ab205811 GSH/GSSG Ratio Detection Assay Kit II (Fluorometric – Green)

For the measurement of glutathione (GSH/GSSG) in a variety of biological samples.
This product is for research use only and is not intended for diagnostic use.

Δ Note: The indicator dye is water soluble, whereas the probe in ab138881 is DMSO soluble.

Quick Assay Procedure

Δ Note: this procedure is provided as a quick reference for experienced users. Follow the detailed procedure when performing the assay for the first time.

- Prepare GSH standard dilution [0.1563 – 10 µM] and (if appropriate) the GSSG standard dilution [0.0781 – 5 µM].
- Prepare 100X Thiol Green Stock solution by adding 100 µL of ddH_2O into the vial of Thiol Green Indicator WS.
- Prepare samples (including deproteinization step) in optimal dilutions to fit the standard curve readings.
- Set up plate in duplicate for GSH standard (50 µL), GSSG standard (50 µL), and samples (50 µL).
- Prepare GSH Assay Mixture (GAM) by diluting 100 µL of 100X Thiol Green Stock solution in 10 mL Assay Buffer.
- Prepare Total Glutathione Assay Mixture (TGAM) by diluting GSSG probe in 5 mL GAM solution.
- Add 50 µL of GAM solution to GSH standard and sample wells.
- Add 50 µL of TGAM solution to GSSG standard and sample wells.
- Incubate plate at RT for 10 – 60 minutes protected from light.
- Monitor fluorescence at Ex/Em = 490/520 nm with a fluorescence microplate reader.

Materials Supplied and Storage

Store kit at -20°C in the dark immediately on receipt and check below for storage for individual components.
The kit is designed for one time use only.

Item	Quantity	Storage temperature (before prep)	Storage temperature (after prep)
Thiol Green Indicator WS	1 vial	-20°C	-20°C
Assay Buffer	25 mL	-20°C	-20°C
GSH Standard (62 µg; lyophilized)	1 vial	-20°C	-20°C
GSSG Standard (124 µg; lyophilized)	1 vial	-20°C	-20°C
GSSG Probe (lyophilized)	1 vial	-20°C	-20°C

Materials Required, Not Supplied

These materials are not included in the kit, but will be required to perform this assay:

- Microplate reader capable of measuring fluorescence at Ex/Em = 490/520 nm
- Black 96 well plate with clear flat bottom
- Dounce homogenizer (if using tissue)
- Mammalian Lysis Buffer 5X (ab179835) to homogenize cell or tissue samples. Alternatively, you can use PBS/0.5% NP-40
- Deproteinizing Sample Preparation Kit – TCA (ab204708): for deproteinization of cell and tissues lysates
- (Optional) Trichloroacetic acid (TCA)
- (Optional) Sodium bicarbonate ($NaHCO_3$)
- 10 kDa Spin Column (ab93349): for deproteinization of fluid samples

Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

Assay Buffer: Ready to use as supplied.

Thiol Green Indicator WS 100X: Reconstitute in 100 µL of ddH_2O to generate a 100X stock solution. Protect from light and keep on ice while in use.

GSH Standard (lyophilized; 62 µg): Reconstitute in 200 µL of Assay Buffer to generate a 1 mM (1 nmol/µL) GSH Standard stock solution. Aliquot so that you have enough volume to perform the desired number of assays. Keep on ice while in use.

GSSG Standard (lyophilized; 124 µg): Reconstitute in 200 µL of ddH_2O to generate a 1 mM (1 nmol/µL) GSSG Standard stock solution. Aliquot so that you have enough volume to perform the desired number of assays. Keep on ice while in use.

GSSG Probe: Prepare as instructed in the Assay Procedure section related to the preparation of TGAM.

Standard Preparation

- Always prepare a fresh set of standards for every use.
- Diluted standard solutions are unstable and must be prepared immediately before use. Do not store for future use.

