

ab 65322

Glutathione Detection Assay Kit (Fluorometric)

Instructions for Use

For the rapid, sensitive and accurate measurement of Glutathione levels in various samples.

This product is for research use only and is not intended for diagnostic use.

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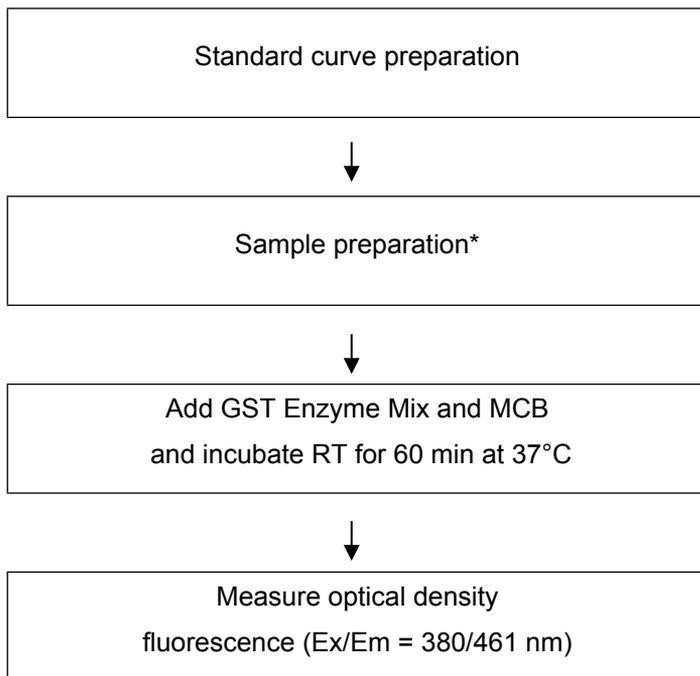
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1. BACKGROUND

Glutathione Detection Assay Kit (Fluorometric) (ab65322) provides a simple *in vitro* assay for detecting total glutathione changes during cellular response to toxicity, apoptosis and other conditions. The assay uses the dye MCB Probe, which forms an adduct with glutathione in a reaction catalyzed by glutathione-S-transferase (GST). The unbound MCB is almost nonfluorescent, whereas the dye emits a fluorescent blue light (Ex/Em = 380/461 nm) when bound to reduced or oxidized glutathione. The assay detects both reduced and oxidized glutathione.

Glutathione (GSH) 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.

2. ASSAY SUMMARY



*Samples might require deproteinization.

3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store kit at -20°C in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in section 5.

Aliquot components in working volumes before storing at the recommended temperature. **Reconstituted components are stable for 2 months.**

5. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)	Storage Condition (After Preparation)
GSH Cell Lysis Buffer	25 mL	-20°C	4°C
MCB Probe	200 µL	-20°C	-20°C
GST Enzyme Mix	200 µL	-20°C	-20°C
GSH Standard	1 vial	-20°C	-20°C

6. MATERIALS REQUIRED, NOT SUPPLIED

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

- MilliQ water or other type of double distilled water (ddH₂O)
 - PBS
 - Microcentrifuge
 - Pipettes and pipette tips
 - Fluorescent microplate reader – equipped with filter for Ex/Em = 380/461 nm
 - 96 well plate: black plates for fluorometric assay
 - Heat block or water bath
 - Dounce homogenizer or pestle (if using tissue)
- For deproteinization step, additional reagents are required:
- Perchloric acid (PCA) 4M, ice cold
 - Potassium Hydroxide (KOH) 2M
 - 10 kD Spin Columns (ab93349) – for fluid samples, if not performing PCA precipitation

7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not use kit or components if it has exceeded the expiration date on the kit labels.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

8. TECHNICAL HINTS

- **This kit is sold based on number of tests. A ‘test’ simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**
- Keep enzymes, heat labile components and samples on ice during the assay.
- Make sure all buffers and solutions are at room temperature before starting the experiment.
- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Make sure you have the right type of plate for your detection method of choice.
- Make sure the heat block/water bath and microplate reader are switched on.

9. REAGENT PREPARATION

- Briefly centrifuge small vials at low speed prior to opening.

9.1 **GSH Cell Lysis Buffer:**

Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C.

9.2 **MCB Probe:**

Ready to use as supplied. Warm by placing in a 37°C bath for 1 – 5 minutes to thaw the DMSO solution before use.

NOTE: DMSO tends to be solid when stored at -20°C, even when left at room temperature, so it needs to melt for few minutes at 37°C. Aliquot probe so that you have enough volume to perform the desired number of assays. Store at -20°C protected from light and moisture. Keep on ice while in use.

9.3 **GST Enzyme Mix:**

Ready to use as supplied. Aliquot GST Enzyme Mix/reagent so that you have enough volume to perform the desired number of assays. Store at -20°C. Keep on ice while in use.

9.4 **GSH Standard:**

Reconstitute the 1mM GSH Standard in 100 µL of ddH₂O to generate a 10 µg/µL standard stock solution. Aliquot standard so that you have enough volume to perform the desired number of assays or freeze immediately after each use. Store at -20°C. Keep on ice while in use.

