Glutathione Assay Kit

Item No. 703002

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	96 Well Quantity/Size	480 Well Quantity/Size
703010	GSH MES Buffer (2X)	1 vial/60 ml	1 vial/60 ml
703014	GSSG Standard	1 vial/2 ml	2 vials/2 ml
703016	GSH Co-Factor Mixture	1 vial	5 vials
703018	GSH Enzyme Mixture	1 vial/0.2 ml	5 vials/0.2 ml
703012	GSH DTNB	4 vials	6 vials
400014	96-Well Solid Plate (Colorimetric Assay)	1 plate	5 plates
400012	96-Well Cover Sheet	1 cover	5 covers

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Fax: 734-971-3641

Email: techserv@caymanchem.com

Hours: M-F 8:00 AM to 5:30 PM EST

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed at 4°C and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader capable of measuring an absorbance of 405-414 nm
2. An adjustable pipettor, repeating pipettor, and an eight channel pipettor (optional)
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable
4. Metaphosphoric acid and triethanolamine (described on page 12)
5. 2-Vinylpyridine (optional, described on page 13)

INTRODUCTION

Background

Glutathione (GSH) is a tripeptide (γ-glutamylcysteinylglycine) widely distributed in both plants and animals.[1,2] GSH serves as a nucleophilic co-substrate to glutathione transferases in the detoxification of xenobiotics and is an essential electron donor to glutathione peroxidases in the reduction of hydroperoxides.[2,3] GSH is also involved in amino acid transport and maintenance of protein sulfhydryl reduction status.[4,5] Concentration of GSH ranges from a few micromolar in plasma to several millimolar in tissues such as liver.[6-9]

About This Assay

Cayman's GSH Assay utilizes a carefully optimized enzymatic recycling method, using glutathione reductase, for the quantification of GSH (see Figure 1, page 6).[10-12] The sulfhydryl group of GSH reacts with DTNB (5,5'-dithio-*bis*-2-(nitrobenzoic acid), Ellman's reagent) and produces a yellow colored 5-thio-2-nitrobenzoic acid (TNB). The mixed disulfide, GSTNB (between GSH and TNB) that is concomitantly produced, is reduced by glutathione reductase to recycle the GSH and produce more TNB. The rate of TNB production is directly proportional to this recycling reaction which is in turn directly proportional to the concentration of GSH in the sample. Measurement of the absorbance of TNB at 405-414 nm provides an accurate estimation of GSH in the sample.

GSH is easily oxidized to the disulfide dimer GSSG. GSSG is produced during the reduction of hydroperoxides by glutathione peroxidase. GSSG is reduced to GSH by glutathione reductase and it is the reduced form that exists mainly in biological systems. Since glutathione reductase is used in the Cayman GSH assay, both GSH and GSSG are measured and the assay reflects total glutathione. The kit can also be used to measure only GSSG by following the protocol given on page 13. GSH measurement can be done in plasma, serum, erythrocyte lysates, tissue samples, and cultured cells using this kit. However, plasma and serum samples will have to be concentrated before assaying, and nearly all samples will require deproteination (see page 12 for more details).

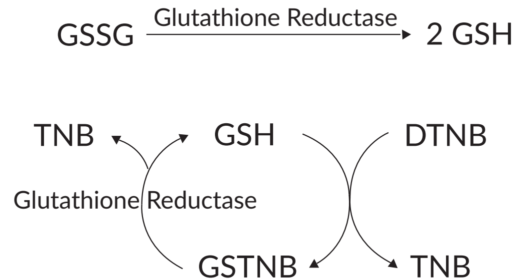

Figure 1. GSH recycling

PRE-ASSAY PREPARATION

Reagent Preparation

1. **GSH MES Buffer (2X) - (Item No. 703010)**

   The buffer consists of 0.4 M 2-(N-morpholino)ethanesulphonic acid, 0.1 M phosphate, and 2 mM EDTA, pH 6.0. Dilute 60 ml of the buffer with 60 ml of HPLC-grade water before use. Hereafter, MES Buffer refers to this diluted buffer.