Dilution of GSH standard:

1. Prepare a 10 µM (10 pmol/µL) GSH standard by diluting 5 µL of GSH 1 mM stock into 495 µL of Assay Buffer.
2. Using the 10 µM GSH standard, prepare standard curve dilution as described in the table in microcentrifuge tubes:

Standard#	Sample to dilute	Volume of standard in well (µL)	Assay Buffer (µL)	Final volume (µL)	End conc. of GSH in well (µM)
1	10 µM	300	0	300	10
2	Std. #1	100	100	200	5
3	Std. #2	100	100	200	2.5
4	Std. #3	100	100	200	1.25
5	Std. #4	100	100	200	0.625
6	Std. #5	100	100	200	0.3125

7	Std. #6	100	100	200	0.1563
8 (blank)	None	0	200	200	0

Each dilution has enough standard to set up duplicate readings (2 x 50 µL).
Δ **Note**: Diluted GSH standard solutions are unstable. Use within 4 hours.

Dilution of GSSG standard:

1. Prepare a 10 µM (10 pmol/µL) GSSG standard by diluting 5 µL of GSSG 1 mM stock into 495 µL of Assay Buffer.
2. Using the 10 µM GSSG standard, prepare standard curve dilution as described in the table in microcentrifuge tubes:

Standard#	Sample to dilute	Volume of standard in well (µL)	Assay Buffer (µL)	Final volume (µL)	End conc. of GSSG in well (µM)
1	10 µM	200	200	400	5
2	Std. #1	100	100	200	2.5
3	Std. #2	100	100	200	1.25
4	Std. #3	100	100	200	0.625
5	Std. #4	100	100	200	0.3125
6	Std. #5	100	100	200	0.1563
7	Std. #6	100	100	200	0.0781
8 (blank)	None	0	200	200	0

Each dilution has enough standard to set up duplicate readings (2 x 50 µL).
Δ **Note**: Diluted GSSG standard solutions are unstable. Use within 4 hours.
Δ **Note**: The concentration of GSH standard solutions are TWICE the concentrations of GSSG standard solutions. Glutathione disulfide (GSSG) will be reduced and form 2 molecules of glutathione (GSH). 1 mol GSSG = 2 moles GSH

<u>**Sample Preparation**</u>
General sample information:
We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.
We recommend that you use fresh samples for the most reproducible assay. If you cannot perform the assay at the same time, we suggest that you snap freeze your samples in liquid nitrogen upon extraction and store them immediately at -80°C. When you are ready to test your samples, thaw them on ice. Be aware, however, that this might affect the stability of your samples and the readings can be lower than expected. Avoid multiple freeze-thaws.
Δ **Note**: DTT and/or 2-mercaptoethanol cannot be used in samples. These reducing agents can interfere with the Thiol Green Dye and generate fluorescent background.
Δ **Note**: Triton X-100 autofluorescence, increasing background. We recommend avoiding this buffer or including background control wells if it is used in sample lysis buffer.

Cell (adherent or suspension) samples:

1. Harvest the amount of cells necessary for each assay. Try starting with ~10,000 – 20,000 cells/well with adherent cells, and 50,000 – 100,000 cells with suspension cells; initial dilution range = 1:10 – 1:1,000.
2. Wash cells with cold PBS.
3. Resuspend cells in 100 µL of ice cold 1X Mammalian Lysis Buffer (alternatively you can use PBS/0.5% NP-40).
4. Homogenize cells quickly by pipetting up and down a few times.
5. Centrifuge sample for 15 minutes at 4°C at top speed using a cold microcentrifuge to remove any insoluble material.
6. Collect supernatant and transfer to a clean tube. Sample should be clear.
7. Keep on ice.
8. Cell samples may contain enzymes that can interfere with the analysis. Remove enzymes from sample by using Deproteinizing Sample Kit – TCA (ab204708). Alternatively, you can perform the TCA/$NaHCO_3$ deproteinization protocol described below.

