Elite™ Glutathione GSH/GSSG Ratio Rapid Assay Kit (Green Fluorescence)

CATALOG NUMBER: CA-G076, 200 assays

Description

Glutathione is a tripeptide that contains L-cysteine, L-glutamic acid and glycine. It is the smallest intracellular protein thiol molecule in the cells, which prevents cell damage caused by reactive oxygen species such as free radicals and peroxides. Glutathione exists in reduced (GSH) and oxidized (GSSG) states. Reduced glutathione (GSH) is a major tissue antioxidant that provides reducing equivalents for the glutathione peroxidase (GPx) catalyzed reduction of lipid hydroperoxides to their corresponding alcohols and hydrogen peroxide to water. In the GPx catalyzed reaction, the formation of a disulfide bond between two GSH molecules generates oxidized glutathione (GSSG). The enzyme glutathione reductase (GR) recycles GSSG to GSH with the simultaneous oxidation of β-nicotinamide adenine dinucleotide phosphate (β-NADPH2). In healthy cells, more than 90% of the total glutathione pool is in the reduced form (GSH). When cells are exposed to increased levels of oxidative stress, GSSG accumulates and the ratio of GSSG to GSH increases. An increased ratio of GSSG-to-GSH is an indication of oxidative stress. The monitoring of reduced and oxidized GSH in biological samples is essential for evaluating the redox and detoxification status of the cells and tissues against oxidative and free radicals mediated cell injury.

There are quite a few reagents or assay kits available for quantitating thiols in biological systems. However, all the commercial kits either lack sensitivity or have tedious protocols. The Elite™ Glutathione GSH/GSSG Ratio Assay Kit provides an ultrasensitive assay to quantitate GSH in the sample. The kit uses a proprietary non-fluorescent dye that becomes strongly fluorescent upon reacting with GSH. With a one-step fluorimetric method, the kit can detect as little as 1 picomole of GSH or GSSG in a 100 µl assay volume (10 nM; Figures 1 and 2). The assay can be performed in a convenient 96-well or 384-well microtiter-plate format and readily adapted to automation without a separation step. Its signal can be easily read by a fluorescence microplate reader at Ex/Em = 490/520 nm.

Kit Components

- **Component A**: Thiolite™ GreenWS — 1 vial
- **Component B**: Assay Buffer — 1 bottle (25 ml)
- **Component C**: GSH Standard — 1 vial (62 µg)
- **Component D**: GSSG Probe — 1 bottle (lyophilized powder)
- **Component E**: GSSG Standard — 1 vial (124 µg)
- **Component F**: Cell/Tissue Lysis Buffer — 1 bottle (10 ml)

Storage

Keep **Component A** in freezer (-20 °C) and avoid exposure to light; **Component C, D, E** at -20 °C; **Component B, &F** at 4 °C. All components are stable for 6 months after receipt if stored properly.

Shelf Life

All reagents are stable for 6 months after receipt when stored properly at the recommended conditions.

Materials required (but not supplied)

- 96 microplates: Tissue culture microplate with black wall and clear bottom is recommended
- Fluorescence microplate reader

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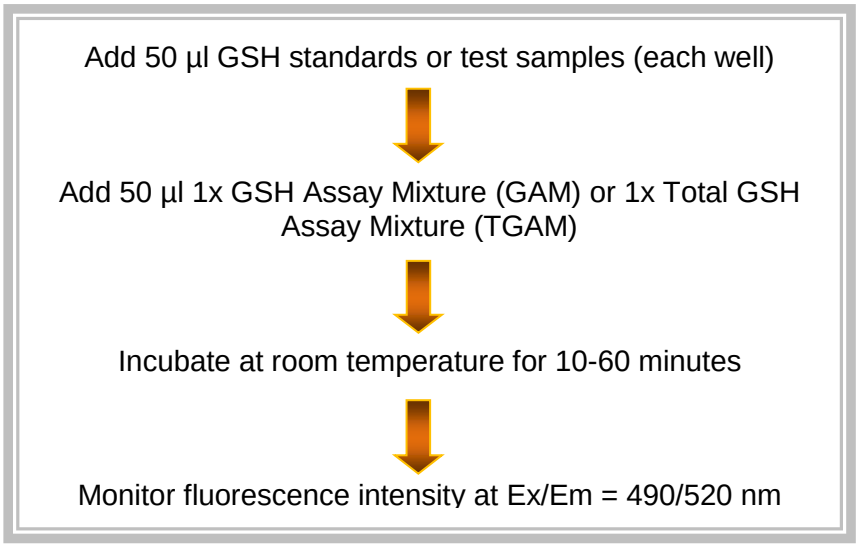
Assay Protocol (for 100 assays in 96-well plate)

Brief Summary of the Assay



1. **Preparation of GSH standard stock solution:**

   Add 200 µl of Assay Buffer (**Component B**) into the vial of GSH Standard (**Component C**) to make 1 mM (1 nmol/µl) GSH standard stock solution.