10. STANDARD PREPARATION

- Always prepare a fresh set of standards for every use.
- Diluted standard solution is unstable and must be used immediately.

10.1 Prepare a 0.1 $\mu\text{g}/\mu\text{L}$ standard GSH solution by diluting 10 μL of the reconstituted GSH Standard in 990 μL GSH Cell Lysis Buffer.

10.2 Using 0.1 $\mu\text{g}/\mu\text{L}$ standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

Standard #	Volume of Standard (μL)	GSH Cell Lysis Buffer (μL)	Final volume standard in well (μL)	End [Glutathione] in well
1	0	300	100	0 $\mu\text{g}/\text{well}$
2	6	296	100	0.2 $\mu\text{g}/\text{well}$
3	12	288	100	0.4 $\mu\text{g}/\text{well}$
4	18	282	100	0.6 $\mu\text{g}/\text{well}$
5	24	276	100	0.8 $\mu\text{g}/\text{well}$
6	30	270	100	1.0 $\mu\text{g}/\text{well}$

Each dilution has enough amount of standard to set up duplicate readings (2 x 100 μL).

11. SAMPLE PREPARATION

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. If you cannot perform the assay at the same time, we suggest that you complete the Sample Preparation step as well as the Deproteinization step before storing the samples. Alternatively, if that is not possible, we suggest that you snap freeze cells or tissue in liquid nitrogen upon extraction and store the samples 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.

11.1 Cell (adherent or suspension) samples:

- 11.1.1 Harvest the amount of cells necessary for each assay (initial recommendation = 1×10^6 cells).
- 11.1.2 Wash cells with cold PBS.
- 11.1.3 Resuspend cells in 100 μL of GSH Cell Lysis Buffer/Cell Lysis Buffer.
- 11.1.4 Homogenize cells quickly by pipetting up and down a few times. Incubate on ice for 10 minutes.
- 11.1.5 Centrifuge sample for 10 minutes at 4°C at top speed using a cold microcentrifuge to remove any insoluble material.
- 11.1.6 Collect supernatant and transfer to a clean tube.
- 11.1.7 Keep on ice.
- 11.1.8 Perform deproteinization step as described in section 11.4.

11.2 Tissue samples:

- 11.2.1 Harvest the amount of tissue necessary for each assay (initial recommendation = 10 mg).
- 11.2.2 Wash tissue in cold PBS.

- 11.2.3 Resuspend tissue in 100 μ L of GSH Cell Lysis Buffer/Cell Lysis Buffer.
- 11.2.4 Homogenize tissue with a Dounce homogenizer sitting on ice, with 10 – 15 passes.
- 11.2.5 Centrifuge samples for 10 minutes at 4°C at top speed using a cold microcentrifuge to remove any insoluble material.
- 11.2.6 Collect supernatant and transfer to a clean tube.
- 11.2.7 Keep on ice.
- 11.2.8 Perform deproteinization step as described in section 11.5.

11.3 Plasma, Serum and Urine and other biological fluids:

Liquid samples can be assayed directly, or diluted with GSH Cell Lysis Buffer/Cell Lysis Buffer.

Plasma, serum and urine samples generally contain high amount of proteins, so they should be deproteinized as described in section 11.5.

Alternatively, you can use 10kD Spin column (ab93349) to deproteinize biological fluids.

11.4 Deproteinization step:

Prepare samples as specified in protocol. You should have a clear protein sample after homogenization and centrifugation. Keep your samples on ice.

- 11.4.1 Add ice cold PCA 4 M to a final concentration of 1 M in the homogenate solution and vortex briefly to mix well. **NOTE:** *high protein concentration samples might need more PCA.*
- 11.4.2 Incubate on ice for 5 minutes.
- 11.4.3 Centrifuge samples at 13,000 x g for 2 minutes at 4°C in a cold centrifuge and transfer supernatant to a fresh tube. Measure volume of supernatant.
- 11.4.4 Precipitate excess PCA by adding ice-cold 2M KOH that equals 34% of the supernatant to your sample (for instance, 34 μ L of 2 M KOH to 100 μ L sample) and vortexing briefly. This will neutralize the sample and precipitate excess PCA.

- 11.4.5 After neutralization, it is very important that pH equals 6.5 – 8 (use pH paper to test 1 μ L of sample). Any left over PCA will interfere with the assay. If necessary, adjust pH with 0.1 M KOH.
- 11.4.6 Centrifuge at 13,000 x g for 15 minutes at 4°C and collect supernatant.

Samples are now deproteinized, neutralized and PCA has been removed. The samples are now ready to use in the assay.

Sample Recovery

The deproteinized samples will be diluted from the original concentration.

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

$$\% \text{ original concentration} = \frac{\text{Initial sample volume}}{(\text{Initial sample volume} + \text{vol PCA} + \text{vol KOH})}$$

NOTE: We suggest using different volumes of sample to ensure readings are within the Standard Curve range.

12. ASSAY PROCEDURE and DETECTION

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- It is recommended to assay all standards, controls and samples in duplicate.

