Amplite™ Fluorimetric Glutathione GSH/GSSG Ratio Assay Kit

Green Fluorescence

Ordering Information	*Storage Conditions*	*Instrument Platform*
Product Number: 10056 (100~200 assays)	Keep at -20 °C Avoid exposure to moisture and light	Fluorescence microplate readers

Introduction

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-$NADPH_2$). 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. Our Amplite™ Fluorimetric 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 Key Features

Broad Application:	Can be used for a variety of biological systems (e.g., plasma, urine and cell extracts).
Sensitive:	Detect as low as 1 picomole of glutathione.
Continuous:	Readily adapted to automation without a separation step.
Convenient:	Formulated to have minimal hands-on time. No wash is required.
Non-Radioactive:	No special requirements for waste treatment.

Kit Components

Components	Amount
Component A: Thiolite™ Green	1 vial
Component B: Assay Buffer	1 bottle (25 mL)
Component C: GSH Standard	1 vial (62 µg)
Component D: DMSO	1 vial (200 µL)
Component E: GSSG Probe	1 bottle (lyophilized powder)
Component F: GSSG Standard	1 vial (124 µg)

Protocol for 100 assays (96-well plate)

Brief Summary

Prepare Thiolite™ Green reaction mixture (50 µL) → Add GSH standards and/or GSSG standards or test samples (50 µL) → Incubate at RT for 10 to 60 minutes → Monitor the fluorescence increase at Ex/Em = 490/520 nm

Note: Thaw all the kit components at room temperature before starting the experiment.

1. Prepare 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.

Ordering: sales@aatbio.com; Tel: 800-990-8053 or 408-733-1055; Fax: 408-733-1304
Technical Support: support@aatbio.com; Tel: 408-733-1055

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

2. Prepare GSSG standard stock solution:

Add 200 µL of ddH_2O into the vial of GSSG Standard (Component F) 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. Prepare 100X Thiolite™ Green stock solution:

Add 100 µL of DMSO (Component D) into the vial of Thiolite™ Green (Component A) to make 100X Thiolite™ Green stock solution.

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

4. Prepare GSH Assay Mixture (GAM):

Add 100 µL of 100X Thiolite™ Green stock solution (from Step 3) into 10 mL of Assay Buffer (Component B), and mix them well by vortexing.

Note1: This GSH assay mixture (GAM) is enough for two 96-well plates. It is unstable at room temperature, and should be used promptly within 2 hours and avoid exposure to light.

Note2: Alternatively, one can make GSH Assay Mixture by adding 100X Thiolite™ Green stock solution with Assay Buffer proportionally.

5. Prepare 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.

Note1: This Total GSH assay mixture (TGAM) is enough for one 96-well plates. It is unstable at room temperature, and should be used promptly within 2 hours and avoid exposure to light.

Note2: Alternatively, one can make a 25X GSSG Probe by adding 200 µL of ddH_2O into the bottle of Component E, and then prepare the TGAM 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. Prepare serially diluted GSH standards (0 to 5 µM):

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

7. **Prepare 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)**					
GSH 1	GSH 1	TS….	TS….			GSSG 1	GSSG 1	TS….	TS….		
GSH 2	GSH 2	….	….			GSSG 2	GSSG 2	….	….		
GSH 3	GSH 3					GSSG 3	GSSG 3				
GSH 4	GSH 4	BL				GSSG 4	GSSG 4				
GSH 5	GSH 5					GSSG 5	GSSG 5				
GSH 6	GSH 6					GSSG 6	GSSG 6				
GSH 7	GSH 7					GSSG 7	GSSG 7				
BL	BL					BL	BL				

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

Table 2 Reagent composition for wells

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 to5 µM into wells from GS1 to GS7 in duplicate. Add the serially diluted GSSG standard from 0.01 µM to5 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 GSSG 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 with 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.*

