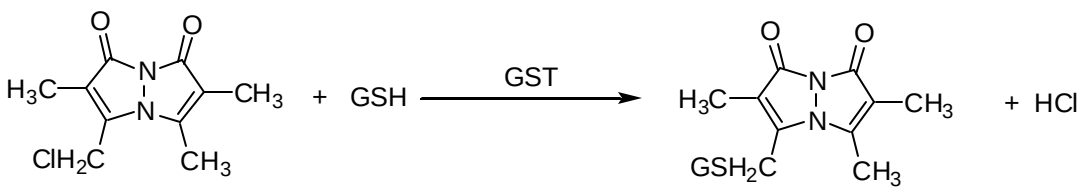
MarkerGene™ Live Cell Glutathione Transferase Activity Kit
Product M1045
Product Information Sheet (1045-006)

NOTE: The following information is given as a viable methodology for use of the MarkerGene™ Live Cell Glutathione Transferase Activity Kit. Users may determine their own best conditions for use dependent on the specific conditions present in their experiment.

I. OVERVIEW

Glutathione (GSH), is a tripeptide (γ-glutamyl-cysteinyl-glycine) that represents the major free thiol in most living cells. It is involved in many biological processes including detoxification of xenobiotics, removal of hydroperoxides, and maintenance of the oxidation state of protein sulfhydryls. It is the key antioxidant present in animal tissues, and diminished glutathione levels have been observed in the early stages of apoptosis. High glutathione transferase levels have also been implicated in Multiple Drug Resistance (MDR) studies.

Older methods for determining glutathione utilize 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB or Ellman's reagent) in an oxidation or oxidation-reduction scheme. But these methods have been largely replaced by a widely used method for determining glutathione levels in living cells and for measuring glutathione-S-transferase activity. The Live Cell Glutathione Assay kit can be used for intracellular glutathione S-transferase detection as well as measuring glutathione levels by simply adding a thiol-reactive probe, monochlorobimane (mCB), to the cell culture medium or lysate to form blue fluorescent GSH-mCB complexes. Unlike other bimanes such as monobromobimane, monochlorobimane appears to form an adduct exclusively with GSH. Unbound monochlorobimane is essentially nonfluorescent, but upon formation of glutathione-mCB adducts inside the cell, it fluoresces strongly (Ex/Em: 380/460), allowing for fluorometric measurement of GSH levels. The formation of these fluorescent adducts is catalyzed by glutathione-S-transferase, making mCB a useful tool for detecting GST activity and measuring GSH in live cells, plasma, erythrocyte lysates, tissue homogenates, cell lysates and in flow cytometry systems. Monochlorobimane is shown to be highly selective for glutathione in rodent cells, but can have reduced labeling of glutathione in human cells due to its lower affinity for human glutathione *S*-transferases.



The kit contains enough reagents for up to 100 assays, including lysis buffer, fluorescent reagent, standards, and a detailed protocol for use. With this assay technique, background fluorescence levels are inherently low because before interacting with cells containing the enzyme, the dye is virtually non-fluorescent. For more information about this assay, see the references below or contact our technical services department for further information.

II. MATERIALS

A.) **Monochlorobimane (mCB)**: 25mM solution of monochlorobimane in anhydrous DMSO (200µL).

B.) **Cell Lysis Buffer**: 25mM Tris-phosphate (pH 7.8) containing 10% glycerol, 1% Triton X-100, 1mg/ml BSA, 2mM EGTA (15mL).

C.) **L-Glutathione (GSH,** g**-glutamyl-cysteinyl-glycine)**: 1.0mM L-Glutathione in D-PBS buffer (see below) (2 mL).

D.) **Glutathione S-transferase (GST)**: 50U/ml solution (200µL).

E.) **Storage and Handling**. Handle all reagents with care. Store mCB and GST at -20°C; store GSH and Cell Lysis Buffer at -4°C. In case of contact with skin or eyes, wash thoroughly with soap and cold-water. Reagents should be stable for at least 6 months following purchase. Limit freeze-thaw cycles for GST Reagent if possible.

