Glutathione (GSH)

Colorimetric Assay kit

(96 Tests)

Zellbio GmbH (Germany)

CAT No. ZX-44100-96

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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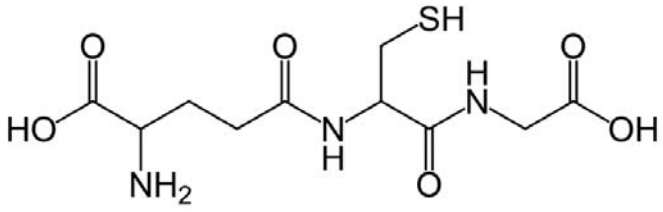
Introduction

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 also 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 colorimetric substrate that reacts with the free thiol group on GSH to yield a highly colored product.

The concentration of Oxidized Glutathione (GSSG) is measured by blocking any free GSH in the sample using 2-Vinylpyridine (2VP _ not supplied). The Total GSH level is determined in the samples which are not treated with 2VP. The Free GSH concentration is calculated from the difference between the Total GSH and the GSSG.

The concentration of GSH can be measured either as an endpoint read of the color developed at 405 nm or as a kinetic method by measuring the rate of color development at 405 nm. 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*
Oxidized Glutathione Standard (250 µM)	85 µL
Detection Reagent Concentrate	250 µL
Assay Buffer	55 mL
NADPH Concentrate	250 µL
Glutathione Reductase Concentrate	250 µL
Clear Half Area 96 Well Plate	1 plate

Storage instruction

All reagents should be stored at 4° C until the expiration date of the kit.

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.

2-Vinylpyridine (2VP). (Prepared fresh by adding 27 µL of 2-vinylpyridine (such as Sigma Catalog Number 132292) to 98 µL of ethanol).

Double distilled water (ddH_2O)

Phosphate Buffer Saline (PBS)

Microplate/ELISA reader capable of reading optical absorption at 405-412 nm

Centrifuge, Vortex mixer

Precision pipettes, multichannel/repeater pipettes 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 (SSA) is a strong acid solution and should be treated like any other laboratory acid.

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 plenty amount of water.

Dimethyl sulfoxide (DMSO), used in the Detection Reagent Substrate, is a powerful aprotic organic solvent that has been shown to enhance 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 reservoirs of multichannel/repeater pipets.**

General remarks

- Equilibrate all kit components to room temperature (RT) 30 minutes before use.
- The instruction must be strictly followed.
- The reading of Microplate/ELISA reader must be set at the appropriate wavelength.
- 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 should be deproteinized with 5% SSA, to remove any protein thiols which slow the oxidation of free GSH (details in Sample Preparation).

Assay protocol

Reagent preparation

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

ii. **Colorimetric Detection Solution:** Prepare a 1:10 dilution of Detection Reagent Concentrate with Assay Buffer (mix 250 µL of Reagent Detection Concentrate with 2.25 mL of Assay Buffer).

iii. **Reaction Mix:** Prepare a 1:1:10 dilutions of NADPH concentrate and Glutathione Reductase Concentrates with Assay Buffer (mix 250 µL of NADPH concentrate and 250 µL of Glutathione Reductase Concentrates with 2 mL of Assay Buffer). Unused Reaction Mixture can be stored at 4°C up to 2 days.

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Sample preparation

After collecting the sample, extraction should be immediately carried out in accordance with 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 of the SSA treated centrifuged supernatants must have their SSA concentration brought down to 1% SSA by dilution with Assay Buffer before conducting the assay.

To measure Oxidized Glutathione (GSSG), reduced Glutathione (GSH) in the sample must be blocked by treatment with 2-vinylpyridine (2VP). SSA-treated samples should be treated with 2VP by addition of 5 µL of 2VP solution for every 250 µL of sample and incubated for 1 hour at RT. 2VP-treated samples must be read off a standard curve made with 2VP-treated standards.

All samples and standards must be in Sample Diluent before starting the assay and must be 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.

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 RBC's 2 times by suspending in 3 volumes of isotonic saline (0.9%), centrifuging at 600 x g for 10 minutes and discarding the saline wash.
- Thoroughly mix 250 µL RBC's 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.

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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 method.
- Incubate the cells at 4°C for 10 minutes.
- Centrifuge at 14,000 rpm for 10 minutes at 4°C and collect supernatant.

➢ 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 sample and weigh up.

❖ **For samples requiring a protein determination**

- Homogenize at 10 mg/250 µL in cold PBS (100 mM, pH 7).
- Centrifuge at 14,000 rpm for 10 minutes at 4°C and remove an aliquot of the supernatant for protein determination.
- 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.

