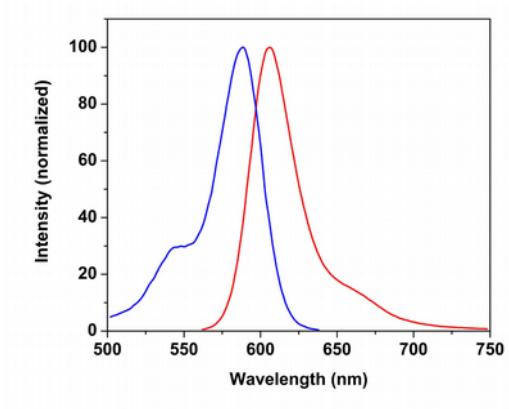
Name :	CAS: - LysoSensor ™, Protonex™
Catalog Number :	FP-GCZ160, 1 mg (M)
Molecular Weight :	MW= 984.03
Soluble in:	DMSO
Absorption / Emission :	$\lambda_{exc}\backslash\lambda_{em}$ (MeOH) = 575 / 597 nm

Also available as:

-Succinimidyl ester (SE) MW: 953.06
FP-GDA080

-Latex Beads conjugate
FP-GDA090

Absorption and Emission spectra at pH 4.0



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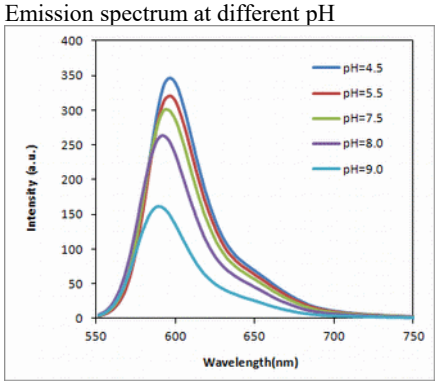
Acidic pH Sensor 600 (Red) dye has a demonstrated pH-dependent fluorescence which fluorescence, unlike most of the existing fluorescent dyes, dramatically increases as pH decreases from neutral to acid. The lack of fluorescence outside the cell eliminates the wash steps, and together its brightly red fluorescence in acidic compartments enables the specific detection of cellular acidic compartments with reduced signal variability and improved accuracy for imaging or flow applications. This dye provides a powerful tool to monitor acidic cell compartments such as phagosomes, lysosomes and endosomes, and is an excellent replacement to pHrodo™. The spectral properties are similar to those of SR101(Texas Red), making his use suitable with the common filter set of TR/SR101. It can be also used for multiplexing cellular functional analysis with green dyes such as GFP, Fluo-8, calcein, or FITC-labeled antibodies.

Emission spectrum at different pH



Assay Protocol

This is a guideline, and should be modified according to each specific needs.
Treat cells as desired before making the working solution.

1. Prepare a 1 to 10 mM Acidic pH Sensor 600 stock solution in DMSO. Make 0.1 to 10 µM working solution by diluting the DMSO stock solution into Hanks solution with 20 mM Hepes buffer (HHBS) or buffer of your choice.
2. Treat cells as desired.
3. Incubate the cells with Acidic pH Sensor 600 working solution for 15min to 2 hours at 37 ºC.
4. Replace the dye-loading solution with HHBS buffer.
5. Analyze the cells with a proper fluorescence instrument fitted with the correct filter set

Labeling Protocol (using the Succinimidyl esters (SE) of Acidic-pH Sensors)

●Introduction

Succinimidyl esters (NHS, SE) are proven to be the best reagents for amine modifications because the amide bonds that are formed are essentially identical to, and as stable as the natural peptide bonds. These reagents are generally stable and show good reactivity and selectivity with aliphatic amines.
There are few factors that need be considered when SE compounds are used for conjugation reaction:
1). Solvents: For the most part, reactive dyes should be dissolved in anhydrous dimethylformamide (DMF) or dimethylsulfoxide (DMSO).
2). Reaction pH: The labeling reactions of amines with succinimidyl esters are strongly pH dependent. Amine-reactive reagents react with non-protonated aliphatic amine groups, including the terminal amines of proteins and the □-amino groups of lysines. Thus amine acylation reactions are usually carried out above pH 7.5. Protein modifications by succinimidyl esters can typically be done at pH 8.5-9.5.
3). Reaction Buffers: Buffers that contain free amines such as Tris and glycine and thiol compounds must be avoided when using an amine-reactive reagent. Ammonium salts (such as ammonium sulfate and ammonium acetate) that are widely used for protein precipitation must also be removed (such as via dialysis) before performing dye conjugations.
4). Reaction Temperature: Most conjugations are done at room temperature. However, either elevated or reduced temperature may be required for a particular labeling reaction.