2. **GSSG Standard - (Item No. 703014)**

   Each vial contains 2 ml of 25 µM GSSG in MES Buffer. This standard is ready to use as supplied. *NOTE: GSSG is provided as a standard instead of GSH. Under the assay conditions, GSSG is immediately reduced to GSH thereby providing the necessary standard.* The standard is stable for at least one year if stored as supplied at 0-4°C.

3. **GSH Co-Factor Mixture - (Item No. 703016)**

   The vials contain a lyophilized powder of $NADP^+$ and glucose-6-phosphate. Reconstitute the contents of the vial with 0.5 ml of water and mix well. This is enough Co-Factor Mixture for 96 wells. Prepare additional vials as needed. The reconstituted reagent will be stable for two weeks if stored at 0-4°C.

4. **GSH Enzyme Mixture - (Item No. 703018)**

   The vials contain glutathione reductase and glucose-6-phosphate dehydrogenase in 0.2 ml buffer. Carefully open the vial without spilling any liquid from the cap. Add 2 ml of diluted MES Buffer to the vial, replace the cap, and mix well. This is enough Enzyme Mixture for 96 wells. Prepare additional vials as needed. The reconstituted Enzyme Mixture will be stable for two weeks if stored at 0-4°C.

5. **GSH DTNB - (Item No. 703012)***

   Each vial contains a lyophilized powder of DTNB (5,5'-dithio-*bis*-(2-nitrobenzoic acid), Ellman's reagent). Reconstitute the contents of the vial with 0.5 ml of water and mix well. The reconstituted reagent must be used within 10 minutes.

*Reconstitution of this reagent should be done just prior to its addition to the Assay Cocktail described on page 17. Four to six vials of this reagent are provided to reconstitute each time the Assay Cocktail is prepared.

Sample Preparation

CAUTION: *Thiol compounds such as mercaptoethanol, dithiothreitol, etc., or thiol alkylating agents such as N-ethylmaleimide should not be added to the samples at any stage of sample collection or preparation. If the samples contain any of these compounds they are unsuitable for GSH quantification.*

Tissue Homogenate

1. Prior to dissection, either perfuse or rinse tissue with a PBS (phosphate buffered saline) solution, pH 7.4, to remove any red blood cells and clots.
2. Homogenize the tissue in 5-10 ml of cold buffer (*i.e.*, 50 mM MES or phosphate, pH 6-7, containing 1 mM EDTA) per gram tissue.
3. Centrifuge at 10,000 x g for 15 minutes at 4°C.
4. Remove the supernatant and store on ice.
5. The supernatant will have to be deproteinated before assaying (see page 12). If not assaying on the same day, the sample will still have to be deproteinated, and then stored at -20°C. The sample will be stable for at least six months.

Cell Lysate

1. Collect cells by centrifugation (*i.e.*, 1,000-2,000 x g for 10 minutes at 4°C). For adherent cells, do not harvest using proteolytic enzymes; rather use a rubber policeman.
2. The cell pellet can be homogenized or sonicated in 1-2 ml of cold buffer (*i.e.*, 50 mM MES or phosphate, pH 6-7, containing 1 mM EDTA).
3. Centrifuge at 10,000 x g for 15 minutes at 4°C.
4. Remove the supernatant and store on ice.
5. The supernatant will have to be deproteinated before assaying (see page 12). If not assaying on the same day, the sample will still have to be deproteinated, and then stored at -20°C. The sample will be stable for at least six months.

