Glutathione (GSH)

Fluorometric Assay kit

(480 Tests)

Zellbio GmbH (Germany)

CAT No. ZX-33100-480

www.zellx.de

Sample Types Validated for:

Whole Blood, Serum, Plasma, Erythrocytes, Urine, Cell Lysates and Tissue Samples

!!! Caution: This product is for Research Use Only. Not for *in-vitro* Diagnostics !!!

Table of Contents

Please read this insert completely prior to using the product.

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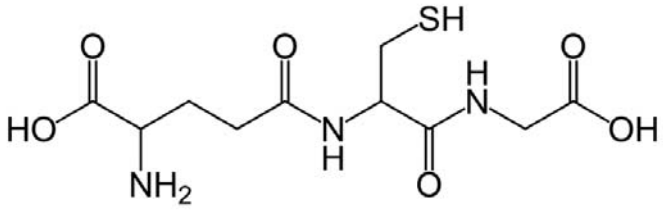
Introduction

Background

Glutathione (GSH) is a major non-protein thiol with a concentration of 0.5 – 10 mM in mammalian cells, which serves as an intracellular reducing substance to protect the cells against oxidative stress.



Glutathione Peroxidase (GPx) catalyzes the reduction of hydrogen peroxide (H_2O_2), using reduced GSH, and produces oxidized GSH dimer (GSSG).

$$H_2O_2 + 2\ GSH \xrightarrow{GPx} H_2O + GSSG$$

GSH plays a critical role in many other biological processes, such as protein and DNA synthesis, and amino acids transport.

Assay principle

The ZellX® Glutathione kit simultaneously quantifies the level of Glutathione (GSH), and oxidized Glutathione (GSSG) in a variety of samples without extra steps of separation or washing. The assay utilizes a Fluorescent Detection reagent which covalently binds to the free thiol group on GSH yielding a highly fluorescent product.

Free GSH is read first after 15 minutes, followed by addition of a reaction mixture that converts all the GSSG into free GSH, which then reacts with the excess Fluorescent Detection reagent to yield the signal related to Total GSH content. The total concentration of GSH generated in the sample is calculated from the generated signal.

The concentration of GSH can be measured at 510 nm with the excitation at 390 nm after 15 min incubation of Fluorescent Detection Reagent with sample/standard. A GSSG standard is provided to generate a standard curve for the assay and all samples should be read off the standard curve.

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General information

Materials supplied in the Kit

Component	*Quantity*
Glutathione Standard (250 µM)	300 µL
Fluorescent Detection Reagent	4 vial
Assay Buffer	200 mL
NADPH Concentrate	1.4 mL
Glutathione Reductase Concentrate	1.4 mL
DMSO	20 mL
Oxidized Glutathione Control	300 µL
Black Half Area 96 Well Plate	5 plate

Storage instruction

All the reagents must be at 4° C stored until the expiration date of the kit. DMSO Can be stored tightly capped at RT as it will freeze at 4° C.

Materials required but not supplied

Aqueous 5-sulfo-salicylic acid dihydrate (SSA) solution at 5% weight/volume (1g of SSA per 20 mL of water). We recommend Sigma-Aldrich Catalog Number S2130.

Double distilled water (ddH_2O)

Phosphate Buffer Saline (PBS)

Fluorescence 96 well plate reader capable of reading fluorescent emission at 510 nm, with excitation at 390 nm

Centrifuge, Vortex mixer

Precision pipettes and multichannel/repeater pipette and disposable pipette tips

Disposable 1.5-2 mL microtubes for sample preparation

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Precautions

This kit should only be used by qualified personnel who have had laboratory safety instruction. The complete insert should be read and understood before attempting to use the product.

Sulfosalicylic acid is a strong acid solution and should be treated like any other laboratory acid..

