Technical Bulletin

Glutathione Assay Kit

Catalogue number CS0260

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.

Glutathione is present inside cells mainly in the reduced form (90–95% of the total glutathione). Oxidation of glutathione leads to the formation of glutathione disulfide (GSSG). Intracellular GSH status appears to be a sensitive indicator of the overall health of a cell, and of its ability to resist toxic challenge. High levels of GSH in the cell may indicate pathological changes.

The Glutathione Assay Kit offers all the required reagents for a simple and quick assay to measure the level of total glutathione (GSSG + GSH) in a biological sample. In addition, the kit provides 5-sulfosalicylic acid, which is required to deproteinize the biological sample.

The kit has been tested on samples prepared from mammalian tissues such as liver, kidney, brain, spleen, and heart muscle, on human plasma and erythrocytes, and on cell lines such as HeLa, A549, Jurkat, U937, A431, COS, CHO, and NIH 3T3.

Components

The kit is sufficient for 700 assays.

- Assay Buffer 5× for Glutathione — 30 ml
  Catalogue Number (A5103)
  500 mM potassium phosphate, pH 7.0 containing 5 mM EDTA
- Glutathione Reductase — 20 units
  Catalogue Number G2424
  400 units per ml of glutathione reductase from baker's yeast in 3.6 M ammonium sulfate, pH 7.0, containing 0.1 mM dithiothreitol
- Glutathione Reduced, Standard — 0.3 mg
  Catalogue Number G4544
- 5,5′-Dithiobis(2-nitrobenzoic acid) [DTNB] — 8 mg
  Catalogue Number D8130
- 5-Sulfosalicylic Acid — 2.5 g
  Catalogue Number S2130
- NADPH — 25 mg
  Catalogue Number MUS84
- Dimethyl Sulfoxide (DMSO) — 7.5 ml
  Catalogue Number D8418

Equipment Required but Not Provided

- 96-well plate
- Plate reader
- Multi-channel pipette

Precautions and Disclaimer

For R&D use only. Not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability

Upon arrival of the kit, the components should be placed at the following temperatures:

DTNB (Catalogue Number D8130), 5-Sulfosalicylic Acid (Catalogue Number S2130), and DMSO (Catalogue Number D8418) should be stored at room temperature.

The Assay Buffer 5× for Glutathione (Catalogue Number A5103), Glutathione Reductase (Catalogue Number G2424), and Glutathione Reduced, Standard (Catalogue Number G4544) should be stored at 2–8 °C.

NADPH (Catalogue Number MUS84) should be stored at –20 °C.

Preparation Instructions

Reagent Preparation

Use ultrapure (17 MΩ·cm or equivalent) water for the preparation of reagents.

Stock Solutions

DTNB Stock Solution (1.5 mg/ml) – Dissolve the contents of the bottle (8 mg) of DTNB (Catalogue Number D8130) with 5.33 ml of DMSO (Catalogue Number D8418) to make a 1.5 mg/ml solution. The solution may be stored in aliquots at –20 °C for up to 3 months.

NADPH Stock Solution (40 mg/ml) – Dissolve the contents of the vial of NADPH (25 mg) in 0.625 ml of water to give a 40 mg/ml solution. The solution may be stored at –20 °C for up to 6 months.

5% 5-Sulfosalicylic Acid (SSA) Solution – Dissolve the contents of the bottle of 5-sulfosalicylic acid (2.5 g) in 50 ml of water. Ensure the powder is completely dissolved. Keep at 2–8 °C.

Glutathione (GSH) Standard Stock Solution (10 mM) Dissolve the contents of the vial of Glutathione Reduced, Standard in 0.1 ml of water. The solution may be stored at –20 °C for at least 3 months.

Working Solutions

The volumes prepared are sufficient for at least 48 reactions of 200 µl performed in a 96 well plate.

1× Assay Buffer (12 ml) - 100 mM potassium phosphate buffer, pH 7.0, with 1 mM EDTA. Dilute 2.4 ml of Assay Buffer 5× (Catalogue Number A5103) five-fold by addition of 9.6 ml of water.

Enzyme Solution (6 units/ml, 0.25 ml) – Dilute 3.8 µl of Glutathione Reductase (Catalogue Number G2424, 400 units/ml) to a final volume of 250 µl with 1× Assay Buffer.

NADPH Solution (0.16 mg/ml, 2.5 ml) – Add 10 µl of NADPH Stock Solution (40 mg/ml) to 2.5 ml of 1× Assay Buffer.

