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ProductInformation

Glutathione Assay Kit, Fluorimetric

Catalog Number **CS1020**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

Reduced glutathione (GSH), a tripeptide (γ-glutamyl-cysteinylglycine), is the major free thiol in most living cells and is involved in many biological processes such as detoxification of xenobiotics, removal of hydroperoxides, and maintenance of the oxidation state of protein sulfhydryls. It is the key antioxidant in animal tissues.[1] Glutathione is present inside cells primarily in the reduced form (90–95% of the total glutathione). The remainder is present in the oxidized form (glutathione disulfide, GSSG). Intracellular GSH status appears to be a sensitive indicator of the overall health of a cell and its ability to resist toxic challenge. High levels of GSH in the cell may indicate pathological changes.[2]

The kit assay utilizes a thiol probe (monochlorobimane), which can freely pass through the plasma membrane. The free, unbound probe shows very little fluorescence, but when bound to reduced glutathione in a reaction that is catalyzed by glutathione S-transferase (GST) it forms a strongly fluorescent adduct.[3]

The Glutathione Assay Kit, Fluorimetric includes all the reagents required for fast and easy measurement of reduced glutathione in cell or tissue extracts,[4] or directly in growing cells.[5] Staurosporine is supplied with the kit to enable induction of apoptosis in cells. Since the amount of reduced glutathione in staurosporine induced cells is significantly lower than in non-induced cells, this can serve as a control system[6] (see Appendix).

The kit was tested on samples prepared from mammalian cells such as HeLa, BHK, NIH 3T3, A431, Jurkat, PYS-2, COS-1, L-8, HepG2, A549, CHO, and U937 cell lines. It was also tested on tissue extracts prepared from rat liver, spleen, brain, kidney, lung, and skeletal muscle. In addition the kit was tested for the *in-situ* measurement (cell based assay) of reduced glutathione levels in BHK, A431, and CHO cell lines.

Components

The kit is sufficient for 200 assays in 96 well plates.

Assay Buffer Catalog Number A8605	50 ml
Substrate Solution Catalog Number S8196	1.0 ml
Lysis Buffer 10× Catalog Number L6168	1.5 ml
Glutathione S-Transferase Catalog Number G9170	1.0 ml
Staurosporine Ready Made Catalog Number S6942	100 µl
Glutathione, reduced Catalog Number G4251	300 mg

Equipment and reagents needed but not supplied

- 96 well plates, Nunc® FluoroNunc™, black (Catalog Number P8741)
- 96 well plates, Greiner, black flat bottom (Catalog Number M0937)
- Plate fluorimeter
- Dulbecco's Phosphate Buffered Saline (PBS, Catalog Number D8537)
- An isotonic buffer such as 25 mM Hepes, pH 7.4, containing 250 mM sucrose for tissue extract preparation
- Ultra-Turrax® T-25 or equivalent (for tissue extract preparation)
- Multichannel pipette (optional)
- Microcentrifuge

Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

1× Lysis Solution – Dilute an aliquot of the Lysis Buffer 10× (Catalog Number L6168) 10-fold with Assay Buffer (Catalog Number A8605).

Reduced Glutathione Standard Solution – Prepare 1 ml of 100 mM Reduced Glutathione Standard Solution by dissolving 30.7 mg of Glutathione, reduced (Catalog Number G4251) in 1 ml of Assay Buffer (Catalog Number A8605). The 100 mM Reduced Glutathione Standard Solution may be frozen in aliquots at –20 °C for up to 30 days or kept at 2–8 °C for 3 days.

For the assay, prepare a fresh 1 mM Reduced Glutathione Standard Solution by diluting 10 µl of the 100 mM Reduced Glutathione Standard Solution with 990 µl of Assay Buffer (100-fold dilution). The diluted 1 mM Reduced Glutathione Standard Solution may be used for only 2 hours due to oxidation by atmospheric oxygen.

Sample Preparation for Cell and Tissue Extracts –

A. Cell Extract Preparation

The procedure is for cell extract preparations from $1–6 \times 10^7$ cells (in suspension or adherent cells) in a 150 cm^2 tissue culture flask.