Dimethyl sulfoxide is a powerful aprotic organic solvent that enhances the rate of skin absorption of skin-permeable substances. Wear protective gloves when using the solvent especially when it contains dissolved chemicals. **NOTE: DMSO can dissolve certain plastics used in reagent reservoirs for holding solutions for multichannel pipettes**.

Fluorescent Detection Reagent should be stored at 4°C in the desiccated pouch. Allow desiccated pouch to warm to room temperature prior to opening. It will react with strong nucleophiles. Buffers containing the preservatives sodium azide, Proclin™ and Kathon™ will react with the reagent.

Reconstituted Fluorescent Detection Reagent in DMSO should be stored at 4°C in the desiccated pouch, and used within the next 2 months. The background on the reconstituted Fluorescent Detection Reagent will increase slowly over time but the increase will not affect the assay results.

General remarks

- Equilibrate all kit components to room temperature (RT) 30 minutes before use.
- The instruction must be strictly followed.
- Pipette tips should not be used more than once to prevent cross contamination.
- Reagents of different batches should not be mixed or used after their expiration dates.
- All samples must be deproteinized with 5% SSA, to remove any protein thiols which slows the oxidation of free GSH (details in Sample Preparation).

Assay protocol

Reagent preparation

i. **Assay Buffer:** Prepare 1:2 dilution of Assay Buffer by diluting the supplied Assay Buffer Concentrate with an equal volume of ddH_2O. Mix thoroughly. Assay Buffer can be stored at 4°C for 3 months.

ii. **Sample Diluent:** Prepare 1:5 dilution of 5% SSA with assay buffer (1 part 5% SSA with 4 parts assay buffer), and mix it well. The pH of the Sample Diluent must be > 6. Sample Diluent can be stored at 4°C for one month.

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iii. **Fluorescent Detection Solution:** Allow the ziploc bag to warm completely to room temperature prior to opening, then remove the vial of Fluorescent Detection Reagent. Add 3.5 mL of the provided DMSO to the each vial and vortex thoroughly. Store any unused reconstituted Detection Reagent at 4°C in the ziploc pouch with desiccant and use within 2 months.

iv. **Reaction Mix:** Prepare a 1:1:10 dilution of NADPH Concentrate and Glutathione Reductase Concentrate with Assay Buffer (mix 1.25 mL of NADPH Concentrate and 1.25 mL of Glutathione Reductase Concentrates with 10 mL of Assay Buffer). Unused Reaction Mixture can be stored at 4°C up to 2 days.

v. **Control (optional):** Add 5 µL of Oxidized Glutathione Control to 245 µL of Sample Diluent, and use within 2 hours of preparation.

The use of Control ensures that the prepared NADPH and Glutathione Reductase system will adequately reduce GSSG to GSH. If this optional control is run it should yield a value for Total Glutathione of approximately 10 ± 2 µM.

Sample preparation

After collecting the sample, extraction should be immediately carried out in accordance with the related instruction. After extraction, experiment should be conducted immediately, otherwise, keep the sample at -70 or -80˚C. Avoid repeated freeze-thaw cycles.

All samples must be treated with the 5% SSA solution. All SSA-treated centrifuged supernatants must have their SSA concentration brought down to 1% SSA by dilution with Assay Buffer before conducting the assay.

All samples and standards must be in Sample Diluent before starting the assay and used within 2 hours of dilution.

I. **Whole Blood, Serum, EDTA or Heparin Plasma, or Urine:**

- Thoroughly mix samples with an equal volume of cold 5% SSA.
- Incubate for 10 min at 4˚C.
- Centrifuge at 14000 rpm for 10 min at 4˚C and collect the supernatant.
- If the supernatant contains particulates, re-centrifuge the supernatant for 15 minutes and collect the clarified supernatant.
- Dilute the supernatant 2:5 with Assay Buffer (2 parts supernatant with 3 parts Assay Buffer).
- At this point the SSA concentration will be 1%.
- Further sample dilutions must be done in Sample Diluent. Treated Whole Blood must be further diluted at least 1:20 for a recommended final dilution of ≥ 1:100. For Treated

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Plasma and Treated Urine a final dilution of ≥ 1:5 is recommended, but further dilutions in Sample Diluent may be necessary.