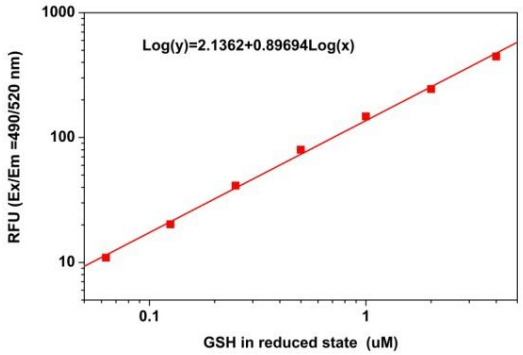

Figure 1. Reduced GSH dose responses were measured in a black 96-well plate with Amplite™ Fluorimetric Glutathione GSH/GSSG Ratio Assay Kit using a Gemini fluorescence microplate reader (Molecular Devices). 50 µL of GSH standards (0.01to 5 µM) or blank control was added into each well, and then 50 µL of GSH Assay Mixture was added. The fluorescence intensity was measured at Ex/Em = 490/520 nm after 30 minutes incubation.

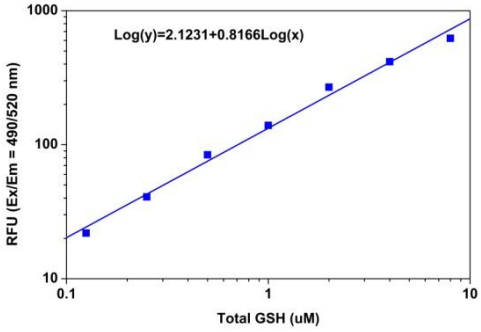

Figure2. Total GSH dose responses were measured with Amplite™ Fluorimetric Glutathione GSH/GSSG Ratio Assay Kit in a black 96-well plate using a Gemini fluorescence microplate reader (Molecular Devices). 50 µL of GSSG standards (0.01 to 5 µM), GSH-containing samples or blank control were added into each 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:
$Log(y) = A + B * Log(x)$
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)**					
458.35	458.35	141	141			634.993	634.993	336	336		
257.558	257.558	….	….			428.513	428.513	….	….		
160.928	160.928					281.123	281.123				
93.101	93.101					151.857	151.857				
54.436	54.436					96.522	96.522				
33.434	33.434					53.277	53.277				
24.155	24.155					34.407	34.407				
13.239	13.239					12.505	12.505				

GSH calibration curve is plotted as shown in Figure 1: Log(y) = 2.1362+0.89694Log(x) (1)
Total GSH calibration curve is plotted as shown in Figure 2: Log(y) = 2.1231+0.8166Log(x) (2)

3. **GSSG Concentration Determination:**

Example 2: Use the data in Example 1
GSH Assay: TS1 = 141-13.24, → [GSH] = 1.034 µM from Equation (1)
Total GSH Assay: TS1 = 336 – 12.5 → [Total GSH] = 3.12 µM from Equation (2)
[GSSG] = ([Total GSH]-[GSH])/2 = (3.12 - 1.034)/2 = 1.042 µM

4. **GSH/GSSG Ratio Determination:**

Example 3: Use the data in Example 1
[GSH]/[Total GSH] = 1.034/3.12 = 33%
[GSH]/[GSSG] = 1.034/1.042 = 0.992

References

1. Mudd SH, Levy HL, Skovby F. Disorder of transsulfuration. In:Scriver CR, Beaudet AL, Sly WS, Valle D, eds. The metabolic andmolecular bases of inherited disease, 7th ed. New York: McGraw-Hill, 1995:1229–1327.
2. Meister A. Selective modification of glutathione metabolism. Science 1983;220:472–7.
3. Gahl WA, Bashan N, Tietze F, Bernardini I, Schulman JD. Lysosomal cystine transport is defective in cystinosis. Science 1982; 217:1263–5.
4. Segal S, Thier SO. Cystinuria. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. The metabolic and molecular bases of inherited disease, 7th ed. New York: McGraw-Hill, 1995:3581–3601.
5. Gahl WA, Ingelfinger J, Mohan P, Bernardini I, Hyman PE, Tangerman A. Intravenous cysteamine therapy for nephropathic cystinosis. Pediatr Res 1995; 38:579–84.