Glutathione Colorimetric Detection Kit

For the quantitative determination of glutathione (GSH) and oxidized glutathione (GSSG) in whole blood, serum, plasma, erythrocytes, urine, cell lysates and tissue samples

Cat. No. KT-727

For Research Use Only.
PRODUCT INFORMATION

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BACKGROUND
Glutathione (L-γ-glutamyl-L-cysteinylglycine; GSH) is the highest concentration non-protein thiol in mammalian cells and is present in concentrations of 0.5 – 10 mM. GSH plays a key role in many biological processes, including the synthesis of proteins and DNA, the transport of amino acids, and the protection of cells against oxidation. Harmful hydrogen peroxide cellular levels are minimized by the enzyme glutathione peroxidase (GP) using GSH as a reductant.

![Glutathione Structure](image)

The oxidized GSH dimer, GSSG, is formed from GSH and peroxide by the GP reaction (see below). An important role of GSSG in the NFκB activating signal cascade is suggested by the facts that the potent NFκB inducer, tetradecanoyl phorbol acetate, increases intracellular GSSG levels and GSSG/GSH ratios.

![Glutathione Peroxidase Reaction](image)

Glutathione S-transferases (GST) are an important group of enzymes that catalyze the nucleophilic addition of GSH to electrophiles. They are encoded by 5 gene families; 4 encode cytosolic GST and one encodes the microsomal form of GST. They have been implicated in a number of diseases. In asthma arachidonic acid is converted to unstable leukotriene A4 (LTA4). LTA4 is either hydrated to form LTB4 or it is conjugated to GSH by a GST, leukotriene C4 synthase, to form leukotriene C4. LTC4 and its derivative LTD4 are important molecules in bronchial asthma. Leukotriene C4 synthase is therefore an important therapeutic target. It has also been shown that increased expression of GSTs can lead to drug resistance. Three glutathione adducts of the drug melphalan, used to treat ovarian cancer and multiple myeloma, have been isolated from reactions involving human microsomal GSTs.
**PRINCIPLE**
The Glutathione kit is designed to quantitatively measure glutathione (GSH), and oxidized glutathione (GSSG) present in a variety of samples. No separation or washing is required. Please read the complete kit insert before performing this assay. A GSSG calibrator is provided to generate a calibration curve for the assay and all samples should be read off the calibration curve.

The kit utilizes a colorimetric substrate that reacts with the free thiol group on GSH to yield a highly colored product. Supplied reagents are in solution and require simple dilution for use in the assay. By using 2-Vinylpyridine (not supplied) to block any free GSH in the sample, Oxidized Glutathione (GSSG) can be determined. Any samples that have not been treated with 2-Vinylpyridine will yield Total GSH levels. The Free GSH concentration in the sample is calculated from the difference between the Total GSH determined and the GSH generated from Oxidized Glutathione for the 2-Vinylpyridine treated samples. The concentration of GSH can be determined either as an endpoint read of the color developed at 405 nm or by measuring the rate of color development at 405 nm.

Our Fluorescent Glutathione kits allow the measurement of both Free and Oxidized Glutathione with higher sensitivity in the same sample in the same well without using 2-Vinylpyridine.

**COMPONENTS**

**Clear 96 Well Plate** 4 plates

**Oxidized Glutathione Calibrator** 350 µL
Oxidized Glutathione at 250 µM in a special stabilizing solution.

**Detection Reagent Concentrate** 1 mL
Detection substrate in DMSO.

**Assay Buffer** 225 mL
A phosphate buffer containing chelators and stabilizers.

**NADPH Concentrate** 1 mL
Reduced β-nicotinamide adenine dinucleotide 2'-phosphate (NADPH) as a stable solution.

**Glutathione Reductase Conc.** 1 mL
Glutathione Reductase (GR) as a stable solution.

**STORAGE**
All components of this kit should be stored at 4°C until the expiration date of the kit.

**OTHER MATERIALS REQUIRED**
Distilled or deionized water.

Repeater pipet with disposable tips capable of dispensing 25 µL.