II. **Erythrocytes, Red Blood Cells:**

- Collect blood with heparin or EDTA.
- Centrifuge the sample, remove and discard the plasma and white cell layer.
- Wash the RBCs 2 times by suspending in 3 volumes of isotonic saline (0.9%), centrifuging at 600 g for 10 minutes and discarding the saline wash.
- Thoroughly mix 250 µL RBCs with 1 mL of cold 5% SSA.
- Incubate for 10 minutes at 4°C.
- Centrifuge at 14000 rpm for 10 min at 4˚C and collect the supernatant.
- Dilute the supernatant 1:4 with Assay Buffer (1 part supernatant with 3 part Assay Buffer).
- At this point the SSA concentration will be 1%.
- The samples have been diluted 1:20 at this point. Further dilutions must be made in Sample Diluent.
- NOTE: Human RBC's require a final dilution of 1:100-1:200 to read within the standard curve.

III. **Cell lysate:**

- Collect 1-10 × 10^6 cells and wash with 1 mL cold PBS.
- Re-suspend cell pellets in cold 5% SSA (at 1-10 × 10^6 cells/mL)
- Lyse and deproteinize the cells by vigorous vortexing, freeze-thaw cycling or other suitable disruption methods.
- Incubate the cells at 4°C for 10 minutes.
- Centrifuge at 14,000 rpm for 10 minutes at 4°C and collect the supernatant.
- Dilute the supernatant 1:5 with Assay Buffer (1 part supernatant with 4 parts Assay Buffer).
- At this point the SSA concentration will be 1%.
- Further sample dilutions must be done in Sample Diluent and need to be determined by the end-user since it will be dependent upon the cell type and number of cells used. The recommended final dilution is ≥ 1:20

➢ Samples that have been frozen will contain lysed cells. The PBS wash may contain substantial amounts of GSH and/or GSSG.

IV. **Tissue sample:**

- Wash fresh tissue with cold PBS to remove blood, and blot it on a filter paper.
- Incise the sample and weigh up.
- **For samples requiring a protein determination.**
- Homogenize at 10 mg/250 µL in cold PBS (100 mM, pH 7).

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- Centrifuge at 14,000 rpm for 10 minutes at 4°C, and remove an aliquot of the supernatant for protein determination. **Protein concentration can be easily determined using the ZellX® BCA Assay, CAT No. ZX-44105-96.**
- Thoroughly mix a second aliquot of the supernatant with an equal volume of cold 5% SSA.
- Incubate for 10 minutes at 4°C.
- Centrifuge at 14,000 rpm for 10 minutes at 4°C to remove precipitated protein. Collect the supernatant.
- Dilute the supernatant 2:5 with Assay Buffer (2 parts supernatant with 3 parts Assay Buffer).
- At this point the SSA concentration will be 1%.

❖ **For samples not requiring a protein determination**

- Homogenize at 10 mg/250 µL in cold 5% SSA.
- Incubate for 10 minutes at 4°C
- Centrifuge at 14,000 rpm for 10 minutes at 4°C to remove precipitated protein. Collect the supernatant.
- Dilute the supernatant 1:5 with Assay Buffer (1 part supernatant with 4 parts Assay Buffer).
- At this point the SSA concentration will be 1%.
- Further sample dilutions must be done in Sample Diluent and need to be determined by the end-user since it will be dependent upon the tissue type and the amount of tissue used.

All the samples must be used within 2 hours of dilution.