Plasma and Erythrocyte lysate

1. Collect blood using an anticoagulant such as heparin, citrate, or EDTA.
2. Centrifuge the blood at 700-1,000 x g for 10 minutes at 4°C. Pipette off the top yellow plasma layer without disturbing the white buffy layer. Store plasma on ice.
3. Remove the white buffy layer (leukocytes) and discard.
4. Lyse the erythrocytes (red blood cells) in four times its volume of ice-cold HPLC-grade water.
5. Centrifuge at 10,000 x g for 15 minutes at 4°C.
6. Collect the supernatant (erythrocyte lysate) and store on ice.
7. The plasma and erythrocyte lysate will have to be deproteinated before assaying (see page 12). If not assaying on the same day, the samples will still have to be deproteinated, and then stored at -20°C. The samples will be stable for at least six months. *NOTE: Plasma samples contain glutathione levels below the detection limit of the assay and thus can not be measured directly.* Before assaying, add TEAM reagent to the deproteinated plasma sample (see page 12), concentrate by lyophilization, and then reconstitute the sample with MES Buffer to one third of its original volume. You will only be able to determine the total GSH content. We do not guarantee the accuracy of the GSSG content due to the many manipulations that the plasma sample has endured.

Serum

1. Collect blood without using an anticoagulant such as heparin, citrate, or EDTA. Allow blood to clot for 30 minutes at 25°C.
2. Centrifuge the blood at 2,000 x g for 15 minutes at 4°C. Pipette off the top yellow serum layer without disturbing the white buffy layer. Store serum on ice.
3. The serum will have to be deproteinated before assaying (see page 12). If not assaying on the same day, the sample will still have to be deproteinated, and then stored at -20°C. The sample will be stable for at least six months. *NOTE: Serum samples contain glutathione levels below the detection limit of the assay and thus can not be measured directly.* Before assaying, add TEAM reagent to the deproteinated serum sample (see page 12), concentrate by lyophilization, and then reconstitute the sample with MES Buffer to one third of its original volume. You will only be able to determine the total GSH content. We do not guarantee the accuracy of the GSSG content due to the many manipulations that the serum sample has endured.

Recommended procedure for deproteination of samples:

Almost all biological samples used for GSH measurement contain large amounts of proteins, *e.g.*, erythrocyte lysate, tissue homogenates, etc. It is necessary to remove as much protein as possible from the sample to avoid interferences due to particulates and sulfhydryl groups on proteins in the assay. Samples that are low in protein (<1 mg/ml) and are devoid of particulates can be assayed directly.

1. MPA Reagent: Dissolve 5 g of metaphosphoric acid (Sigma-Aldrich 239275 or VWR Item No. AA33267-22) in 50 ml water. The MPA solution is stable for four hours at 25°C.
2. Add an equal volume of the MPA Reagent to the sample and mix by vortexing. Allow the mixture to stand at room temperature for five minutes and centrifuge at >2,000 g for at least two minutes (a microfuge will be sufficient for the centrifugation). Carefully collect the supernatant without disturbing the precipitate. The supernatant can be stored at this stage for long periods of time (up to six months) at -20°C without any degradation of GSH or GSSG. Do not add TEAM Reagent until you are ready to assay the sample.
3. TEAM Reagent: Prepare a 4 M solution of triethanolamine (Sigma-Aldrich, Item No. T58300) in water by mixing 531 µl of triethanolamine with 469 µl of water. The TEAM solution is stable for four hours at 25°C.
4. Add 50 µl of TEAM Reagent per ml of the supernatant and vortex immediately. The TEAM Reagent will increase the pH of the sample. The sample is ready for assay of total GSH (*i.e.*, both oxidized and reduced). Any necessary dilutions of the sample should be done at this stage with MES Buffer.

Sample preparation for exclusive measurement of GSSG:

Quantification of GSSG, exclusive of GSH, is accomplished by first derivatizing GSH with 2-vinylpyridine.[13] This can be achieved as follows:

1. Prepare a 1 M solution of 2-vinylpyridine (Sigma-Aldrich, Item No. 13229-2) in ethanol by mixing 108 µl of 2-vinylpyridine and 892 µl of ethanol.
2. Add 10 µl of the 2-vinylpyridine solution per ml of sample from step 4 of deproteination of sample. Mix well on a vortex mixer and incubate at room temperature for about 60 minutes and assay the sample.* This procedure can derivatize up to 1 mM GSH. More concentrated samples should be diluted with MES Buffer before derivatization.