1. Collect the cells from the flasks. For more than one flask collect the cells from each flask separately.
   - For cells in suspension centrifuge at 600 × *g* for 5 minutes.
   - For adherent cell lines - remove the cells from the flask with Trypsin/EDTA solution. Add 20 ml of growth medium (to neutralize the trypsin) and then centrifuge at 600 × *g* for 5 minutes.
2. Wash the cells with PBS and centrifuge at 600 × *g* for 5 minutes.
3. Discard the supernatant, suspend the pellet in 1 ml of PBS, and transfer to microcentrifuge tubes.
4. Centrifuge at 600 × *g* for 5 minutes. Discard the supernatant. The packed cell volume should be approximately 60-100 µl for the indicated cell counts ($1–6 \times 10^7$ cells).
5. To the cell pellet add 150 µl of 1× Lysis Solution per ~65 µl of packed cell volume (for a different volume of packed cells adjust the volume of 1× Lysis Solution accordingly). Vortex to mix.
6. Incubate for 15 minutes on ice and centrifuge for 10 minutes at 16,000 × *g*.
7. Keep the supernatant liquid and discard the pellet.

B. Tissue Extract Preparation

Prepare a tissue homogenate using an isotonic buffer (25 mM Hepes, pH 7.4, containing 250 mM sucrose).

1. Add 4 ml of the isotonic buffer per 1 gram of washed tissue.
2. Homogenize the tissue with an Ultra-Turrax T-25 homogenizer for 20 seconds and then centrifuge at 1,000 × *g*.
3. Discard the nuclear pellet and add a tenth volume of Lysis Buffer 10× (Catalog Number L6168) to the supernatant.
4. Incubate for 10 minutes on ice and then centrifuge 10 minutes at 16,000 × *g*. Use the supernatant for the assay.

Storage/Stability

The kit is shipped on wet ice and storage at –20 °C is recommended.

Procedure

A. Assay for reduced glutathione in cell or tissue extracts (endpoint reaction)

The procedure for the fluorimetric measurement of both the samples and standards is performed at the same time. It is recommended to test the unknown samples in duplicates.

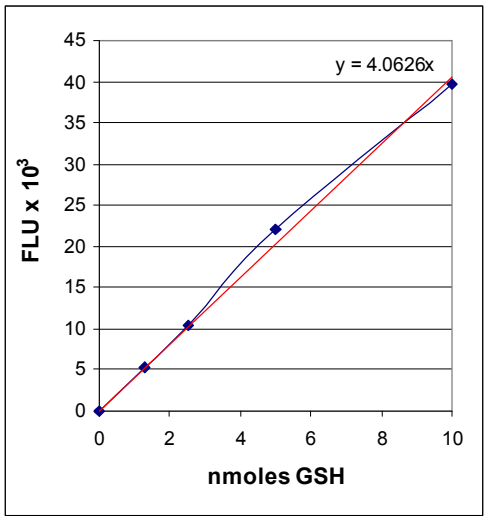
1. Set the fluorimeter plate reader filter at an excitation wavelength of 360±40 nm and emission wavelength of 485±20 nm. Optimize the sensitivity according to the instrument available.
   Note: The optimal wavelengths for measuring fluorescence:
   $\lambda_{excitation}$ = 390 nm
   $\lambda_{emission}$ = 478 nm.
2. For a reduced glutathione standard curve, add the amounts of 1 mM Reduced Glutathione Standard Solution (see Table 1) to separate wells of the 96 well plate (Catalog Number P8741).
3. Place a suitable amount of unknown sample in the other wells (perform in duplicate):
   1-6 µl of the extract obtained from cells or
   10-20 µl of the tissue extracts
   (protein content in the range of 5-20 mg/ml)
   prepared according to the Sample Preparation for Cell and Tissue Extracts section.
4. Add the Assay Buffer (Catalog Number A8605) and GST (Catalog Number G9170) reagents (see Table 1).

Table 1.
Reaction/Standard Curve Schemes

Sample	1 mM Reduced Glutathione Standard Solution (µl)	Unknown Sample (µl)	Assay Buffer (µl)	GST Enzyme (µl)	Substrate Solution (µl)
Blank	–	–	92.5	5	2.5
GSH Standard 1.3 nmoles	1.3	–	91.0	5	2.5
GSH Standard 2.5 nmoles	2.5	–	90.0	5	2.5
GSH Standard 5 nmoles	5	–	87.5	5	2.5
GSH Standard 10 nmoles	10	–	82.5	5	2.5
Test Sample	–	1–30	92.5 minus volume of sample	5	2.5

5. Start the reaction by addition of the 2.5 µl of Substrate Solution (Catalog Number S8196), preferably with a multichannel pipette and incubate the plate at 37 °C for 60 minutes.
6. Read the plate in the plate reader.
7. Use the values obtained for the GSH standards to plot a standard curve. The amount of reduced glutathione present in the sample may be determined from the standard curve (linear regression analysis will indicate the amount of fluorescence per nmole of standard).