Tissue samples:

1. Harvest the amount of tissue necessary for each assay (initial recommendation = 20 mg; initial dilution recommendation = 1/10 – 1/100).
2. Wash tissue in cold PBS.
3. Resuspend tissue (20 mg) in 400 µL of ice-cold Mammalian Lysis Buffer (alternatively you can use PBS/0.5% NP-40)).
4. Homogenize tissue with a Dounce homogenizer with 10 – 15 passes.
5. Centrifuge sample for 15 minutes at 4°C at top speed using a cold microcentrifuge to remove any insoluble material.
6. Collect supernatant and transfer to a clean tube. Sample should be clear.
7. Keep on ice.
8. Tissue samples may contain enzymes that can interfere with the analysis. Remove enzymes from sample by using Deproteinizing Sample Kit – TCA (ab204708). Alternatively, you can perform the TCA/$NaHCO_3$ deproteinization protocol described below.

Plasma, Serum, Blood, and Urine:

Biological fluid samples generally contain high amount of proteins which can interfere with the assay. Remove enzymes from the sample by using Deproteinizing Sample Kit – TCA (ab204708), TCA protocol, or using a 10 kD Spin Column (ab93349). Add sample to the spin column, centrifuge at 10,000 x *g* for 10 minutes at 4°C and collect the filtrate.

Alternative Deproteinization protocol:

For this step you will need additional reagents:

- Trichloroacetic acid (TCA)
- Sodium bicarbonate

1. Add 1 volume ice cold 100% (w/v) TCA into 5 volumes of sample and vortex briefly to mix well.
2. Incubate on ice for 5 – 10 minutes.
3. Centrifuge samples at 12,000 x *g* for 5 minutes at 4°C in a cold centrifuge and transfer supernatant to a fresh tube. Measure volume of supernatant.

∆ **Note**: Samples can be stored at -80°C for 1 month if needed.

4. Neutralize the sample by adding $NaHCO_3$ to supernatant and vortex briefly. Add $NaHCO_3$ drop by drop until pH equals 4 – 6 (use pH paper to test 1 µL of the sample). Any leftover TCA will interfere with the assay.

∆ **Note**: Avoid adding neutralization buffer to pH > 7 as GSH is very labile and easily oxidizes at pH > 7.

5. Centrifuge at 13,000 x *g* for 15 minutes at 4°C and collect supernatant.
6. Samples are now deproteinized, neutralized, and TCA has been removed. The samples are ready to use in the assay.

Sample Recovery

The deproteinized sample will be diluted from the original concentration.

To calculate the dilution factor of your final sample, simply apply the following formula:

$$\% \, original \, concentration = \frac{initial \, sample \, volume}{(initial \, sample \, vol \, + \, TCA \, vol \, + \, NaHCO3 \, vol} \, x \, 100$$

Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature just prior to use and gently agitate.
- Assay all standards, controls and samples in duplicate.

1.1 Reaction wells set up:

- GSH Standard wells = 50 µL GSH standard dilutions.
- GSSG Standard wells = 50 µL GSSG standard dilutions (skip this if only GSH is being measured)
- Sample wells = 50 µL samples

See example plate layout for reduced glutathione (GSH only) and total glutathione (GSH + GSSG) detection in the table below:

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				
GSH8	GSH8					GSSG8	GSSG8				

GSH = GSH Standards, GSSG = GSSG Standards, TS = Test Samples, BL = Assay buffer

∆ **Note**: When just GSH assay is needed, fill ONLY the wells in two left columns (Panel A) according to table above. When Total GSH assay is needed, fill the wells in both Panel A (left) and Panel B (right) according to table above.

1.2 GSH Assay Mixture (GAM) set up [GSH detection]:

Prepare GAM by adding 100 µL of 100X Thiol Green Stock solution into 10 mL of Assay Buffer. This is enough for two 96-well plates.

∆ **Note**: GAM solution is unstable at room temperature and should be used promptly within 2 hours. It is stable at 4°C for 4 hours. Avoid light exposure.

∆ **Note**: For measuring the GSH/GSSG ratio there is enough reagent to perform 100 tests. If the kit is used to measure GSH only, there is enough reagent provided in the kit to perform 200 tests.

1.3 Total Glutathione Assay Mixture (TGAM) set up [GSH + GSSG detection]:

Prepare TGAM by adding 5 mL of GAM solution into the bottle of the GSSG Probe and mix well by vortexing. This is enough for one 96-well plate.

