ab138881 GSH/GSSG Ratio Detection Assay Kit (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.

For overview, typical data and additional information please visit: www.abcam.com/ab138881
(use abcam.cn/ab138881 for China, or abcam.co.jp/ab138881 for Japan)

Materials Supplied and Storage

- Store kit at -20°C in the dark immediately on receipt and check below for storage for individual components. Kit can be stored for 1 year from receipt, if components have not been reconstituted.
- Aliquot components in working volumes before storing at the recommended temperature.
- Avoid repeated freeze-thaws of reagents.

Δ **Note**: For measuring GSH only, there is enough reagent provided in the kit to perform 200 tests.

Item	Quantity	Storage temp. (before prep)	Storage temp. (after prep)
DMSO	400 µL	-20°C	-20°C
Thiol Green Indicator	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:

- 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 kD Spin Column (ab93349): for deproteinization of fluid samples

1. Reagent Preparation: Briefly centrifuge small vials at low speed prior to opening.

1.1 DMSO: Ready to use as supplied.

1.2 Assay Buffer: Ready to use as supplied.

1.3 Thiol Green Indicator 100X: Reconstitute in 200 µL of DMSO (section 6.1) to generate a 50X stock solution. Add DMSO drop by drop, vortexing in between, to prevent precipitation. Aliquot so that you have enough volume to perform the desired number of assays. Protect from light and keep on ice while in use.

1.4 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.

1.5 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.

1.6 GSSG Probe: Reconstitute in 200 µL of ddH_2O to generate a 25X probe stock solution. Aliquot so that you have enough volume to perform the desired number of assays. Keep on ice while in use.

2. Standard Preparation: Prepare a fresh set of standards for every use. Do not store for future use.

2.1 Dilution of GSH standard:

2.1.1 Prepare a 10 µM (10 pmol/µL) GSH standard by diluting 5 µL of GSH 1 mM stock into 495 µL of Assay Buffer.

2.1.2 Using the 10 µM GSH standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

Standard#	Sample to dilute	Volume of standard in well (µL)	Assay Buffer (µL)	End conc. of GSH in well (µM)
1	10 µM	300	0	10
2	Std. #1	100	100	5
3	Std. #2	100	100	2.5
4	Std. #3	100	100	1.25
5	Std. #4	100	100	0.625
6	Std. #5	100	100	0.3125
7	Std. #6	100	100	0.1563
8 (blank)	None	0	200	0

2.2 Dilution of GSSG standard:

2.2.1 Prepare a 10 µM (10 pmol/µL) GSSG standard by diluting 5 µL of GSSG 1 mM stock into 495 µL of Assay Buffer.

2.2.2 Using the 10 µM GSSG standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

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

Δ **Note**: Each dilution has enough standard to set up duplicate readings (2 x 50 µL).
Δ **Note**: Diluted GSH and 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.

3. Sample Preparation

Perform several dilutions of your sample. Use fresh samples. If you cannot perform the assay at the same time, snap freeze your samples in liquid nitrogen upon extraction and store them at -80°C.

When you are ready to test your samples, thaw them on ice. This might affect the stability of samples and 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 autofluoresces, increasing background. We recommend avoiding this buffer or including background control wells if it is used in sample lysis buffer.

3.1 Cell (adherent or suspension) samples:

1. Harvest the amount of cells necessary for each assay (initial recommendation = $10^7 – 10^8$ cells); 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 – 1,000.
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 - 0.5% NP40 can be made up in PBS pH6.0 for lysis).
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. Keep on ice.
7. 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 a TCA/$NaHCO_3$ deproteinization step described in Section 3.4

3.2 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 - 0.5% NP40 can be made up in PBS pH6.0 for lysis).
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. Keep on ice.
7. 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 a TCA/$NaHCO_3$ deproteinization step described in Section 3.4

3.3 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 described in section 8.4, 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.

3.4 Alternative Deproteinization protocol:

For this step you need extra reagents: Trichloroacetic acid (TCA) and Sodium bicarbonate

1. Add 1 volume ice cold 100% (w/v) TCA into 5 volumes of sample and vortex briefly to mix.
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.

Δ **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.

Δ **Note**: Avoid adding neutralization buffer to pH > 7 as GSH is 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:

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

4. Assay Procedure: Use materials and reagents at room temperature. Assay all in duplicate.

4.1 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)					
GSH1	GSH1	TS	TS			GSSG1	GSSG1	TS	TS		
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

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

4.2.1 Prepare GAM by diluting the 50X Thiol Green Stock solution (Step 1.3) in Assay Buffer at 1:50 dilution and mix well by vortexing.

Δ **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.
Δ **Note**: For measuring GSH only, there is enough reagent provided in the kit to perform 200 tests.

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

4.3.1 Prepare TGAM by diluting the 25X GSSG Probe Stock solution (Step 1.6) in GAM (Step 4.2) at 1:25 dilution and mix well by vortexing.

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

4.4 Run GSH and Total Glutathione assay:

4.4.1 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.

4.4.2 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.
4.4.3 Incubate at room temperature for 10 – 60 minutes protected from light.
4.4.4 Monitor fluorescence at Ex/Em = 490/520 nm with a fluorescence microplate reader.

4.5 Protocol for 384-well plate assay:
4.5.1 Reaction well set up: use 25 µL standards and 25 µL samples.
4.5.2 Follow same procedure as for 96-well plate until step 4.3.
4.5.3 GSH detection: add 25 µL sample + 25 µL GAM into each well.
4.5.4 Total GSH + GSSG detection: add 25 µL sample + 25 µL TGAM into each well.
4.5.5 Incubate at room temperature for 10 – 60 minutes protected from light.
4.5.6 Monitor fluorescence at Ex/Em = 490/520 nm with a fluorescence microplate reader.

Data analysis

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.
4. Draw a smooth curve through these points to construct 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: $Log(y) = A + B * Log(x)$
6. If the samples were diluted prior to reaction well set up, multiply the concentrations by the dilution factor. Multiply by alternative deproteinization (section 3.4) 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 samples were diluted prior to reaction well set up, multiply concentrations by the dilution factor. Multiply by alternative deproteinization (section 3.4) dilution factor if performed.
9. Concentration of oxidized glutathione disulfide (GSSG)(µM) in the test samples is calculated:
   GSSG = (Total Glutathione – GSH)/2 GSH = calculated from steps 5 - 6.
10. GSH/GSSG Ratio Determination: Ratio = [GSH]/[GSSG]
   [GSH] = concentration from steps 5-6. [GSSG] = concentration from steps 7-8.

FAQs/Troubleshooting

The signal I get for GSH is higher than total glutathione. What happened?
GSH (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 II (Fluorometric – Green) (ab205811)?
The working principle and detection of both products is the same. The only difference is that the detection probe included in ab205811, Thiol Green Indicator WS, is water soluble, whereas the indicator in this product is soluble in DMSO only.

Copyright © 2021 Abcam. All Rights Reserved. The Abcam logo is a registered trademark. All information / detail is correct at time of going to print.
For all technical or commercial enquiries please go to:
www.abcam.com/contactus
www.abcam.cn/contactus (China)
www.abcam.co.jp/contactus (Japan)