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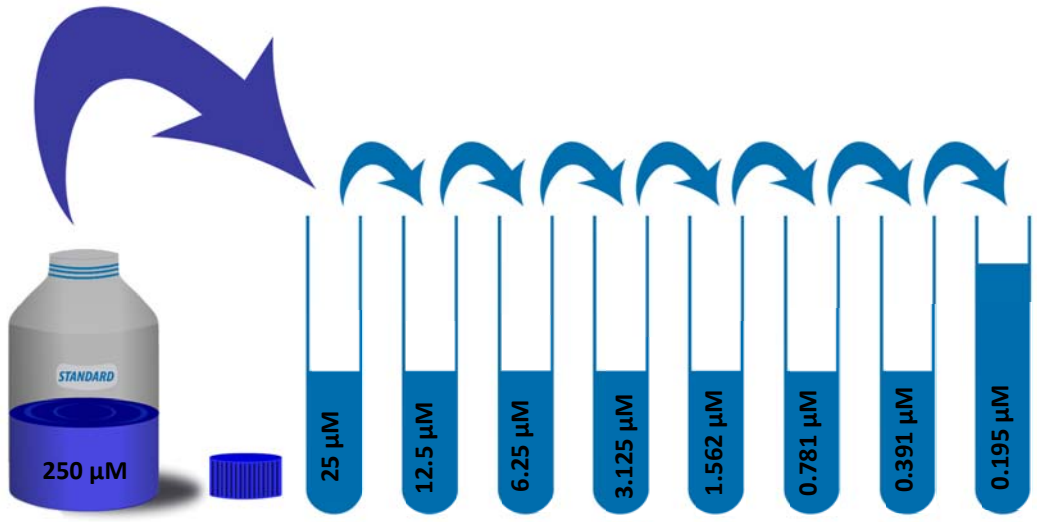
Standard preparation

- Prepare a 1:10 dilution of Standard with Sample Diluent (mix 50 µL of Standard with 450 µL of Sample Diluent), and label as the Standard No.8 (25 µM GSH).
- Make series of lower dilutions as described in the table.
- The Sample Diluent is used as the 0 µM standard.

No.	*Concentration Total GSH*	*Material needed*
Standard No.8	25 µM	50 µL Standard + 450 µL sample diluent
Standard No.7	12.5 µM	250 µL Standard No.8 + 250 µL sample diluent
Standard No.6	6.25 µM	250 µL Standard No.7 + 250 µL sample diluent
Standard No.5	3.125 µM	250 µL Standard No.6 + 250 µL sample diluent
Standard No.4	1.56 µM	250 µL Standard No.5 + 250 µL sample diluent
Standard No.3	0.781 µM	250 µL Standard No.4 + 250 µL sample diluent
Standard No.2	0.391 µM	250 µL Standard No.3 + 250 µL sample diluent
Standard No.1	0.195 µM	250 µL Standard No.2 + 250 µL sample diluent
Standard No.0	0 µM	250 µL Sample Diluent



All standard must be used within 1 hours of preparation

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Assay Procedure

Free and total Glutathione use the standards and samples diluted with Sample Diluent as described previously.

1. Pipette 50 µL of samples or standards or control into duplicate wells in the plate.
2. Pipette 50 µL of Sample Diluent into duplicate wells as the Zero standard.
3. Add 25 µL of Fluorescent Detection Solution to each well using a multichannel/repeater pipette.
4. Gently tap the sides of the plate to ensure adequate mixing of the reagents.
5. Incubate at room temperature for 15 minutes.
6. Read the fluorescent signal from each well at 510 nm with excitation at 370-410 nm (first read). This data will be used to determine the Free GSH concentration.
7. Add 25 µL of Reaction Mix to each well using a multichannel/repeater pipet.
8. Gently tap the sides of the plate to ensure adequate mixing of the reagents.
9. Incubate at room temperature for 15 minutes.
10. Read the fluorescent emission at 510 nm with excitation at 370-410 nm (second read). This data will be used to determine the Total GSH concentration.