   Note: The unused 1 mM GSH standard stock solution should be divided into single use aliquots and stored at -20 °C.

2. **Preparation of GSSG standard stock solution:**

   Add 200 µl of ddH_2O into the vial of GSSG Standard (**Component E**) to make 1 mM (1 nmol/µl) GSSG standard stock solution.

   Note: The unused GSSG standard stock solution should be divided into single use aliquots and stored at -20 °C.

3. **Preparation of 100x Thiolite™ GreenWS stock solution:**

   Add 100 µl of ddH_2O into the vial of Thiolite™ GreenWS (**Component A**) to make 100X Thiolite™ GreenWS stock solution.

   Note: The unused Thiolite™ GreenWS stock solution should be divided into single use aliquots, stored at -20 °C, and kept from light.

4. **Preparation of 1x GSH assay mixture (GAM):**

   Add 100 µl of 100X Thiolite™ GreenWS stock solution (from Step 3) into 10 ml of Assay Buffer (**Component B**), and mix them well.

   Note: *always use freshly made 1x GSH assay mixture (GAM) and do not keep it for overnight use.*

5. **Preparation of Total GSH Assay Mixture (TGAM):**

   Add 5 ml of GAM (from Step 4) into the bottle of GSSG Probe (Component E), and mix them well.

   Note: *This total GSH assay mixture (TGSM) is enough for one 96-well plate. It is unstable at room termperature, and should be used promptly within 2 hours and avoid exposure to light.*

   Note: *Alternatively, one can prepare a 25x GSSG Probe by adding 200 µl of ddH_2O into the bottle of Component E, and then prepare the TGSM assay mixture by mix the stock solution with GAM (from step 4) proportionally. Aliquot and store the unused 25x GSSG Probe stock solution at -20 °C, and avoid freeze-thaw cycles.*

6. **Preparation of serially diluted GSH standards (0 to 5 µM):**

   6.1. Add 10 µl of GSH standard stock solution (from Step 1) into 990 µl of Assay Buffer (Component B) to generate 10 µM (10 pmol/µl) GSH standard solution.

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Note: Diluted 10 µM GSH standard solution is unstable. Use within 4 hours.

6.2. Take 200 µl of 10 µM GSH standard solution to perform 1:2 serial dilutions to get 5, 2.5, 1.25, 0.625, 0.3125, 0.1563, 0.0781, and 0 µM serially diluted GSH standards.

6.3. Add GSH standards and GSH-containing test samples into a solid black 96-well microplate as shown in Table 1 and Table 2. When just GSH assay is needed, fill ONLY the wells in two left columns (Panel A) according to Table 1. Skip Step 7 and go directly to Step 8.

Note: Treat cells or tissue samples as desired (***see Appendix A***).

7. Preparation of serially diluted GSSG standards (0 to 5 µM):

7.1. Add 10µl of GSSG standard stock solution (from Step 2) into 990 µl of Assay Buffer (Component B) to generate 10 µM (10 pmol/µl) GSSG standard solution.

Note: Diluted GSSG standard solution is unstable. Use within 4 hours.

7.2. Take 200 µl of 10 µM GSSG standard solution to perform 1:2 serial dilutions to get 5, 2.5, 1.25, 0.625, 0.3125, 0.1563, 0.0781, and 0 µM serially diluted GSSG standards. The concentrations of Total GSH standard solutions should be twice the concentrations of GSSG standard solutions as 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.1563, and 0 µM.

7.3. Add GSSG standards and GSH-containing test samples into a solid black 96-well microplate as shown in Tables 1 and 2. When Total GSH assay is needed, fill the wells in both Panel A (left) and Panel B (right) according to Table 1.

Note: Treat cells or tissue samples as desired.