*2-Vinylpyridine inhibits color development in the assay to some extent. Hence, it is essential to prepare the standards also the same way by adding 2-vinylpyridine (*i.e.*, add 5 µl of 2-vinylpyridine solution per Standard tube) and incubating to the same length of time as the sample.

Tissue Homogenization using the Precellys 24 Homogenizer

- Freeze organs immediately upon collection and then store at -80°C. Snap-freezing of tissues in liquid nitrogen is preferred.
- Add cold buffer to sample (*i.e.*, 50 mM MES or phosphate, pH 6-7, containing 1 mM EDTA).
- Homogenize the tissue sample using the Precellys 24 according to appropriate settings:
- Spin the tissue homogenates at 10,000 x g for 15 minutes at 4°C.
- Collect supernatant and assay samples according to the kit booklet protocol. Samples may need to be diluted appropriately for assay and should be normalized using a protein assay.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. A typical layout of standards and samples to be measured in duplicate is given below in Figure 2. We suggest you record the contents of each well on the template sheet provided (see page 26).

	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33
B	B	B	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34
C	C	C	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35
D	D	D	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36
E	E	E	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37
F	F	F	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38
G	G	G	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39
H	H	H	S8	S8	S16	S16	S24	S24	S32	S32	S40	S40

A-H = Standards
S1-S40 = Sample wells

Figure 2. Sample plate format

Pipetting Hints

- It is recommended that an adjustable pipette be used to deliver reagents to the wells.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- All reagents must be equilibrated to room temperature before beginning the assay.
- The volume of sample and standards added to the wells is 50 µl and the final volume of the assay is 200 µl in all the wells.
- It is not necessary to use all the wells on the plate at one time. However, a standard curve must be run simultaneously with each set of samples.
- Add TEAM Reagent to the deproteinated samples (see deproteination procedure, page 12).
- Use diluted MES Buffer in the assay.
- If the expected concentration of GSH in the sample is not known or if it is expected to be beyond the range of the standard curve, it is prudent to assay the sample at several dilutions.
- It is recommended that the samples and standards be assayed at least in duplicate.
- Prepare the Assay Cocktail just before its addition to the wells.
- Addition of the Assay Cocktail to the wells must be done as quickly as possible. The time difference in addition between the first well to the last should not be more than two minutes.

Standard Preparation

Take eight clean test tubes and mark them A-H. Aliquot the GSSG Standard (Item No. 703014) and MES Buffer to each tube as described in Table 1.

Tube	GSSG Standard (µl)	MES Buffer (µl)	Final Concentration (µM GSSG)	Equivalent Total GSH (µM)*
A	0	500	0	0
B	5	495	0.25	0.5
C	10	490	0.5	1.0
D	20	480	1.0	2.0
E	40	460	2.0	4.0
F	80	420	4.0	8.0
G	120	380	6.0	12.0
H	160	340	8.0	16.0

Table 1. Glutathione standards
*Under the assay conditions GSSG is reduced to produce 2 mole equivalents of GSH.

Performing the Assay

1. Add 50 µl of Standard (tubes A-H) per well in the designated wells on the plate (see **Sample Plate Format**, Figure 2, page 14).
2. Add 50 µl of sample to each of the sample wells.
3. Cover the plate with the plate cover provided.
4. Prepare the Assay Cocktail by mixing the following reagents in a 20 ml vial: MES Buffer (11.25 ml), reconstituted Cofactor Mixture (0.45 ml), reconstituted Enzyme Mixture (2.1 ml), water (2.3 ml), and reconstituted DTNB (0.45 ml). *NOTE: The volumes of reagents given are for the use of the entire plate. Adjust the volumes of the reagents accordingly if only a part of the plate is used. Prepare fresh Assay Cocktail and run a standard curve each time the assay is performed. Use the Assay Cocktail within 10 minutes of preparation.*
5. Remove the plate cover and add 150 µl of the freshly prepared Assay Cocktail to each of the wells containing standards and samples using a multichannel pipette. Replace the plate cover. Incubate the plate in the dark on an orbital shaker until measured as outlined below.
6. GSH concentration of the samples can be determined either by the End Point Method or the Kinetic Method. The End Point Method is adequate for most purposes. However, if the levels of cysteine or other thiols in the samples are expected to be significant compared to GSH, the Kinetic Method should be used.