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

2-Vinylpyridine (2VP) is used to block any free GSH or other thiols present in the treated samples. 2VP is prepared by adding 27 µL of 2-vinylpyridine (such as Sigma Catalog Number 132292) to 98 µL of ethanol. Use immediately and discard remaining unused solutions.

A 96 well plate reader capable of reading optical absorption at 405-412 nm.

Software for converting raw optical density readings from the plate reader and carrying out four parameter logistic curve (4PLC) fitting. Contact your plate reader manufacturer for details.

**PRECAUTIONS**
As with all such products, 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.

Sullosalicylic acid 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 copious amounts of water.

Dimethyl sulfoxide 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.

**SAMPLE TYPES**

GSH is identical across species and we expect this kit may measure GSH from sources other than human. The end user should evaluate recoveries of GSH in samples from other species being tested.

If samples need to be stored after collection, we recommend storing them at -70°C or lower, preferably after being frozen in liquid nitrogen. This assay has been validated for human whole blood, serum, EDTA and heparin plasma, urine, and isolated erythrocytes. Most cell lysates and tissue homogenates should also be compatible. Samples containing visible particulate should be centrifuged prior to using.

All samples will be deproteinized with 5% SSA, please see sample specific information below for details. This treatment removes any protein thiols present in the samples and also slows oxidation of free GSH.

**SAMPLE PREPARATION**

All samples must be treated with the SSA solution. All of the SSA treated centrifuged supernatants must have their SSA concentration brought down to 1% SSA by dilution with Assay Buffer. Further dilutions of the sample, using Sample Diluent, may be necessary to allow the GSH concentration to be measurement in the assay. Detailed instructions follow.

**All samples and calibrators must be in Sample Diluent before starting the assay**

To measure Oxidized Glutathione in samples, 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 \( \mu \)L of 2VP solution for every 250 \( \mu \)L of sample. 2VP treated samples must be read off a calibration curve made with 2VP-treated calibrators. **Use all samples within 2 hours of dilution.**

**Whole Blood, Serum, EDTA or Heparin Plasma, or Urine**

Thoroughly mix sample 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. Collect the supernatant. If the supernatant contains particulates, re-centrifuge the supernatant for 15 minutes and collect the clarified second supernatant. Samples can be stored in aliquots at ≤ -70°C or analyzed immediately. At this point the SSA concentration will be 2.5%.

The supernatant must be diluted 1:2.5 with Assay Buffer by mixing one part with 1.5 parts of Assay Buffer to bring the SSA concentration to 1%. The sample will have been diluted 1:5 at this point.

All final dilutions are made in Sample Diluent. Treated Whole Blood must be further diluted at least 1:20 for a recommended final dilution of ≥ 1:100. For Treated Plasma and Treated Urine a final dilution of ≥ 1:5 is recommended, but further dilutions in Sample Diluent may be necessary.

**Tissue Samples**

Fresh tissue is washed with ice cold PBS to remove blood then blotted on filter paper before recording wet weight. **NOTE:** Samples that have been frozen will contain lysed cells. The PBS wash may contain substantial amounts of GSH and/or GSSG.

- **For Samples Where a Protein Determination is to be Obtained:** Homogenize at 10 mg/250 \( \mu \)L in ice cold 100mM phosphate buffer, 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. The supernatant must be diluted 1:2.5 with Assay Buffer by mixing one part with 1.5 parts of Assay Buffer. The SSA concentration will be 1%.

- **For Samples Not Requiring a Protein Determination:** Homogenize at 10 mg/250 \( \mu \)L in ice cold 5% SSA, incubate at 10 minutes at 4°C, then centrifuge at 14,000 rpm for 10 minutes at 4°C to remove precipitated protein. Collect the supernatant.
The supernatant must be diluted 1:5 with Assay Buffer by mixing one part with 4 parts of Assay Buffer. The SSA concentration will be 1%.

Further sample dilutions must be determined by the end-user since it will be dependent upon the tissue type and the amount of tissue used. These dilutions must be made in the prepared Sample Diluent.