Table 1. Layout of GSH standards, GSSG standards, and test samples in a solid black 96-well microplate

Panel A (GSH)						**Panel B (GSSG)**					
BL	BL	TS	TS			BL	BL	TS	TS		
GSH1	GSH1					GSSG1	GSSG1				
GSH2	GSH2					GSSG2	GSSG2				
GSH3	GSH3					GSSG3	GSSG3				
GSH4	GSH4					GSSG4	GSSG4				
GSH5	GSH5					GSSG5	GSSG5				
GSH6	GSH6					GSSG6	GSSG6				
GSH7	GSH7					GSSG7	GSSG7				

Note: GS= GSH Standards; GSSG= GSSG Standards; BL=Blank Control; TS=Test Samples.

Table 2. Reagent composition for each well

GSH and/or GSSG Standards	Blank Control	Test Sample
Serial Dilutions: 50 µl	Assay Buffer: 50 µl	50 µl

Note: Add serially diluted GSH standards from 0.01 µM to 5 µM into wells from GS1 to GS7 in duplicate. Add the serially diluted GSSG standard from 0.01 µM to 5 µM into wells from GSSG1 to GSSG7 in duplicate. Add TS into wells in both Panel A and Panel B.

8. **Run GSH and Total GSH assay:**

8.1. Add 50 µl of GSH Assay Mixture (GAM, from Step 4) into the wells in Panel A (left) of GSH standard, blank control, and test samples (from Step 6.3) to make the total assay volume of 100 µl/well.

Note: For a 384-well plate, add 25 µl of sample and 25 µl of GSH Assay mixture into each well.

8.2. If total GSH (in reduced and oxidized states) assay is needed, add 50 µl of Total GSH Assay Mixture (TGAM from Step 5) into the wells in Panel B (right) of GSH standard, blank control, and test samples (From Step 6.3) to make the total assay volume of 100 µl/well.

Note: For a 384-well plate, add 25 µl of sample and 25 µl of Total GSH Assay Mixture into each well.

8.3. Incubate the reaction at room temperature for 10 minutes to 1 hour, protected from light.

8.4. Monitor the fluorescence increase at Ex/Em = 490/520 nm using a fluorescence plate reader.

Data Analysis

1. Calibration Curve:

The fluorescence in blank wells (with the assay buffer only) is used as a control, and is subtracted from the values for those wells with the GSH reactions. ***Note:*** The fluorescence background increases with time, thus it is important to subtract the fluorescence intensity value of the blank wells for each data point.

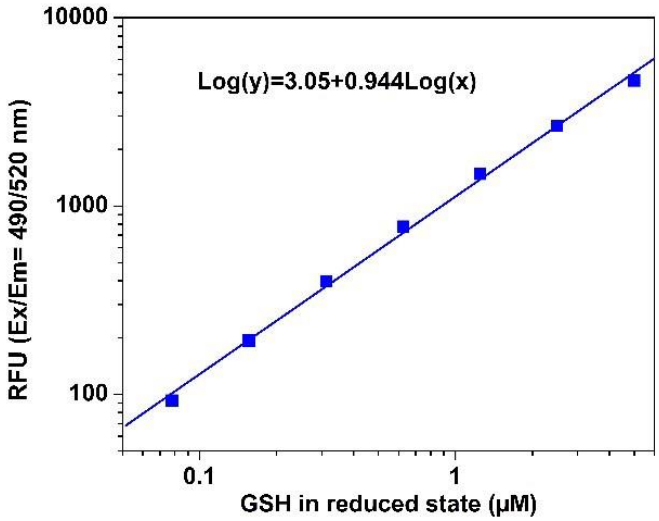

Figure 1. Reduced GSH dose responses were measured in a black 96-well plate with Elite™ Rapid Fluorimetric Glutathione GSH/GSSG Ratio Assay Kit using a Gemini fluorescence microplate reader (Molecular Devices). 50 µl of GSH standards (0.078 to 5 µM) or blank control was added into each well, and then 50 µl of GSH Assay Mixture was added. The fluorescence intensity was measured at Ex/Em = 490/520 nm after 30 minutes' incubation.