Working Mixture (8 ml) – To 8 ml of 1× Assay Buffer, add 228 µl of the diluted Enzyme Solution (6 units/ml) and 228 µl of DTNB Stock Solution (1.5 mg/ml). Mix well. This solution may be kept for up to 3 hours at room temperature.

Glutathione Standard Solutions – Dilute an aliquot of the Glutathione (GSH) Standard Stock Solution (10 mM) 200-fold to 50 µM with the 5% 5-Sulfosalicylic Acid (SSA) Solution. Prepare the 50 µM solution in the 5% SSA Solution fresh for each standard curve. A glutathione solution is considerably more stable in water than in 5% SSA Solution, so the stock solution should always be kept in water at –20 °C.

Prepare the Glutathione Standard Solutions by serial dilution as shown in Table 1. Begin with 50 µl of the 50 µM glutathione solution in the first well and then dilute two-fold each time by taking an aliquot of 25 µl from the previous well and adding it to 25 µl of 5% SSA Solution.

Table 1.
Preparation of Glutathione Standard Solutions by serial dilutions of the 50 µM glutathione solution.

Well Number	1	2	3	4	5
GSH Concentration (µM)	50	25	12.5	6.25	3.125
GSH Solution (µl)	50	25 (from well 1)	25 (from well 2)	25 (from well 3)	25 (from well 4)
5% SSA (µl)	None	25	25	25	25
nmoles GSH in a 10 µl sample	0.5	0.25	0.125	0.0625	0.0312

Procedure

The biological sample is first deproteinized with the 5% 5-Sulfosalicylic Acid Solution, centrifuged to remove the precipitated protein, and then assayed for glutathione (see Appendix, Sample Preparation). The measurement of GSH uses a kinetic assay in which catalytic amounts (nmoles) of GSH cause a continuous reduction of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) to TNB and the GSSG formed is recycled by glutathione reductase and NADPH. The GSSG present will also react to give a positive value in this reaction.

1. $2\ GSH + DTNB \rightarrow GSSG + 2\ TNB$

2. $GSSG + NADPH + H^+ \xrightarrow[\text{reductase}]{\text{Glutathione}} 2\ GSH + NADP^+$

The combined reaction:

$$DTNB + H^+ + NADPH \xrightarrow[GSSG/GSH]{\text{Glutathione reductase}} 2\ TNB + NADP^+$$

The reaction rate is proportional to the concentration of glutathione up to 2 µM. The yellow product, 5-thio-2-nitrobenzoic acid (TNB) is measured spectrophotometrically at 412 nm. The assay uses a standard curve of reduced glutathione to determine the amount of glutathione in the biological sample.

Assay Reaction

1. Set a plate reader to 412 nm with kinetic read at 1-minute intervals for 5 minutes.
2. Set up the reaction scheme according to Table 2. Perform every test in duplicate.

Table 2.
Reaction Scheme

	Mix and incubate 5 minutes			Start
Sample measured	**Sample volume**	**5% SSA**	**Working Mixture**	**NADPH** (0.16 mg/ml)
Reagent Blank	–	10 µl	150 µl	50 µl
Standard curve (various dilutions)	10 µl	–	150 µl	50 µl
Unknown sample	X µl	10-X	150 µl	50 µl

Note: The final concentration of the components in the reaction mixture is 95 mM potassium phosphate buffer, pH 7.0, 0.95 mM EDTA, 0.038 mg/ml (48 µM) NADPH, 0.031 mg/ml DTNB, 0.115 units/ml glutathione reductase, and 0.24% 5-sulfosalicylic acid.

3. The first 2 wells should contain only 10 µl of the 5% 5-Sulfosalicylic Acid Solution as a reagent blank. Add duplicate 10 µl samples of the prepared Glutathione Standard Solutions into separate wells of the plate. Add varying volumes of the unknown sample in duplicate into separate wells (up to 10 µl sample).
   Note: If necessary, bring the final volume of the unknown sample up to 10 µl with 5% SSA Solution.
4. Add 150 µl of the Working Mixture to each well with a multichannel pipette. Mix by pipetting up and down.
5. Incubate 5 minutes at room temperature and then add 50 µl of the diluted NADPH Solution with a multichannel pipette. Mix by pipetting up and down.
6. Use the plate reader to measure the absorbance in each well. Subtract the reagent blank value from every measurement, unless software is used that performs this automatically.

Results

Calculation

Use the values of the Glutathione Standard Solutions to determine the standard curve and calculate the ΔA412/min equivalent to 1 nmole of reduced glutathione per well. Calculate the nmoles of GSH in the unknown sample.

$$\text{nmoles GSH per ml of sample} = \frac{\Delta A_{412}/\text{min(sample)} \times \text{dil}}{\Delta A_{412}/\text{min(1 nmole)} \times \text{vol}}$$

where:

ΔA_{412}/min(sample) = slope generated by sample (after subtracting the values generated by the blank reaction).