●Storage

Upon receipt, SE-Acidic pH Sensor dyes should be stored at <-15°C, and kept from light and moisture. The reconstituted DMSO stock solution can be stored at <-15°C for less than two weeks. The protein conjugate should be stored at > 0.5 mg/mL in the presence of a carrier protein (e.g., 0.1% bovine serum albumin). The conjugate solution could be stored at 4°C for two months without significant change when stored in the presence of 2 mM sodium azide and kept from light. For longer storage, the protein conjugates could be lyophilized or divided into single-used aliquots and stored at ≤ –60 °C, and protected from light.

●**Sample Labeling Protocol**

Note: This protocol was developed to label Goat anti-mouse IgG . You might need further optimization for your particular proteins.

1. **Prepare protein labeling stock solution** (Solution A), typically 5mg/ml in buffer pH8.5

Mix 100µL of a reaction buffer (e.g., 1 M sodium carbonate solution or 1 M phosphate buffer with pH ~9.0) with 900µL of the target protein solution (e.g. antibody, protein concentration >2 mg/ml if possible).

Note 1: The pH of the protein solution (Solution A) should be 8.5 ±0.5. If the pH of the protein solution is lower than 8.0, adjust the pH to the range of 8.0-9.0 using 1 M sodium bicarbonate solution or 1 M pH 9.0 phosphate buffer.

Note 2: The protein should be dissolved in 1X phosphate buffered saline (PBS), pH 7.2-7.4. If the protein is dissolved in Tris or glycine buffer, it must be dialyzed against 1X PBS, pH 7.2-7.4, to remove free amines or ammonium salts (such as ammonium sulfate and ammonium acetate) that are widely used for protein precipitation.

Note 3: Impure antibodies or antibodies stabilized with bovine serum albumin (BSA) or gelatin will not be labeled well. The presence of sodium azide or thimerosal might also interfere with the conjugation reaction. Sodium azide or thimerosal can be removed by dialysis or spin column for optimal labeling results.

Note 4: The conjugation efficiency is significantly reduced if the protein concentration is less than 2 mg/mL. For optimal labeling efficiency the final protein concentration range of 2-10 mg/mL is recommended.

2. **Prepare dye stock solution** (Solution B):

Add anhydrous DMSO into the vial of Acidic pH Sensor dye SE to make a 10-20 mM stock solution. Mix well by pipetting or vortex.

Note 1: Prepare the dye stock solution (Solution B) before starting the conjugation. Use promptly. Extended storage of the dye stock solution may reduce the dye activity. Solution B can be stored in freezer for two weeks when kept from light and moisture. Avoid freeze-thaw cycles.

Note 2: The Acidic pH Sensor Green 500 SE is not very soluble in DMSO, it is OK to use its suspension in the conjugation buffer.

3. **Determine the optimal dye/protein ratio** (optional):

Note: Each protein requires distinct dye/protein ratio, which also depends on the properties of dyes. Over labeling of a protein could detrimentally affects its binding affinity while the protein conjugates of low dye/protein ratio gives reduced sensitivity. We recommend you experimentally determine the best dye/protein ratio by repeating Steps 4 and 5 using a serial different amount of labeling dye solutions. In general 4-6 dyes/protein are recommended for most of dye-protein conjugates.

3.1 **Use 10:1 molar ratio of Solution B (dye)/Solution A (protein)** as the starting point:
Add 5 µl of the dye stock solution (Solution B, assuming the dye stock solution is 10 mM) into the vial of the protein solution (95 µl of Solution A) with effective shaking. The concentration of the protein is ~0.05 mM assuming the protein concentration is 10 mg/mL and the molecular weight of the protein is ~200KD.

Note: The concentration of the DMSO in the protein solution should be <10 %.

3.2 Run conjugation reaction (see Step 4 below).

3.3 Repeat #3.2 with the molar ratios of Solution B/Solution A at 5:1; 15:1 and 20:1 respectively.

3.4 Purify the desired conjugates using premade spin columns (see Step 5)

3.5 Calculate the dye/protein ratio (DOS) for the above 4 conjugates (see Point ●6).

3.6 Run your functional tests of the above 4 conjugates to determine the best dye/protein ratio to scale up your labeling reaction.

4. **Run conjugation reaction (labeling)**:

4.1 Add the appropriate amount of dye stock solution (Solution B) into the vial of the protein solution (Solution A) with effective shaking.

Note: The best molar ratio of Solution B/Solution is determined from Step 3.6. If Step 3 is skipped, we recommend to use 10:1 molar ratio of Solution B (dye)/Solution A (protein).

4.2 Continue to rotate or shake the reaction mixture at room temperature for 30-60 minutes.

5● Purify the conjugation

The following protocol is an example of dye-protein conjugate purification by using a Sephadex G-25 column.

5.1 Prepare Sephadex G-25 column according to the manufacture instruction.

5.2 Load the reaction mixture (directly from Step 4) to the top of the Sephadex G-25 column.

5.3 Add PBS (pH 7.2-7.4) as soon as the sample runs just below the top resin surface.

5.4 Add more PBS (pH 7.2-7.4) to the desired sample to complete the column purification. Combine the fractions that contain the desired dye-protein conjugate.