**Erythrocytes, Red Blood Cells (RBC’s)**
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.

After the 2 washes, mix 250µL RBC’s with 1mL of cold 5% SSA. Incubate for 10 minutes at 4°C and centrifuge at 14,000 rpm for 10 minutes at 4°C. Collect the supernatant. At this point the SSA concentration will be 4%. The supernatant must be diluted 1:4 with Assay Buffer by mixing one part with 3 parts of Assay Buffer. The SSA concentration will now be 1% and the sample will have been diluted 1:20 at this point. Further dilutions are made in Sample Diluent for a recommended final dilution of ≥1:40.

**Cell Lysates**
Washed cell pellets are resuspended at 1-10x10^6 cells/mL in cold 5% SSA (we used Jurkats at 5x10^6 cells/mL) and are lysed and deproteinized by vigorous vortexing, freeze/thaw cycling or other suitable disruption method. Incubate cells at 4°C for 10 minutes followed by centrifugation for 10 minutes at 14,000 rpm and 4°C. **NOTE:** Samples that have been frozen will contain lysed cells. The PBS wash may contain substantial amounts of GSH and/or GSSG.

The deproteinized supernatants must be diluted 1:5 with Assay Buffer by mixing one part with 4 parts of Assay Buffer. The SSA concentration will be 1%. The sample will have been diluted 1:5 at this point. 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.

**Use all samples within 2 hours of dilution.**

**REAGENT PREPARATION**
Allow the kit reagents to come to room temperature for 30 minutes. We recommend that all calibrators and samples be run in duplicate to allow the end user to accurately determine GSH concentrations. Ensure that all samples have reached room temperature and have been diluted as appropriate prior to running them in the kit.

**Sample Diluent**
Prepare the Sample Diluent by diluting one part 5% SSA 1:5 with four parts Assay Buffer and vortex thoroughly. The pH of the Sample Diluent must be > 6. Sample Diluent can be stored at 4°C for one month.

**2-Vinylpyridine Treatment**
To measure Oxidized Glutathione, free GSH must be blocked by alkylation. To 250 µL of SSA treated samples, calibrators or Sample Diluent add 5 µL of the ethanolic solution of 2VP and allowed to incubate at room temperature for 1 hour. The 2VP treated samples and calibrators 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. The 2VP treated Sample Diluent is used for the zero calibrator. **Samples treated with 2VP should be read off a calibration curve generated with 2VP treated calibrators.**

**Colorimetric Detection Reagent**
Prepare the Colorimetric Detection Reagent by diluting one part Colorimetric Detection Reagent Concentrate 1:10 with nine parts Assay Buffer. See Colorimetric Detection Reagent Dilution Table for suitable volumes.

### Colorimetric Detection Reagent Dilution Table

<table>
<thead>
<tr>
<th></th>
<th>1/2 Plate</th>
<th>One Plate</th>
<th>Two Plates</th>
<th>Four Plates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colorimetric Detection Concentrate</td>
<td>140 µL</td>
<td>260 µL</td>
<td>500 µL</td>
<td>1 mL</td>
</tr>
<tr>
<td>Assay Buffer</td>
<td>1.26 mL</td>
<td>2.34 mL</td>
<td>4.5 mL</td>
<td>9 mL</td>
</tr>
<tr>
<td>Total Colorimetric Reagent Volume</td>
<td>1.4 mL</td>
<td>2.6 mL</td>
<td>5 mL</td>
<td>10 mL</td>
</tr>
</tbody>
</table>
Reaction Mixture

Prepare the Reaction Mixture by diluting one part each NADPH and Glutathione Reductase Concentrates 1:10 into eight parts Assay Buffer. See Reaction Mix Dilution Table for suitable volumes. Store any unused Reaction Mixture at 4°C for no more than 2 days.