ΔA_{412}/min(1 nmole) = slope calculated from standard curve for 1 nmole of GSH

dil = dilution factor of original sample

vol = volume of sample in the reaction in ml

Appendix

Biological Sample Preparation

Reagents:

- Dulbecco's Phosphate Buffered Saline (PBS) (Catalogue Number D8537)
- Liquid nitrogen

Equipment:

- Microcentrifuge
- Microcentrifuge tubes
- Pestle and glass tube, Potter-Elvehjem (PTFE in glass homogenizer), 3 ml (Catalogue Number P7734)
- Overhead electric motor
- Thermostatic bath (37 °C)
- Pestle and mortar

Tissue Extracts

1. Samples that contain a lot of blood should be washed twice with PBS before flash freezing.
2. Flash freeze the tissue in liquid nitrogen immediately after excision from the subject. This tissue can then be ground in a pestle and mortar with liquid nitrogen to prepare a fine powder.
3. Take an aliquot of the powder (0.1–0.3 g) and add 3 volumes of 5% 5-Sulfosalicylic Acid (SSA) Solution (0.3–0.9 ml) and vortex. Add another seven volumes of the 5% SSA Solution. Homogenize the sample with a 3 ml PTFE pestle in glass tube until an even suspension is achieved.
4. Leave at 2–8 °C for 10 minutes and then centrifuge at 10,000 × g for 10 minutes.
5. Measure the volume of the supernatant and use this as the original sample volume in the calculation for glutathione determination. Keep the sample at 2–8 °C.
6. For the assay procedure, dilute the sample 5 to 20-fold to stay in the detection range.

Note: If the assay cannot be performed immediately (within 2 hours), the extract may be stored at −70 °C for up to 10 days.

Blood (red blood cells or plasma)

Best results are obtained with fresh blood samples.

1. Separate the red blood cells from plasma by centrifugation at 600 × g for 10 minutes. The pellet contains the red blood cells and the supernatant is the plasma fraction.
2. Wash the red blood cells twice with 3 volumes of PBS (Suspend in PBS and then centrifuge for 10 minutes at 600 × g).
3. Take a 200 µl aliquot of the red blood cell pellet or plasma (from step 1) and add 200 µl of 5% SSA Solution.
4. Vortex each sample vigorously and leave for 10 minutes at 2–8 °C.
5. Centrifuge at 10,000 × g for 10 minutes.
6. Measure the volume of the supernatant and use this as the original sample volume in the calculation for glutathione determination. Keep the samples at 2–8 °C.
7. For the assay of red blood cells, dilute the sample ~10-fold to stay in the detection range. In order to obtain a significant value of GSH when measuring its level in plasma, more than 10 µl of the supernatant from step 6 may be needed. Alternatively, the absorbance may be recorded for a longer time period.

Note: If the assay cannot be performed immediately (within 2 hours), the extract may be stored at −70 °C for up to 10 days.

Cell Extracts

Best results are obtained with fresh cell samples.

1. Wash at least 10^8 cells with PBS, then suspend to a cell density of 1×10^8 cells per ml in PBS and transfer the cells to a microcentrifuge tube.
2. Centrifuge the cells at 600 × g to obtain a packed cell pellet. Remove the supernatant.
3. Measure the volume of the pellet, add 3 volumes of the 5% SSA Solution to the packed cell pellet and vortex.
4. Freeze and thaw the suspension twice (use liquid nitrogen to freeze and a 37 °C bath to thaw) and leave for 5 minutes at 2–8 °C.
5. Centrifuge the extract at 10,000 × g for 10 minutes.
6. Measure the volume of the supernatant and use this as the original sample volume in the calculation for glutathione determination. Keep the sample at 2–8 °C.
7. For the assay procedure, there may be a need for sample dilution (up to 20-fold) in order to stay in the detection range.

Note: If the assay cannot be performed immediately (within 2 hours), the extract may be stored at −70 °C for up to 10 days.

References

1. Akerboom, T.P., and Sies, H., Assay of glutathione, glutathione disulfide, and glutathione mixed disulfides in biological samples. Methods Enzymol., 77, 373-382 (1981).
2. Nair, S. et al., Flow cytometric monitoring of glutathione content and anthracycline retention in tumor cells. Cytometry, 12, 336-342 (1991). fluminea (Muller), Comp. Biochem. Physiol. Part C, 131, 133-151 (2002).

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