FOR RESEARCH USE ONLY!

EZCell™ Glutathione Detection Kit (Blue Fluorescence)

05/16

(Catalog # K504-100; 100 assays; Store at -20°C)

I. Introduction:

Glutathione (GSH) represents major low molecular-weight free thiol in living cells. As the key antioxidant in mammalian cells, intracellular GSH forms rapidly eliminated conjugates with electrophilic xenobiotics, free radicals as well as hydroperoxides. Insufficiency of cellular GSH results in oxidative stress damage and mitochondrial degeneration. Diminished levels of glutathione are symptomatic of cell stress thus; it is advantageous to monitor distribution of the intracellular GSH as an indicator of early apoptosis and cell death. BioVision's EZCell™ Glutathione Detection Kit (Blue Fluorescence) utilizes Monochlorobimane (MCB), a non-fluorescent and cell-permeable dye, that forms highly fluorescent adducts with GSH (GSH-MCB) detectable at EX/EM= 380/465 ±20 nm respectively. The reaction is catalyzed by glutathione-S-transferase (GST) and the intensity of the fluorescence reflects the amount of intracellular GSH. Hence, our kit provides a useful tool for fast and easy evaluation of the GSH effectors directly in the living cells.

II. Applications:

- Rapid and sensitive detection of intracellular levels of GSH and activity of GST in live cells
- Screening for compounds affecting intracellular GSH levels

III. Sample Type:

- Live cell cultures (adherent or suspension)

IV. Kit Contents:

Components	K504-100	Cap Code	Part Number
Assay Buffer	100 ml	NM	K504-100-1
Monochlorobimane (MCB)	1 ml	Red	K504-100-2

V. User Supplied Reagents and Equipment:

- A 6-, 12-, 24-, or 96-well plate for culturing cells and opaque white plate for measurements of fluorescence. Alternatively, opaque black or white plates with clear bottoms can be used for culturing and measurements.
- Stock solutions of GSH effectors of interest in media (e.g., Staurosporine, H_2O_2 or other apoptosis-inducing compounds).
- Multi-well spectrophotometer or fluorescent microscope capable of measuring EX/EM=360/460 ±20 nm.

VI. Storage Conditions and Reagent Preparation:

Upon receiving, store kit at -20°C, protected from light. Prior to opening, allow reagents to warm to room temperature then briefly centrifuge the vials. Read the entire protocol before performing the assay. Follow storage instruction for each component of the kit.

- **Assay Buffer:** Ready to use. Store at 4ºC.
- **Monochlorobimane:** Warm the vial to >18 C° prior to use (usually 1–2 min in a 37 °C water bath is sufficient), spin briefly. Store at -20°C protected from light.

VII. Assay Protocol:

The following assay protocol applies to a 96-well format and provides enough reagents for 100 experimental tests including treatments and controls. For other plate formats, proportionally adjust appropriate dispensing volumes.

1. **Preparation of control and experimental wells:** Subculture the cell line of choice in appropriate medium until the density reaches about 5 x 10^5 viable cells/ml. Determine viability (BioVision Cat. K305, K303) and seed 200 µl of cell culture per well. Incubate overnight at 37 °C, 5 % CO_2. Next day, remove the media and proceed to the GSH effector assay.
2. **GSH effector assay:** Your experiment should always consist of parallel negative, positive and experimental wells respectively.
   a. **Experimental wells**: add 200 µl of cell culture media containing your GSH effector of interest (not provided in the kit) at desired concentration (e.g. 200 µM H_2O_2; 5-10 µg/ml Staurosporine)
   b. Add 200 µl of media to each of the **negative and positive** control wells.
   c. Incubate the plate overnight at 37 °C, 5 % CO_2. The incubation time may vary depending on your normal protocol.
   d. Next day, add 10 µl of **Monochlorobimane (MCB)** to each of the **experimental and positive control** wells. Do not add MCB to the **negative control** wells. Incubate the plate for additional 90-120 min at 37 °C, 5 % CO_2.
   e. Remove the dye and treatment by centrifugation at 700 X g for 5 min. Add 200 µl of the Assay Buffer to each well and continue to the preferred method of detection.
3. **Detection of intracellular GSH:** At this point all experimental and control wells can be analyzed under the fluorescent microscope or a plate reader directly in the white opaque plates with clear bottoms used for culturing or transferred into the white opaque plate.
   a. **Fluorescence plate reader**: For adherent cells; read fluorescence directly off the culturing plate. If working with suspension cells; aliquot 200 µl of the culture suspension into the white opaque plate and record fluorescence at EX/EM= 380/465 ±20 nm respectively. Compare level of fluorescence between controls and treated cells. Alternatively, cells can be lysed and lysates analyzed with the BioVision Glutathione Fluorometric Assay Kit (Cat. K251-100).