Figure 1.
Typical Standard Curve



B. Cell based assay for reduced glutathione (kinetic reaction)

The following procedure is for measurement of reduced glutathione *in-situ* as a cell based assay. The fluorimeter wavelength settings are the same as in Procedure A. If possible, read the plates from the bottom. The sensitivity setting should be recalibrated for different plate types.

1. Grow adherent cells (2×10^4 to 1×10^5 per well) in duplicates for ~16 hours in a 96 well black fluorimeter plate with transparent bottomed wells (Catalog Number M0937). Leave the rest of the wells empty for determining the standard curve.
2. Before assaying, wash the cell containing wells twice with 200 µl of PBS and then add to each well 97 µl of Assay Buffer.
3. In the same plate prepare reduced glutathione standards (0–10 nmoles) by the addition of 1.3, 2.5, 5 and 10 µl of 1 mM Reduced Glutathione Standard Solution to the appropriate wells. Bring the volume in each well to 92.5 µl with Assay Buffer and add 5 µl of GST enzyme.
4. Add 2.5 µl of the Substrate Solution to all the wells and incubate the plate at room temperature in the fluorimeter.
5. Measure the fluorescence at 20 minute intervals for up to 3 hours until the readings reach a plateau. Note: Human cell lines contain GST isozymes that do not efficiently utilize the monochlorobimane as a substrate, whereas, rodent cell lines contain GST isozymes that are much more suited to this substrate.[4] Thus the results of direct kinetic assays should be interpreted accordingly.

References

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3. Fernandez-Checa, J.C., and Kaplowitz, N., The use of monochlorobimane to determine hepatic GSH levels and synthesis. Anal. Biochem., **190**, 212-219 (1990).
4. Kamencic, H., *et al*., Monochlorobimane fluorimetric method to measure tissue glutathione, Anal. Biochem., **286**, 35-37 (2000).
5. Sebastia, J., *et al*., Evaluation of fluorescent dyes for measuring intracellular glutathione content in primary cultures of human neurons and Neuroblastoma SH-SY5Y. Cytometry Part A, **51A**, 16-25 (2003).
6. Marchetti, P., *et al*., Redox regulation of apoptosis: impact of thiol oxidation status on mitochondrial function. Eur. J. Immunol., **27**, 289-296 (1997).
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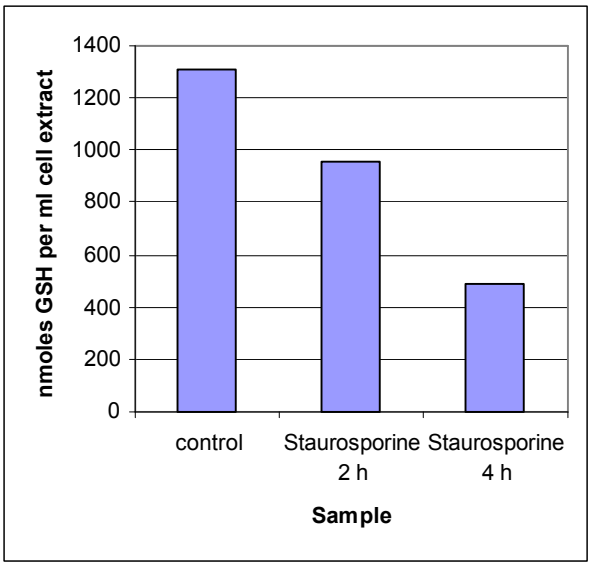
Appendix

Induction of apoptosis with Staurosporine in HeLa cells

1. HeLa cells were grown in three 150 cm^2 flasks to ~80% confluence.
2. Staurosporine Ready Made (Catalog Number S6942) was added to one of the flasks to give a final concentration of 1 μg/ml in the growth medium (i.e., ~1:500 dilution).
3. After 2 hours Staurosporine Ready Made was added to another flask (see step 2).
4. After 4 hours from the beginning of the procedure the cells were collected separately from all 3 flasks.
5. The cells were extracted as described in Cell Extract Preparation procedure and the samples were tested for the level of reduced glutathione.

Figure 2.
Glutathione levels after apoptosis induction in HeLa cells



The results shown in Figure 2 indicate a marked reduction in the reduced glutathione content of the Staurosporine treated cells.