For total GSH only: after adding the Fluorescent Detection reagent, add 25 µL Reaction Mix, and tap the side of the plate to ensure adequate mixing of the reagents, incubate at RT for 15 min and read the fluorescent signal.

Calculation

- Average the duplicate Fluorescent Unit (FLU) readings for each standard and sample.
- Subtract the mean FLUs for the zero standard from all FLU values
  (for example if the FLU value of zero standard, and standard 6 are 0.087, and 1.086 respectively; then the adjusted FLUs equal 0 and 0.999 respectively.)
- Create a standard curve by reducing the data using the four parameter logistic curve (4PLC) fitting routine on the plate reader using the adjusted FLU values
- The concentrations obtained should be multiplied by the dilution factor to obtain sample values.

Free Glutathione (GSH) concentrations are calculated from the data obtained from the first read utilizing the curve fitting routine of the plate reader.

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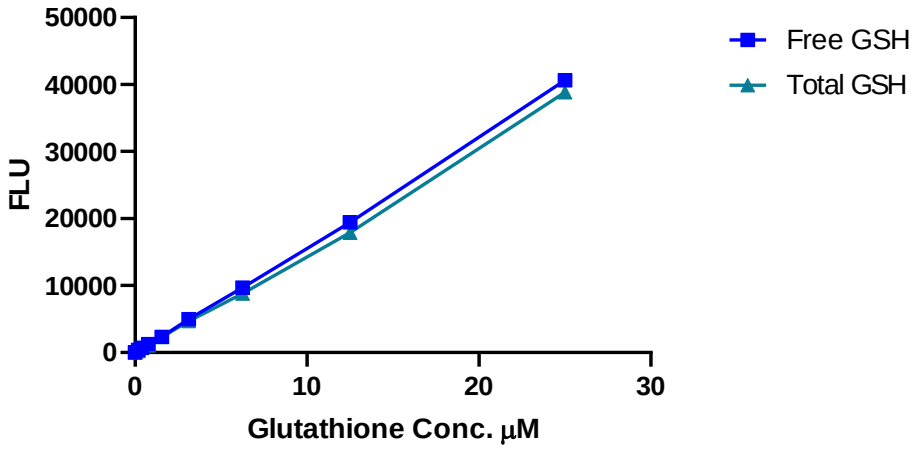
Total Glutathione concentrations of the samples are calculated from the data obtained from the second read utilizing the curve fitting routine of the plate reader. Ensure that the Reaction Mix is added to all the wells used, including the standard and control wells.

Oxidized glutathione (GSSG) concentrations are obtained by subtracting the Free GSH levels from the Total GSH concentrations and dividing by 2.

$$GSSG = \frac{Total\ GSH - Free\ GSH}{2}$$

Note: When Free GSH and Total GSH levels are almost identical, we suggest that you block the free GSH by addition of 2-Vinylpyridine (2VP) to an aliquot of the sample. Add 5 µL of the prepared 2VP to 250 µL of 5% SSA-treated samples and incubate at room temperature for 1 hour. The 2VP-treated samples should then be diluted in Assay Buffer and Sample Diluent according to the dilutions recommended for each sample type prior to using in the assay as described earlier.

2VP is TOXIC and may cause burns. 2VP solutions should be prepared in a fume hood. Use immediately and discard remaining unused solutions by mixing with copious amounts of water.



A typical standard curve of ZellX GSH Assay kit

Run your own standard curves for calculation of results

Assay range

The detection limit of the ZellX® GSH assay was determined as 38 nM in the Free GSH and 42 nM in the Total GSH assays.

Sensitivity

The sensitivity of the ZellX® GSH assay was determined 45 nM for Free GSH and 48 nM for Total GSH assay.

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Precision

Intra-Assay Precision (Precision within an assay): 4 samples were tested 20 times in an assay for total and free GSH.

Inter-Assay Precision (Precision between assays): 4 samples were tested in duplicate on 20 different assays over multiple days for total and free GSH.