   End Point Method: Read the plate at 405-414 nm after 25 minutes.

   Kinetic Method: Read the plate at 405-414 nm at five minutes intervals for 30 minutes.

ANALYSIS

Calculations

End Point Method

1. Calculate the average absorbance from the 25 minutes measurement for each standard and sample.
2. Subtract the absorbance value of the standard A from itself and all other values (both standards and samples). This is the corrected absorbance.
3. Plot the corrected absorbance values (from step 2 above) of each standard as a function of the concentration of GSSG or Total GSH of Table 1 (see Figure 3).

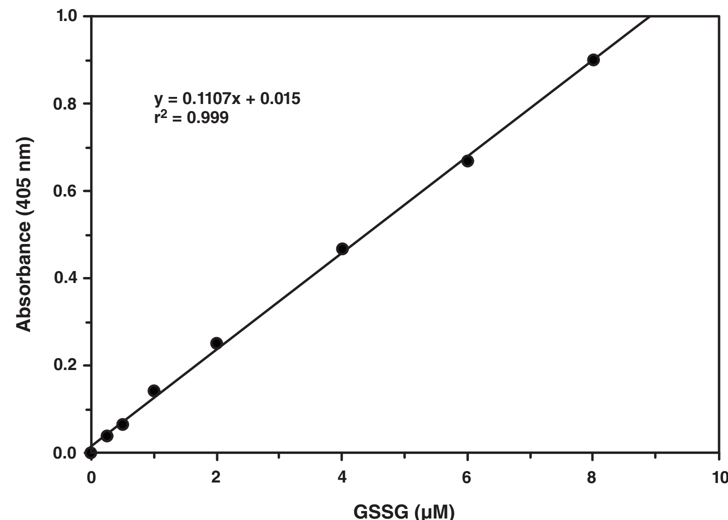

Figure 3. Plot of corrected absorbance at 25 minutes versus GSSG concentration (µM)

4. Calculate the values of GSSG or Total GSH for each sample from the standard curve.

$$[\text{Total GSH}] \text{ or } [\text{GSSG}] = \left[\frac{(\text{Absorbance at 405-414 nm}) - (\text{y-intercept})}{\text{Slope}}\right] \times 2^* \times \text{Sample dilution}$$

**NOTE: If your sample required deproteination, multiply by "2" to account for the addition of MPA Reagent.*

Kinetic Method

1. Plot the average absorbance values of each standard and sample as a function of time and determine the slope for each curve (see Figure 4). This is called i-slope.

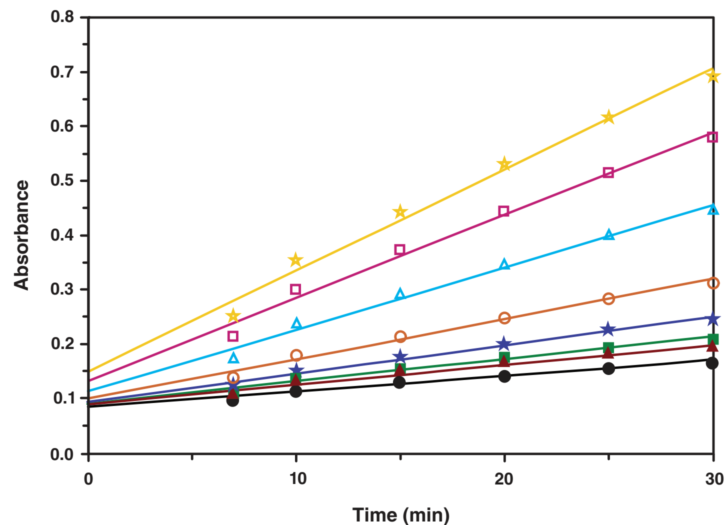

Figure 4. Plot of absorbance versus time for each standard

2. Plot the i-slopes of each standard as a function of the concentration of GSSG or total GSH of Table 1 (see Figure 5, below). The slope of this curve is called f-slope.