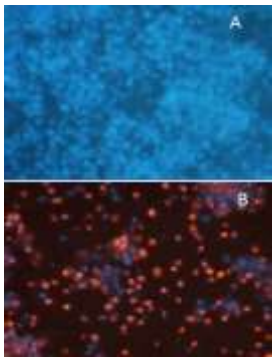
      Note: Fluorescence measured directly in the culturing plates might result in lower RFU values. To increase the RFU readout, increase the number of cells per well or set your plate reader for bottom reading option.

   b. **Microscope analyses:** Compare the fluorescence intensity of experimental and control wells under the fluorescent microscope directly in the tissue culture plate. Optional: To visualize dead cells, add 1 µl of 250 µg/ml solution of **Propidium Iodide** (BioVision Cat.1056) per well.

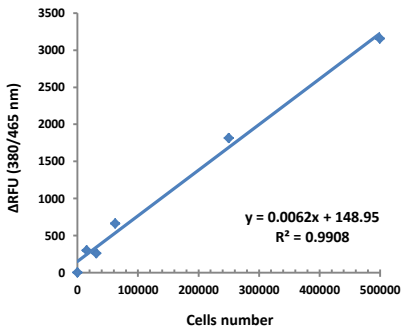
4. **GSH Fluorescence Curve:** Important: To increase the accuracy of your data, GSH fluorescence curve should be prepared from the same cell suspension in parallel with the experimental treatment. Prepare a new standard curve for each experiment and cell line. In a 96-well white plate, prepare a series of dilutions of your cell suspension starting with the same volume and number of cells as in the experimental wells. Dilute the cells by factor of 2. To minimize the error, aliquot at least four (or more) wells per dilution. Incubate the plate overnight at 37 °C, 5 % CO_2. Next day change media in all the wells and supplement half of the wells in each dilution set with 10 µl of **Monochlorobimane (MCB).** Continue the incubation at 37 °C, 5 % CO_2 for the same amount of time as the experimental wells. Remove media from all the wells by centrifugation at 700 X g for 5 min. Wash the cells in 200 µl of the Assay Buffer, spin to remove the wash, add 100 µl of the Assay Buffer per well and measure fluorescence at EX/EM=380/465 ± 20 nm.
5. **Calculations:** Determine background fluorescence by calculating an average from all the wells without MCB. Calculate average fluorescence for each dilution and subtract the background value respectively. Plot the GSH Fluorescence Curve to obtain fluorescence per cell number and the detection limit for your assay.

   The following figures demonstrate typical results with the EZCell™ Glutathione Detection Kit. These data should be used for reference only. Your data will depend on the cell type and tested compounds.

(I)



(II)



(III)

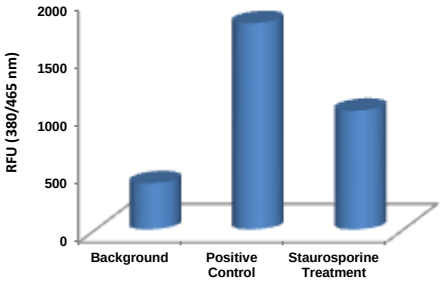


Figure: Effect of Staurosporine on GSH levels reflected by reduction in fluorescence intensity in HeLa cells. HeLa cells were seeded overnight at 5 x 10^5 of viable cells/well. The next day the cell culture was treated with 0.5 µg/ml of Staurosporine and returned to the incubator for the next 24 h. Levels of GSH in the living cells corresponding to the intensity of the signal were determined as described in the Assay Protocol. Fluorescence was measured directly in white opaque plates with clear bottoms used for cell culturing. **(I) Comparison of GSH levels in control and apoptotic cells:** *Panel A:* control, untreated cells with bright uniform GSH staining; *Panel B:* apoptotic cells with diminished blue fluorescence post 24 h treatment with Staurosporine. Dead cells are visualized by red staining of nuclear DNA with Propidium Iodide. **(II) GSH Fluorescence Curve of Jurkat cells prepared for this particular assay.** Detection limit for corresponds to about 65,000 of Jurkat cells per well. Your results may not be identical to these. A new curve must be obtained for each experiment and the cell line. **(III) Reduced levels of fluorescence in apoptotic Jurkat cells**. Decrease in RFU values between cells treated overnight with Staurosporine vs untreated positive control. Background values correspond to the cell culture without MCB treatment.

VIII. RELATED PRODUCTS

Glutathione Colorimetric Assay Kit (K261)
Glutathione (GSH/GSSG/Total) Fluorometric Assay Kit (K264)
GST Fluorometric Activity Assay Kit (K260)
Staurosporine (1048)
Hydrogen Peroxide Assay Kit (K265)
Glutathione Fluorometric Assay Kit (K251)
GST Colorimetric Activity Assay Kit (K263)
Propidium Iodide (1056)
GST Inhibitor-2 (Ethacrynic acid))
Intracellular Hydrogen Peroxide Detection Kit (cell-based) (K204)

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