Amplite™ Fluorimetric Glutathione GSH/GSSG Ratio Assay Kit *Green Fluorescence*

Catalog number: 10056
Unit size: 200 Tests

Component	Storage	Amount
Component A: Thiolite™ Green	Freeze (< -15 °C), Minimize light exposure	1 vial
Component B: Assay Buffer	Freeze (< -15 °C)	1 bottle (25 mL)
Component C: GSH Standard	Freeze (< -15 °C)	1 vial (62 µg)
Component D: DMSO	Freeze (< -15 °C)	1 vial (400 µL)
Component E: GSSG Probe	Freeze (< -15 °C), Minimize light exposure	1 bottle (lyophilized powder)
Component F: GSSG Standard	Freeze (< -15 °C), Minimize light exposure	1 vial (124 µg)

OVERVIEW

When cells are exposed to increased levels of oxidative stress, GSSG will accumulate and the ratio of GSH to GSSG will decrease. The glutathione redutase recycles GSSG to GSH with simultaneous oxidation of b-nicotinamide adenine dinuclecotide phosphate. The monitoring of GSH/GSSG ratio and the quantification of GSSG in biological samples are essential for evaluating the redox and detoxification status of cells and tissues in relation to the protective role of glutathione against oxidative and free-radical-mediated cell injury. There are a few reagents or assay kits available for the quantitation of thiols in biological systems. However, all the commercial kits either lack sensitivity or have tedious protocols. Our Amplite™ Fluorimetric GSH/GSSG Ratio 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 thiol. The kit provides a sensitive, one-step fluorimetric method to detect as little as 1 picomole of cysteine or GSH in a 100 µL assay volume. The assay can be performed in a convenient 96-well or 384-well microtiter-plate format and easily adapted to automation without a separation step. Its signal can be easily read by a fluorescence microplate reader.

AT A GLANCE

Important With regards to GSH:GSSG determination, the following equation is important to remember:
GSSG -> Reduction -> 2 GSH
For each redox of GSSG, two moles of GSH is produced. Thus, when determining [Total GSH] during data analysis, it is important to remember that [Total GSH] = 2 x [GSSG]. For instance, if [GSSG] standard for a particular well is 5 uM, [Total GSH] for that well should be calculated using 10 uM.

Protocol Summary

1. Prepare GSH standards and/or GSSG standards or test samples (50 µL)
2. Add GSH working solution (50 µL)
3. Incubate at RT for 10 to 60 minutes
4. Monitor the fluorescence increase at Ex/Em = 490/520 nm

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

KEY PARAMETERS

Fluorescence microplate reader

Excitation	490 nm
Emission	520 nm
Cutoff	510 nm
Recommended plate	Solid black

PREPARATION OF STOCK SOLUTIONS

Unless otherwise noted, all unused stock solutions should be divided into single-use aliquots and stored at -20 °C after preparation. Avoid repeated freeze-thaw cycles.

1. GSH standard solution (1 mM)
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 solution.

2. GSSG standard solution (1 mM)
Add 200 µL of ddH_2O into the vial of GSSG Standard (Component F) to make 1 mM (1 nmol/µL) GSSG standard solution.

3. Thiolite™ Green stock solution (100X)
Add 100 µL of DMSO (Component D) into the vial of Thiolite™ Green (Component A) to make 100X Thiolite™ Green stock solution. **Note:** One might add 200 µL of DMSO to make 50 X Thiolite™ Green stock solution for better solubility.

PREPARATION OF STANDARD SOLUTION

For convenience, use the Serial Dilution Planner:
https://www.aatbio.com/tools/serial-dilution/10056

GSH or GSSG standard
Prepare serially diluted GSH standards (0 to 10 µM): Add 10µL of GSH standard stock solution to 990 µL of Assay Buffer (Component B) to generate 10 µM GSH standard solution (GSH7). Note: Diluted GSH standard solution is unstable. Use within 4 hours. Take 10 µM GSH standard solution to perform 1:2 serial dilutions to get 5, 2.5, 1.25, 0.625, 0.3125, and 0.1563 serially diluted GSH standards (GSH6-GSH1). Prepare serially diluted GSSG standards (0 to 5 µM):Add 10µL of GSSG standard stock solution into 990 µL of Assay Buffer (Component B) to generate 10 µM GSSG standard solution. Note: Diluted GSSG standard solution is unstable. Use within 4 hours. Take 10 µM GSSG standard solution to perform 1:2 serial dilutions to get 5, 2.5, 1.25, 0.625, 0.3125, 0.1563, and 0.0781 serially diluted GSSG standards (GSSG7-GSSG1).