∆ **Note**: For a smaller volume of TGAM, one can make 25X GSSG Probe by adding 200 µL of ddH_2O into the bottle of GSSG Probe, and then prepare the TGAM assay mixture by mixing the stock solution with GSH Assay Mixture (GAM) proportionally. Prepare only as much GAM as needed. Aliquot and store the unused 25X GSSG probe stock solution at -20°C and avoid freeze-thaw cycles.

∆ **Note**: TGAM solution is unstable at room temperature and should be used promptly within 2 hours. Avoid light exposure.

1.4 Run GSH and Total Glutathione assay:

1. For GSH detection, add 50 µL of GSH Assay Mixture (GAM) into each GSH standard and sample well (Panel A) to make the total assay volume 100 µL/well.
2. For Total GSH + GSSG (reduced and oxidized), add 50 µL of Total Glutathione Assay Mixture (TGAM) into each GSSG standard and sample well (Panel B) to make total assay volume 100 µL/well.
3. Incubate at room temperature for 10 – 60 minutes protected from light.
4. Monitor fluorescence at Ex/Em = 490/520 nm with a fluorescence microplate reader.

1.5 Protocol for 384-well plate assay:

1. Reaction well set up: use 25 µL standards and 25 µL samples.
2. Follow same procedure as for 96-well plate until step 1.3.
3. GSH detection: add 25 µL sample/standard + 25 µL GAM into each well.
4. Total GSH + GSSG detection: add 25 µL sample/standard + 25 µL TGAM into each well.
5. Incubate at room temperature for 10 – 60 minutes protected from light.
6. Monitor fluorescence at Ex/Em = 490/520 nm with a fluorescence microplate reader.

Data Analysis

Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiply the concentration found by the appropriate dilution factor.

1. Average the duplicate reading for each standard, control and sample.
2. Subtract the mean value of Panel A blank (Standard #8) from all Panel A standards and sample readings. Subtract the mean value of Panel B blank (Standard #8) 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 + GSSG.

Δ **Note**: GSSG is converted to Total GSH at a rate of 1 mole of GSSG to 2 moles of GSH. Thus, the end concentration of GSSG in the standard dilution needs to be multiplied by a factor of 2 to determine the Total GSH concentration for plotting the standard curve.

GSSG + NADPH → 2GSH + NADP

4. Draw the best smooth curve through these points to construct the standard curves. Most plate reader software or Excel can plot these values and curve fit. 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.

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

$$Log(y) = A + B * Log(x)$$

Δ **Note**: The equation is generated by most instrument software.

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. Concentration of oxidized glutathione disulfide (GSSG) (µM) in the test samples is calculated as:

   GSSG = (Total Glutathione – GSH)/2

   GSH = calculated from steps 5 - 6.
9. GSH/GSSG Ratio Determination:

   Ratio = [GSH]/[GSSG]

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

   [GSSG] = concentration as calculated from steps 7-9.

FAQs / Troubleshooting

The signal I get for GSH is higher than total glutathione. What happened?

SH (reduced glutathione) is very easy to oxidize, especially at pH > 7. If the GSH standard signal is lower than the GSSG signal, or the concentration of GSH in the sample is higher than the total glutathione, then the GSH has been oxidized. Ensure pH of sample is not >7, keep samples and reagents on ice when not in use, protect the indicator dye from light, and work as rapidly as possible.

Alternatively, it could be that there is no statistically significant difference between the GSH and the total glutathione concentrations, indicative of little to no GSSG in the sample. If such is the case, the GSH concentration may be greater than the total glutathione concentration due to experimental bias.

What is the difference between this product and GSH/GSSG Ratio Detection Assay Kit (Fluorometric – Green) (ab138881)?

The working principle and detection of both products is the same. The only difference is that the detection probe included in this kit, Thiol Green Indicator WS, is water soluble, whereas the indicator in ab138881 is soluble in DMSO only

What cell lysis buffer can I use?

0.5% NP40 can be made up in PBS pH6.0 for lysis. The assay buffer has no detergent, so it can't be used for lysing the cells.

What components could interfere with this kit?

DTT or 2-mercaptoethanol can interfere with Component A so cannot be used. Methanol should not interfere with this kit.

Technical Support

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