<table>
<thead>
<tr>
<th>Reaction Mix Dilution Table</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

CALIBRATOR PREPARATION

For the measurement of Oxidized Glutathione (GSSG), a 50 µL aliquot of the 250 µM Oxidized Glutathione Calibrator should be treated with 1 µL of 2VP. 2VP-treated Calibrators are prepared by labeling six test tubes as #1 through #6. Pipet 475 µL of Sample Diluent into tube #1 and 250 µL into tubes #2 to #6. Carefully add 25 µL of the 2VP-treated Calibrator to tube #1 and vortex completely. Take 250 µL of the solution in tube #1 and add it to tube #2 and vortex completely. Repeat this for tubes #3 through #6. The concentration of Oxidized Glutathione in tubes 1 through 6 will be 12.5, 6.25, 3.125, 1.56, 0.781 and 0.391 µM. The concentration of Total GSH in tubes 1 through 6 will be 25, 12.5, 6.25, 3.125, 1.56, and 0.781 µM after addition of the Reaction Mixture. 2VP treated Sample Diluent must be used as a 0 µM calibrator.

To determine Total GSH, the Calibrators are prepared by labeling six test tubes as #1 through #6. Pipet 475 µL of Sample Diluent into tube #1 and 250 µL into tubes #2 to #6. Carefully add 25 µL of the supplied Calibrator to tube #1 and vortex completely. Take 250 µL of the solution in tube #1 and add it to tube #2 and vortex completely. Repeat this for tubes #3 through #6. The concentration of Total GSH in tubes 1 through 6 will be 25, 12.5, 6.25, 3.125, 1.56, and 0.781 µM after addition of the Reaction Mixture. Sample Diluent must be used as a 0 µM calibrator.

Use all Calibrators within 2 hours of preparation.
ASSAY PROTOCOL-END POINT

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

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

1. Use the plate layout sheet on the back page to aid in proper sample and calibrator identification.
2. Pipet 50 µL of either 2VP treated or untreated samples or calibrators into duplicate wells in the plate.
3. Pipet 50 µL of either 2VP treated or untreated Sample Diluent into duplicate wells as the Zero calibrator.
4. Add 25 µL of the Colorimetric Detection Reagent to each well using a repeater pipet.
5. Add 25 µL of the Reaction Mixture to each of the wells using a repeater pipet.
6. Gently tap the sides of the plate to ensure adequate mixing of the reagents.
7. Incubate at room temperature for 20 minutes.
8. Read the optical density at 405 nm. These data will be used to determine either Oxidized Glutathione or Total Glutathione concentration.

ASSAY PROTOCOL-KINETIC

1. Carry out steps 1-4 above.
2. Gently tap the sides of the plate to ensure adequate mixing of the reagents.
3. Add 25 µL of the Reaction Mixture to each of the wells using a repeater and immediately place plate in reader and read optical density at 405 nm every minute for at least 10 minutes. These data will be used to determine Total or Oxidized Glutathione concentration kinetically.

CALCULATION OF RESULTS

Average the duplicate optical density readings for each calibrator and sample. Create a calibration curve by reducing the data using the 4PLC fitting routine on the plate reader, after subtracting the mean ODs for the zero calibrator. The concentrations obtained should be multiplied by the dilution factor to obtain sample values.

Glutathione concentrations (see below) are calculated from the data using the curve fitting routine supplied with the plate reader.

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

Note: 1 GSSG = 2 GSH

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

\[
\text{Total GSH} = \text{Free GSH} + \text{Oxidized GSH (GSSG)}
\]

\[
\text{Oxidized GSH} = \frac{\text{(measured 2VP Treated GSH concentration)}}{2}
\]

\[
\text{Free GSH} = \text{Total GSH Conc. - Oxidized GSH Conc.}
\]
### TYPICAL DATA-TOTAL GLUTATHIONE

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean OD</th>
<th>Net OD</th>
<th>Total Glutathione Conc. (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero</td>
<td>0.086</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Standard 1</td>
<td>1.239</td>
<td>1.153</td>
<td>25</td>
</tr>
<tr>
<td>Standard 2</td>
<td>0.673</td>
<td>0.587</td>
<td>12.5</td>
</tr>
<tr>
<td>Standard 3</td>
<td>0.368</td>
<td>0.282</td>
<td>6.25</td>
</tr>
<tr>
<td>Standard 4</td>
<td>0.224</td>
<td>0.138</td>
<td>3.125</td>
</tr>
<tr>
<td>Standard 5</td>
<td>0.155</td>
<td>0.069</td>
<td>1.56</td>
</tr>
<tr>
<td>Standard 6</td>
<td>0.123</td>
<td>0.037</td>
<td>0.781</td>
</tr>
<tr>
<td>Sample 1</td>
<td>0.360</td>
<td>0.273</td>
<td>6.05</td>
</tr>
<tr>
<td>Sample 2</td>
<td>0.246</td>
<td>0.160</td>
<td>3.65</td>
</tr>
</tbody>
</table>