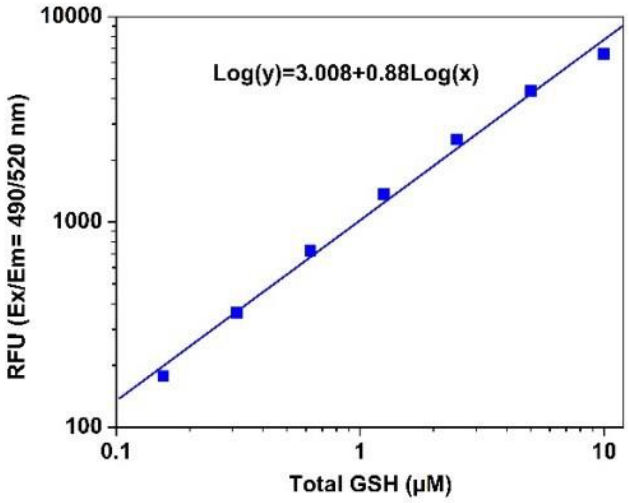

Figure 2. Total GSH dose responses were measured with Elite™ Rapid Fluorimetric Glutathione GSH/GSSG Ratio Assay Kit in a black 96-well plate using a Gemini fluorescence microplate reader (Molecular Devices). 50 µl of GSSG standards (0.078 to 5 µM which is equivalent to 0.156 to 10 µM Total GSH), GSH-containing samples or blank control were added into each well, and then 50 µl of Total GSH Reaction Mixture was added. Fluorescence intensity was measured at Ex/Em = 490/520 nm after 30 minutes' incubation.

2. GSH or Total GSH Concentration Determination:

The change of fluorescence intensity with GSH concentration can be described as a linear regression:

$Log(y) = A + B \times Log(x)$

Note: The equation is generated by most instrument software.

[GSH] can be calculated by the equation from the GSH standard calibration curve as shown in Figure 1.

[Total GSH] can be calculated by the equation from the Total GSH standard calibration curve as shown in Figure 2

Example1: The data from above experiment is shown in the following table.

Panel A (GSH)						Panel B (GSSG)					
4885.264	4885.264	1300.846	1300.846			6861.1375	6861.1375	3026.36	3026.36		
2916.466	2916.466	….	….			4634.0045	4634.0045	….	….		
1727.133	1727.133					2813.1135	2813.1135				
1025.278	1025.278					1648.495	1648.495				
647.0175	647.0175					1008.247	1008.247				
441.613	441.613					646.887	646.887				
341.465	341.465					463.8995	463.8995				
248.9975	248.9975					286.639	286.639				

GSH calibration curve is plotted as shown in Figure 1: Log(y) = 3.05+0.994Log(x)

Total GSH calibration curve is plotted as shown in Figure 2: Log(y) = 3.008+0.88Log(x)

3. GSSG Concentration Determination:

Example 2: Use the data in Example 1

GSH Assay: TS1 = 1300.846, → [GSH] = 0.934 μM from Equation (1)

Total GSH Assay: TS1 = 3026.36 → [Total GSH] = 3.447 μM from Equation (2)

[GSSG] = ([Total GSH]-[GSH])/2 = (3.447 – 0.934)/2 = 1.256 μM.

4. GSH/GSSG Ratio Determination:

Example 3: Use the data in Example 1

[GSH]/[GSSG] = 0.934/1.256 = 0.744

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Appendix A

Sample cell lysis preparation for the assay kits

This protocol serves as a general guide for preparing samples for the assays. For the best results, users need to choose their own method for cell lysis preparation. ***A method of freeze-thaw (or other lysis methods using physical disruption in PBS) is strongly recommended for the preparation of cell lysate, especially when the background noise became an issue for this assay.***

1. Lysis of plant cells

   Homogenize the leaves with the Cell/Tissue Lysis Buffer (**Component F**) at 200 mg/ml, and centrifuge at 2500 rpm for 5-10 minutes. Use the supernatant for the assay.

2. Lysis of bacterial cells

   Collect bacterial cells by centrifugation (i.e. 10,000 x g, 0°C, 15 min). Add Cell/Tissue Lysis Buffer (**Component G**) to the pellet (1 mL per 100 to 10,000,000 cells), and leave at room temperature for 15 minutes. Centrifuge at 2500 rpm for 5 minutes, and use the supernatant for the assay.

3. Lysis of mammalian cells

   Remove the medium from the culture plate (wells). Use about 100 µL Cell/Tissue Lysis Buffer (**Component G**) per 1-5 million cells (or add lysis buffer 100 µL lysis buffer per well in a 96-well cell culture plate), and leave at room temperature for 15 minutes. For the assay, use the lysate directly or centrifuge at 1500 rpm for 5min then use the supernatant.

4. Lysis of tissues

   Weigh ~ 20 mg tissue, then wash with cold PBS buffer. Homogenize the tissue with 400 µl of Cell/Tissue Lysis Buffer (**Component G**) in a micro-centrifuge tube, and centrifuge at 2500 rpm for 5-10 minutes. Use the supernatant for the assay.

   Note: for additional Cell/Tissue Lysis Buffer (Cat# CA-N015g)