PREPARATION OF WORKING SOLUTION

1. GSH working solution (GSH-WS)
Add 100 µL of 100X Thiolite™ Green stock solution into 10 mL of Assay Buffer (Component B) and mix well by vortexing. **Note:** This GSH working solution (GSH-WS) is enough for two 96-well plates. It is unstable at room temperature, and should be used promptly within 2 hours. **Note:** Avoid exposure to light.[/note]

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

2. Total GSH working solution (TGSH-WS)
Add 5 mL of GSH-WS into the bottle of GSSG Probe (Component E) and mix them well by vortexing. **Note:** This Total GSH working solution (TGSH-WS) 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. **Note:** 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 TGSH-WS by mix the stock solution with GSH-WS proportionally.

SAMPLE EXPERIMENTAL PROTOCOL

Table 1. Layout of GSH standards, GSSG standards and test samples in a solid black 96-well microplate. GSH = GSH Standards (GSH1 - GSH7, 0.15 to 10 µM); GSSG = GSSG Standards (GSSG1 - GSSG7, 0.08 to 5 µM); BL=Blank Control; TS=Test Samples

Panel A				Pannel B			
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		

Table 2. Reagent composition for each well.

Well	Volume	Reagent
GSH	50 µL	Serial Dilutions (0.15 to 10 µM)
GSSG	50 µL	Serial Dilutions (0.08 to 5 µM)
BL	50 µL	Assay Buffer
TS	50 µL	Test Sample

1. Prepare GSH standards (GSH), blank controls (BL), and test samples (TS) according to the layout provided in Tables 1 and 2. For a 384-well plate, use 25 µL of reagent per well instead of 50 µL.

2. Add 50 µL of GSH-WS into each well of GSH standards, blank controls and test samples to make the total assay volume 100 µL/well. Use 25 µL for 384-well plates, for total assay volume of 50 µL/well.

3. If Total GSH (in reduced and oxidized states) assay is needed, prepare TGSH-WS and GSSG standards. Add GSSG standards (GSSG), blank controls (BL), and test samples (TS) according to layout provided in Tables 1 and 2. For a 384-well plate, use 25 µL of reagent per well instead of 50 µL. Then add 50 µL of TSGH-WS into each well of GSSG standards, blank controls and test samples to make the total assay volume 100 µL/well (50 µL total assay volume for 384-well plates).

4. Incubate the reaction for 10 to 60 minutes at room temperature, protected from light.

5. Monitor the fluorescence increase at Ex/Em = 490/520 nm with a fluorescence microplate reader.

EXAMPLE DATA ANALYSIS AND FIGURES

Samples producing higher signal than that of the highest standard should further be diluted in buffer and reanalyzed. This dilution factor should then be multiplied to get the concentration.

1. Average the duplicate reading for each standard, control and sample.
2. Subtract the mean value of Panel A blank from all Panel A standards and sample readings. Subtract the mean value of Panel B blank from all Panel B standards and sample readings. Fluorescence background increases with time, thus it is important to subtract the fluorescence intensity value of the blank wells for each data point.
3. Plot the corrected values for each standard as a function of the final concentration (µM) of GSH and/or Total GSH.
4. Draw the best smooth curve through these points to construct the standard curves. Calculate the trendline equation based on your standard curve data (use the equation that provides the most accurate fit).
5. Apply the Panel A corrected sample RFU readings to the GSH standard curve formula to get the GSH concentration (µM) of the test samples.
6. If the samples were diluted prior to reaction well set up, multiply the concentrations by the dilution factor. Multiply by alternative deproteinization dilution factor if performed.
7. Apply the Panel B corrected sample RFU readings to the GSSG standard curve formula to get the total glutathione (GSH + GSSG) concentration (µM) of the test samples.
8. If the samples were diluted prior to reaction well set up, multiply the concentrations by the dilution factor. Multiply by alternative deproteinization dilution factor if performed.
    a. Concentration of oxidized glutathione disulfide (GSSG) (µM) in the test samples is calculated as:
    b. GSSG = (Total Glutathione - GSH)/2
    c. GSH = calculated from steps 5 - 6

GSH/GSSG Ratio Determination:

Ratio = [GSH]/[GSSG]

[GSH] = concentration as calculated from steps 5-6

[GSSG] = concentration as calculated from steps 7-8[note] **0.078 to 5 µM GSSG which is equivalent to 0.156 to 10 µM GSH.**

Figure 1. GSH and Total (GSH+GSSG) dose responses were measured with Amplite™ Fluorimetric Glutathione GSH/GSSG Ratio Assay Kit. Blue line: in the presence of GSH only; Red line: in the presence of 1:1 GSH/GSSG.

DISCLAIMER

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