III. MICROPLATE READER PROTOCOL

For optimal sensitivity using a microplate reader, it is recommended that the fluorophore should be excited using optical filters matching the absorbance and emission wavelengths. For more information about appropriate filter systems, see our web site at http://www.markergene.com/Filter Sets.htm or contact your instrument manufacturer.

Culture adherent cells in the multiwell microplate; they are usually grown for 2-4 days for acceptable cell densities. Wash the cells with D-PBS and make sure enough D-PBS is added to cover the bottom of the wells. Wash nonadherent cells with D-PBS and centrifuge at low speed (<700 x g) to collect the cells without damage. Add the collected cells to wells using enough buffer to cover well bottoms; to reduce dilution of cytotoxic reagents, small buffer volumes are preferred. Treat cells with cytotoxic agents as needed prior to or synchronous with fluorescent reagent staining. If desired, induce apoptosis in cells by a desired method. Concurrently incubate a control culture without such induction. Minimum number of cells per well is about ~200.

A. Measuring Glutathione Levels

Prepare a lysate sample for microplate reader measurement as follows. Collect the cells (1×10^6) by centrifugation at 700 x g for 5-10 minutes at 4°C. Remove the supernatant and resuspend the cell pellet in 1 ml ice-cold D-PBS. Transfer this suspension into a 1.5 mL microcentrifuge tube, and centrifuge at 700 x g for 5 minutes at 4°C. Remove the supernatant and resuspend cells in 100µl ice-cold Cell Lysis Buffer (M1045-002). Incubate on ice for 10 minutes, then centrifuge at high speed (10,000 x g) in an Eppendorf centrifuge for 10 minutes. Transfer the supernatant to a fresh tube and add 2µl of 25mM mCB solution (M1045-001) and 2µl of GST solution (M1045-004) to each sample. Prepare a negative control sample by adding 2µl of 25mM mCB to 100µl of Cell Lysis Buffer (no cells). Incubate all samples at 37°C for 15-30 minutes. Measure the fluorescence in a fluorometer or fluorescence plate reader using Ex/Em = 380/460nm, respectively.

B. Measuring Glutathione-S-Transferase Activity

This procedure allows for measurement of GST activity, both cytosolic and microsomal, through conjugation of mCB with reduced glutathione (GSH). This conjugation results in an increase in fluorescence (EX/EM: 380/460) and the rate of increase is proportional to GST activity in the sample.

Cell Lysates: Collect the cells (1×10^6) by centrifugation at 700 x g for 5-10 minutes at 4°C. For adherent cells, do not use proteolytic enzymes to harvest; use a rubber policeman. Remove the supernatant and resuspend the cell pellet in 1 ml ice-cold D-PBS. Transfer this suspension into a 1.5 mL microcentrifuge tube, and centrifuge at 700 x g for 5 minutes at 4°C. Remove the supernatant and resuspend cells in 100µl ice-cold

Cell Lysis Buffer (M1045-002). Incubate on ice for 10 minutes, then centrifuge at high speed (10,000 x g) in an Eppendorf centrifuge for 10 minutes. Remove the supernatant for assay and store on ice – if not using on the same day, freeze supernatant at -80°C.

Tissue homogenates: Perfuse tissue with D-PBS (containing heparin to remove red blood cells and clots) prior to dissection. Homogenize the tissue in 5mL of cold buffer (such as 100mM potassium phosphate, pH 7.0, containing 2mM EGTA) per gram of tissue. Centrifuge at 10,000 x g for 15-20 minutes at 4°C. Remove the supernatant for assay and store on ice – if not using on the same day, freeze supernatant at -80°C.

In order to separate cytosolic and microsomal GST of tissue homogenates and cell lysates, centrifuge the 10,000 x g supernatant at 100,000 x g for 50-60 minutes at 4°C; the 100,000 x g supernatant should contain the cytosolic GST and the pellet should contain microsomal GST. Suspend the microsomal pellet in cold D-PBS (or other suitable buffer).