Note 1: For immediate use, the dye-protein conjugate need be diluted with staining buffer, and aliquoted for multiple uses.

Note 2: For longer term storage, dye-protein conjugate solution need be concentrated or freeze dried (see below).

6● Characterize the Desired Dye-Protein Conjugate

The Degree of Substitution (DOS) is the most important factor for characterizing dye-labeled protein. Proteins of lower DOS usually have weaker fluorescence intensity, but proteins of higher DOS (e.g. DOS > 6) tend to have reduced fluorescence too. The optimal DOS for most antibodies is recommended between 2 and 10 depending on the properties of dye and protein. For effective labeling, the degree of substitution should be controlled to have 4-10 moles of iFluor™ 647 SE to one mole of antibody. The following steps are used to determine the DOS of iFluor™ 647 SE labeled proteins.

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6.1. Measure absorption:

To measure the absorption spectrum of a dye-protein conjugate, it is recommended to keep the sample concentration in the range of 1-10 $^{.00}$M depending on the extinction coefficient of the dye.

6.2. Read OD (absorbance) at 280 nm and dye maximum absorption:

Measure the absorbance (OD) of the Acidic pH Sensor dyes conjugates at 280 nm (the maximum absorption of protein) and at the λmax for the Acidic pH Sensor dyes. For most spectrophotometers, the sample (from the column fractions) need be diluted with de-ionized water* so that the OD values are in the range of 0.1 to 0.9. To obtain accurate DOS, make sure that the conjugate is free of the non-conjugated dye. See the absorbance maxima, the extinction coefficient, and the correction factor for the pHrodo™ dyes on Table1.

6.3. Calculate DOS using the following equations:

3.1 Calculate protein concentration

$$[\text{Protein}] = \frac{A280 - (OD_{@\text{Dye max.abs.}} \times CF_{280nm})}{EC_{\text{Protein}}} \times \text{dilution factor}$$

3.2 Calculate dye concentration

$$[\text{Dye}] = \frac{OD_{@\text{Dye max.abs.}}}{EC_{\text{Dye}}} \times \text{dilution factor}$$

3.3 Calculate the degree of labeling

$$\text{DOS} = [\text{Dye}] / [\text{Protein}] = [{}^{D}OD_{max} \times EC_{\text{Protein}(280nm)}] / [\, EC_{\text{Dye}} \times (A280 - CF_{280nm} \times A_{max})]$$

[Dye] is the dye concentration, and can be readily calculated from the Bee-Lambert Law: $A=\varepsilon_{dye}CL$.

[Protein] is the protein concentration. This value can be either estimated by the weight (added to the reaction) if the conjugation efficiency is high enough (preferably > 70%) or more accurately calculated by the Beer-Lambert Law: $A=\varepsilon_{protein}CL$. For example, IgG has the ε value to be 203 000 $cm^{-1}M^{-1}$.

EC is molar Extinction Coefficient of the dye or of protein. I.e. EC_{Protein} at 280 nm ($^{P}\varepsilon_{280}$) is 203 000$cm^{-1}M^{-1}$ for IgG.

CF is dye absorption correction factor at 280 nm.

References

1. Sarantis H, Grinstein S. (2012) Monitoring phospholipid dynamics during phagocytosis: application of genetically-encoded fluorescent probes. Methods Cell Biol, 108, 429.
2. Schreiner L, Huber-Lang M, Weiss ME, Hohmann H, Schmolz M, Schneider EM. (2011) Phagocytosis and digestion of pH-sensitive fluorescent dye (Eos-FP) transfected E. coli in whole blood assays from patients with severe sepsis and septic shock. J Cell Commun Signal, 5, 135.
3. Leclerc L, Boudard D, Pourchez J, Forest V, Sabido O, Bin V, Palle S, Grosseau P, Bernache D, Cottier M. (2010) Quantification of microsized fluorescent particles phagocytosis to a better knowledge of toxicity mechanisms. Inhal Toxicol, 22, 1091.
4. Flannagan RS, Grinstein S. (2010) The application of fluorescent probes for the analysis of lipid dynamics during phagocytosis. Methods Mol Biol, 591, 121.

Related products

See also Acidic-pH Tracking dyes FT-97578A (LysoTracker dyes: Blue DND-122, Green DND26, Yellow HCK123, Red DND99)
See also CMAC, CMTMR and CMFDA FT-12662A (Celltracker dyes)

Cell Biology assays > Cell tracing [BE030a]

- 5-CMF, FP-BT5160
- CFDA-SE, FP-52493A

Buffers

- HEPES, 061940

Ordering information

Catalog size quantities and prices may be found at http://www.fluoprobes.com
Please inquire for higher quantities (availability, shipment conditions).

For any information, please ask : FluoProbes® / Interchim; Hotline : +33(0)4 70 03 73 06

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