### TYPICAL DATA-OXIDIZED GLUTATHIONE

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean OD</th>
<th>Net OD</th>
<th>Total Glutathione Conc. (µM)</th>
<th>Oxidized Glutathione Conc. (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero</td>
<td>0.087</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Standard 1</td>
<td>1.086</td>
<td>0.999</td>
<td>25</td>
<td>12.5</td>
</tr>
<tr>
<td>Standard 2</td>
<td>0.619</td>
<td>0.532</td>
<td>12.5</td>
<td>6.25</td>
</tr>
<tr>
<td>Standard 3</td>
<td>0.364</td>
<td>0.277</td>
<td>6.25</td>
<td>3.125</td>
</tr>
<tr>
<td>Standard 4</td>
<td>0.222</td>
<td>0.135</td>
<td>3.125</td>
<td>1.52</td>
</tr>
<tr>
<td>Standard 5</td>
<td>0.156</td>
<td>0.069</td>
<td>1.56</td>
<td>0.781</td>
</tr>
<tr>
<td>Standard 6</td>
<td>0.117</td>
<td>0.030</td>
<td>0.781</td>
<td>0.391</td>
</tr>
<tr>
<td>Sample 1</td>
<td>0.177</td>
<td>0.089</td>
<td>-</td>
<td>1.02</td>
</tr>
<tr>
<td>Sample 2</td>
<td>0.125</td>
<td>0.038</td>
<td>-</td>
<td>0.48</td>
</tr>
</tbody>
</table>
Always run your own calibration curve for calculation of results. Do not use this data.

**Typical Calibration Curves**

![Typical Calibration Curves Graph](image)

**VALIDATION DATA**

**Sensitivity and Limit of Detection**

Sensitivity was calculated by comparing the ODs for twenty wells run for each of the zero and calibrator #6. The detection limit was determined at two (2) standard deviations from the zero along the calibration curve.

Sensitivity was determined as 0.634 µM of Glutathione. This is equivalent to 31.7 pM/well.

The Limit of Detection was determined in a similar manner by comparing the ODs for twenty wells run for each of the zero and a low concentration human urine sample.

The Limit of Detection was determined as 1.78 µM Glutathione. This is equivalent to 89 pM/well.

**Linearity**

Linearity was determined by taking Jurkat cell lysates at 25 x 10^6 cells/mL and one at 1.28 x 10^6 cells/mL, diluted 1:5, and mixed in the ratios given below. The measured concentrations were compared to the expected values based on the ratios used.
Intra Assay Precision
Two whole blood samples and one human urine sample were SSA treated, diluted in 1% SSA Calibrator Diluent and run in replicates of 20 in an assay. The mean and precision of the calculated Total Glutathione concentrations were:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Glutathione Conc. (µM)</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.36</td>
<td>2.1</td>
</tr>
<tr>
<td>2</td>
<td>5.55</td>
<td>3.1</td>
</tr>
<tr>
<td>3</td>
<td>3.30</td>
<td>5.0</td>
</tr>
</tbody>
</table>
Inter Assay Precision
Two whole blood samples and one human urine sample were SSA treated, diluted in 1% SSA Calibrator Diluent and run in duplicates in twenty assays run over multiple days by four operators. The mean and precision of the calculated Total Glutathione concentrations were:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Glutathione Conc. (µM)</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.43</td>
<td>7.5</td>
</tr>
<tr>
<td>2</td>
<td>5.27</td>
<td>8.4</td>
</tr>
<tr>
<td>3</td>
<td>3.08</td>
<td>13.3</td>
</tr>
</tbody>
</table>