12.1 Set up Reaction wells:

- Standard wells = 100 μ L standard dilutions.
- Sample wells = 10 – 100 μ L samples (adjust volume to 100 μ L/well with GSH Cell Lysis Buffer/Cell lysis buffer).

NOTE: for HeLa cells, 20 μ L appears to be the optimum amount of sample.

- 12.2 Add 2 μ l of the GST Enzyme Mix and 2 μ l of MCB into each sample and standard. Mix the plate well. Incubate the plate at 37°C for 1 hour. It can be very informative to acquire the fluorescence data kinetically during the incubation to observe the GSH-MCB adduct formation.

12.3 Measurement:

Measure the fluorescence value in a fluorometer or fluorescence plate reader at Ex./Em. = 360 \pm 20 nm/460 \pm 20 nm.

13. CALCULATIONS

- Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
 - 13.1 Average the duplicate reading for each standard and sample.
 - 13.2 Subtract the mean absorbance value of the blank (Standard #1) from all standard and sample readings. This is the corrected absorbance.
 - 13.3 Plot the corrected absorbance values for each standard as a function of the final concentration of glutathione.
 - 13.4 Draw the best smooth curve through these points to construct the standard curve. 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).

- 13.5 The glutathione concentration in sample can be calculated as follows:

$$C = A/V \text{ } \mu\text{g/ml}$$

Where:

A = Amount of glutathione from Standard Curve (μg).

V = Original sample volume added into sample well (ml).

Glutathione molecular weight: 307 g/mol.

Results can be expressed as $\mu\text{g/mL}$ of sample, $\mu\text{g}/10^6$ cells or for apoptosis assay, as the percentage change in glutathione level in treated versus untreated control samples.

14. TYPICAL DATA

TYPICAL STANDARD CURVE – Data provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed.

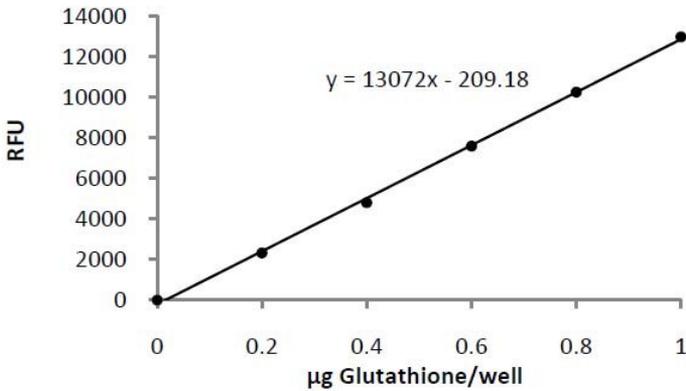


Figure 1. Typical Glutathione standard calibration curve using fluorometric reading.

15. QUICK ASSAY PROCEDURE

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 standard, probe and prepare enzyme mix (aliquot if necessary); get equipment ready.
- Prepare standard curve.
- Prepare samples in duplicate (find optimal dilutions to fit standard curve readings), including deproteinization step.
- Set up plate for standard (100 μ L) and samples (100 μ L) wells).
- Add 2 μ L of GST Enzyme Mix and 2 μ L of MCB to standard and sample wells.
- Incubate plate at 37°C during 60 min protected from light and read fluorescence at Ex/Em = 380/461 nm.

16. TROUBLESHOOTING

Problem	Cause	Solution
Assay not working	Use of ice-cold buffer	Buffers must be at room temperature
	Plate read at incorrect wavelength	Check the wavelength and filter settings of instrument
	Use of a different 96-well plate	Colorimetric: Clear plates Fluorometric: black plate
Sample with erratic readings	Samples not deproteinized (if indicated on protocol)	Use PCA precipitation protocol for deproteinization
	Cells/tissue samples not homogenized completely	Use Dounce homogenizer, increase number of strokes
	Samples used after multiple free/ thaw cycles	Aliquot and freeze samples if needed to use multiple times
	Use of old or inappropriately stored samples	Use fresh samples or store at -80°C (after snap freeze in liquid nitrogen) till use
	Presence of interfering substance in the sample	Check protocol for interfering substances; deproteinize samples
Lower/ Higher readings in samples and Standards	Improperly thawed components	Thaw all components completely and mix gently before use
	Allowing reagents to sit for extended times on ice	Always thaw and prepare fresh reaction mix before use
	Incorrect incubation times or temperatures	Verify correct incubation times and temperatures in protocol

RESOURCES

Problem	Cause	Solution
Standard readings do not follow a linear pattern	Pipetting errors in standard or reaction mix	Avoid pipetting small volumes (< 5 μL) and prepare a master mix whenever possible
	Air bubbles formed in well	Pipette gently against the wall of the tubes
	Standard stock is at incorrect concentration	Always refer to dilutions on protocol
Unanticipated results	Measured at incorrect wavelength	Check equipment and filter setting
	Samples contain interfering substances	Troubleshoot if it interferes with the kit
	Sample readings above/ below the linear range	Concentrate/ Dilute sample so it is within the linear range

17. NOTES

Technical Support

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