❖ **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.

All the samples must be used within 2 hours of dilution

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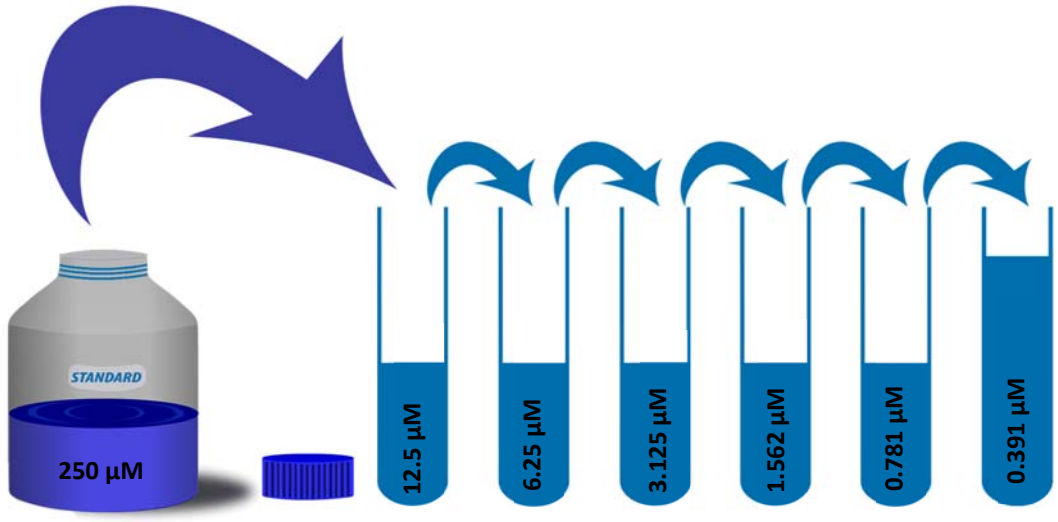
Standard preparation

For the Oxidized Glutathione (GSSG):

- Treat 50 µL of Oxidized Glutathione Standard (250 µM) by 1 µL of 2VP as described earlier.
- Prepare a 1:20 dilution of 2VP-treated Standard with Sample Diluent (mix 25 µL of 2VP-treated standard with 475 µL of Sample Diluent), and label as the Standard No.6 (12.5 µM GS).
- Make series of lower dilutions as described in the table.
- The 2VP-treated Sample Diluent is used as the 0 µM standard.

No.	*Concentration GSSG/Total GSH*	*Material needed*
Standard No.6	12.5 µM / 25 µM	25 µL 2VP-treated Standard + 475 µL Sample Diluent
Standard No.5	6.25 µM / 12.5 µM	250 µL Standard No.6 + 250 µL Sample Diluent
Standard No.4	3.125 µM / 6.25 µM	250 µL Standard No.5 + 250 µL Sample Diluent
Standard No.3	1.56 M / 3.125 µM	250 µL Standard No.4 + 250 µL Sample Diluent
Standard No.2	0.781 µM / 1.56 µM	250 µL Standard No.3 + 250 µL Sample Diluent
Standard No.1	0.391 µM / 0.781 µM	250 µL Standard No.2 + 250 µL Sample Diluent
Standard No.0	0 µM	250 µL 2VP-treated Sample Diluent



All standard must be used within 2 hours of preparation

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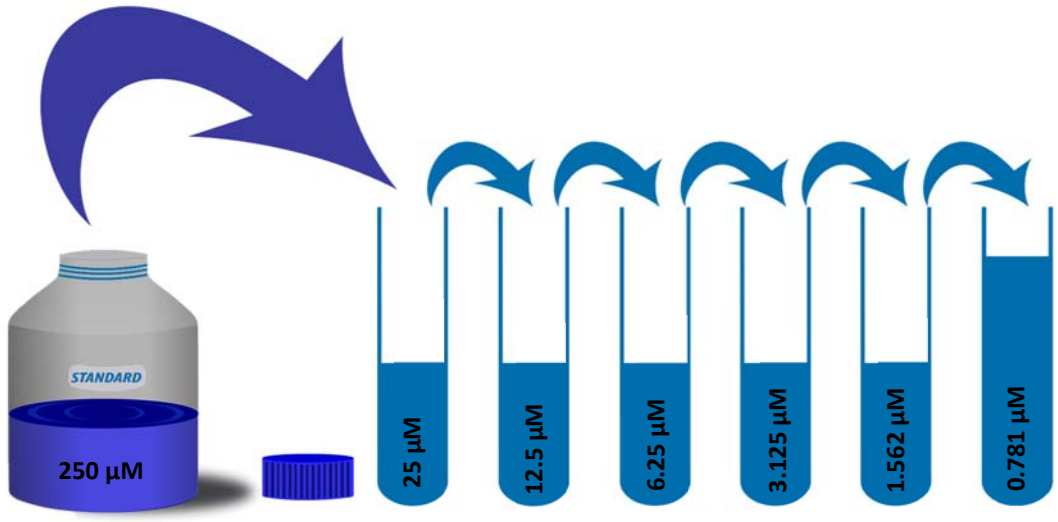
For Total Glutathione (GSH):