Total GSH:

Item	*%CV*
Intra assay	2.3, 4.7, 2.7, 4.7
Inter assay	8.3, 8.0, 10.0, 6.0

Free GSH:

Item	*%CV*
Intra assay	3.0, 3.1, 4.6, 4.0
Inter assay	8.6, 14.7, 7.2, 6.0

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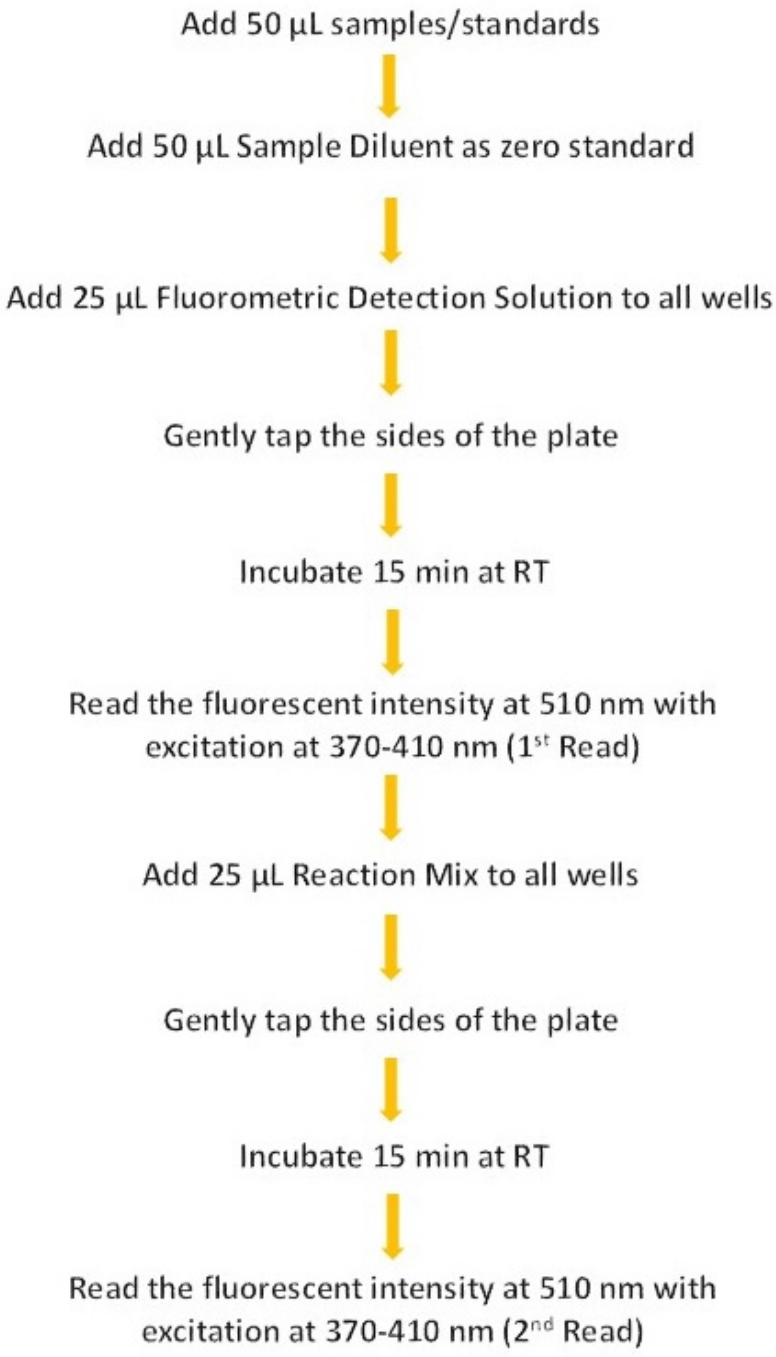
Protocol summary



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