Sample Assay: For background or non-enzymatic controls, add 178µL of Cell Lysis Buffer and 20µL of L-Glutathione (GSH) solution (M1045-003) to three wells of a 96-well plate. For positive controls, add 176µL of Cell Lysis Buffer, 20µL of L-Glutathione (GSH) solution, and 2µL of Glutathione S-transferase (GST) solution (M1045-004) to three wells. For sample wells, add 158µL Cell Lysis Buffer, 20µL of L-Glutathione solution, and 20µL of sample to wells. If necessary, perform serial dilutions of sample in Cell Lysis Buffer before adding to wells of plate to obtain optimal GST activity. Add 2µL of monochlorobimane (mCB) solution (M1045-001) to each well and gently shake the 96-well plate to mix (record time started). Measure the fluorescence once every minute in a fluorescence plate reader using Ex/Em = 380/460nm, respectively, to obtain at least 5-10 time points.

IV. PROTOCOL FOR FLUORESCENCE MICROSCOPY

Culture adherent cells on sterile glass coverslips until acceptable cell densities are reached (usually grown for 2-3 days). Non-adherent cells can also be used. Remove media and wash adherent cells with D-PBS. If non-adherent cells are used, wash with D-PBS, sediment by centrifugation, and place an aliquot of cell suspension on a coverslip. Allow cells to settle at 37°C to glass surface. Place coverslips in small petri dishes (2 per dish) and cover with a working solution (such as 50µM mCB, 10µM glutathione, 140 mM NaCl, 10 mM glucose, 5 mM KCl, 1.8 mM $CaCl_2$, 5 mM HEPES, and 1mg/mL BSA, pH 7.4)[9] and incubate for 15 minutes. After incubation, rinse coverslips with D-PBS (or buffer listed above **without** 50µM mCB) by brief immersion in a separate well containing fresh D-PBS (or buffer without mCB) and carefully invert

and mount coverslip onto a microscope slide, either using a rubber gasket, ring of petroleum jelly, or by placing a drop of media onto the slide. View with fluorescence microscope containing appropriate filter sets for Ex/Em = 380/460nm.

V. FLOW CYTOMETRY USE

Culture cells in suspension or use a rubber policeman to produce a suspension of adherent cells. For analysis by flow cytometry, wash cell suspensions by centrifugation and resuspension in D-PBS. Centrifuge again and resuspend in monochlorobimane working solution (prepare a 0.5mM mCB solution in D-PBS by adding 2µL of 25mM mCB solution per 100µL of D-PBS). Positive results have been confirmed for staining 1 x 10^6 cells (HL-60 cells grown in suspension or D5 cells using a rubber policeman) with ~200µL of a D-PBS solution containing 0.5mM mCB. Control experiments should be performed to ensure appropriate cell staining and cells should be examined by fluorescence microscopy.

VI. NOTES

The suggested tissue culture buffer, D-PBS (200mg/L KCl, 200mg/L KH_2PO_4, 8000mg/L NaCl, and 1150mg/L Na_2HPO_4) or any standard saline buffer can be used, but color additives should be checked for fluorescence interference.

β-mercaptoethanol, dithiothreitol and cysteine are known to interfere with this assay. Avoid using these compounds during sample preparation. Other amino acids are not known to interfere in this assay.

REFERENCES:

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Kit Contents

Description	Quantity	Part No.	Storage
Sample preparation			
Monochlorobimane (mCB): 25mM monochlorobimane in anhydrous DMSO	1 x 200µL	1045-001	F,G
Cell Lysis Buffer: 25mM Tris-phosphate (pH 7.8) containing 10% glycerol, 1% Triton X-100, 1mg/ml BSA, 2mM EGTA	1 x 15mL	1045-002	C,G
L-Glutathione (GSH): 1.0mM solution in D-PBS	1 x 2mL	1045-003	C,G
Glutathione S-transferase (GST): 50U/mL solution	1 x 200µL	1045-004	F,G
Documentation			
MSDS sheets	3	1045-005	N/A
Product Information sheet	1	1045-006	N/A

Notes: F=store at or below -18 ^{o}C; R=store at room temperature; C=store cold (4 ^{o}C); L=light sensitive; D=store desiccated; FL=flammable; G=wear protective clothing/gloves/safety glasses when using; B=avoid breathing dust/fumes.