- Prepare a 1:20 dilution of Standard with Sample Diluent (mix 25 µL of Standard with 475 µL of Sample Diluent), and label as the Standard No.6 (25 µM GSH).
- Make series of lower dilutions as described in the table.
- Sample Diluent must be used as the 0 µM standard.

No.	*Concentration Total GSH*	*Material needed*
Standard No.6	25 µM	25 µL Standard + 475 µL Sample Diluent
Standard No.5	12.5 µM	250 µL Standard No.6 + 250 µL Sample Diluent
Standard No.4	6.25 µM	250 µL Standard No.5 + 250 µL Sample Diluent
Standard No.3	3.125 µM	250 µL Standard No.4 + 250 µL Sample Diluent
Standard No.2	1.56 µM	250 µL Standard No.3 + 250 µL Sample Diluent
Standard No.1	0.781 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 2 hours of preparation

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Assay Procedure

For Oxidized Glutathione (GSSG) use the 2VP-treated standards, 2VP-treated Sample Diluent and 2VP-treated samples diluted with Sample Diluent as described previously.

For Total Glutathione use the standards and samples diluted with Sample Diluent as described previously.

1. Pipette 50 µL of either 2VP-treated or untreated samples or standards into duplicate wells in the plate.
2. Pipette 50 µL of either 2VP-treated or untreated Sample Diluent into duplicate wells as the Zero standard.
3. Add 25 µL of the Colorimetric Detection Solution to each well using a multichannel/repeater pipette.
4. Add 25 µL of the Reaction Mix to each of the wells using a multichannel/repeater pipette.
5. Gently tap the sides of the plate to ensure adequate mixing of the reagents.
6. Incubate at room temperature for 20 minutes.
7. Read the optical density at 405 nm.

➢ These data will be used to determine either Oxidized Glutathione or Total Glutathione concentration as the endpoint measurement.

For kinetic measurement: after adding the Colorimetric Detection Solution; gently tap the side of the plate and add 25 µL of the Reaction Mix to each well using a multichannel/repeater pipette and immediately read the optical density at 405 nm every minute for at least 10 minutes.

➢ These data will be used to determine either Oxidized Glutathione or Total Glutathione concentration kinetically.

Calculation

- Average the duplicate optical density (OD) readings for each standard and sample.
- Subtract the mean ODs for the zero standard from all OD values

  (for example if the OD value of zero standard, and standard 6 are 0.087, and 1.086 respectively; then the adjusted ODs 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 OD values
- The concentrations obtained should be multiplied by the dilution factor to obtain sample values.

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Oxidized Glutathione concentrations of the samples are calculated based on the data obtained from 2VP-treated samples read off a 2VP-treated standard curve. The concentration of Oxidized Glutathione (GSSG) in the samples would be half of the GSH concentration read off the curve.

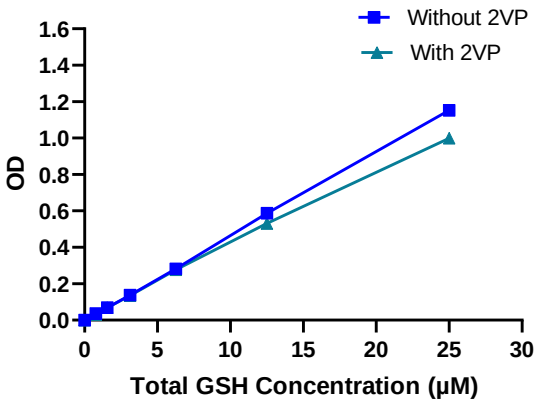
- **1 GSSG = 2 GSH**

Free glutathione (GSH) concentrations are obtained by subtracting the Oxidized Glutathione (GSSG) levels obtained from the 2VP-treated standard and samples from non-treated standards and samples (Total GSH). Concentrations obtained will be in µM of Glutathione.