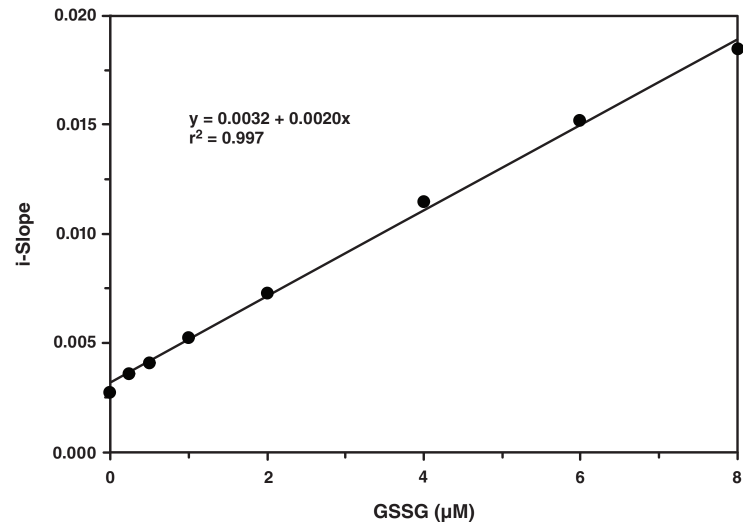

Figure 5. Plot of slope versus GSSG concentration

3. Calculate the values of GSSG or total GSH for each sample from their respective slopes using the slope *versus* GSSG or GSH standard curve.

[Total GSH] or [GSSG] =

$$\left[\frac{\text{(i-Slope for the sample) - (y-intercept)}}{\text{f-Slope}}\right] \times 2^{*} \times \text{Sample dilution}$$

**NOTE: If your sample required deproteination, multiply by "2" to account for the addition of MPA Reagent.*

Performance Characteristics

Precision:

Inter-assay coefficient of variation is 3.6% (n=5). Intra-assay coefficient variation is 1.6% (n=84).

Assay Range:

Under the standardized conditions of the assay described in this booklet, the dynamic range of the kit is 0-16 µM GSH (or 0-8 µM GSSG).

RESOURCES

Interferences

Added thiols such as mercaptoethanol, dithiothreitol, etc., and high levels of cysteine will consume all the DTNB and cause severe interference in the estimation of GSH. Thiol alkylating agents such as N-ethylmaleimide will inhibit glutathione reductase thereby rendering the assay ineffective.

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/ technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
No color development	A. One or more of the constituents of Assay Cocktail was missing B. Standards were not added to the wells	Make sure to add all components to the Assay Cocktail and the wells
Non-linear standard curve	Absorbance values too high (>1.2) at high GSH concentrations	Use the absorbance measured at lower time points; ideally, the highest point on the standard curve should have an absorbance value less than 1.2 AU
No color in the sample above the background	Concentration of GSH in the sample is too low (<0.25 µM)	Concentrate the sample by lyophilization and reconstitute in a smaller volume of MES Buffer than the original volume of the sample; concentration should be performed after deproteination of the sample
Absorbance value of the sample is higher than the highest point of the standard curve	Sample is too concentrated and/or high concentration of other thiols	Dilute the sample and reassay; analyze the data using Kinetic Method; if the absorbance value falls within the range of any of the standards but the i-slope does not, thiols other than GSH are interfering with the assay

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NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.

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