$$Total\ GSH\ (\mu M) = Free\ GSH\ +\ Oxidized\ GSH\ (GSSG)$$

$$Oxidized\ GSH\ (\mu M) = \frac{(measured\ 2VP\ Treated\ GSH\ concentration)}{2}$$

$$Free\ GSH\ (\mu M) = Total\ GSH\ Conc. - Oxidized\ GSH\ Conc.$$



A typical standard curve of ZellX® GSH Assay kit

Run your own standard curves for calculation of results

Assay range

The detection limit of ZellX® GSH assay was determined as 1.78 µM Glutathione (89 pM/well).

Sensitivity

The sensitivity of the ZellX® GSH assay was determined as 0.634 µM Glutathione (31.7 pM/well).

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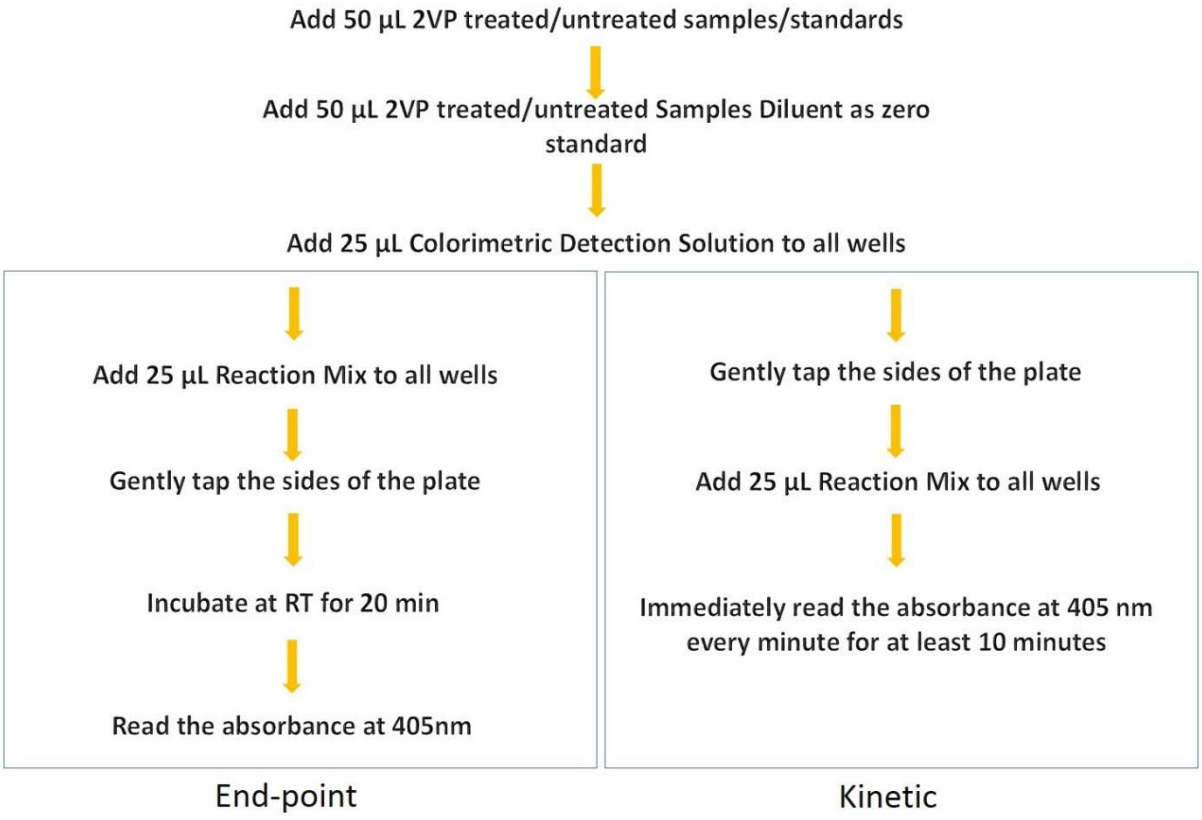
Precision

Intra-Assay Precision (Precision within an assay): 3 samples were tested 20 times in an assay.

Inter-Assay Precision (Precision between assays): 3 samples were tested in duplicate on 20 different assays over multiple days.

Item	*%CV*
Intra assay	2.1, 3.1, 5.0
Inter assay	7.5, 8.4, 13.3

Protocol summary



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References

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