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## Thiol Detection Assay Kit

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Item No. 700340

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## TABLE OF CONTENTS

<b>GENERAL INFORMATION</b>	3	Materials Supplied
	4	Safety Data
	4	Precautions
	4	If You Have Problems
	4	Storage and Stability
	4	Materials Needed but Not Supplied
<b>INTRODUCTION</b>	5	Background
	5	About This Assay
<b>PRE-ASSAY PREPARATION</b>	6	Reagent Preparation
	7	Sample Preparation
<b>ASSAY PROTOCOL</b>	9	Plate Set Up
	11	Standard Preparation
	12	Performing the Assay
<b>ANALYSIS</b>	13	Calculations
	13	Performance Characteristics
	16	Comparison to Ellman's Reagent
<b>RESOURCES</b>	18	Interferences
	20	Troubleshooting
	21	References
	22	Plate Template
	23	Notes
	23	Warranty and Limitation of Remedy

## GENERAL INFORMATION

### Materials Supplied

Item Number	Item	96 wells Quantity/Size	480 wells Quantity/Size
700341	Thiol Assay Buffer (10X)	1 vial/5 ml	1 vial/5 ml
700006	Fluorometric Thiol Detector	1 vial/300 µl	5 vials/300 µl
700343	Thiol GSH Standard	1 vial/10 mg	1 vial/10 mg
700344	Thiol Cysteine Standard	1 vial/10 mg	1 vial/10 mg
400091	Half-Volume 96-Well Plate (black)	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: 734-971-3640  
Email: techserv@caymanchem.com

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 at -20°C and used before the expiration date indicated on the outside of the box.

## Materials Needed But Not Supplied

1. A fluorometer with the capacity to measure fluorescence using an excitation wavelength between 380-390 nm and emission wavelength between 510-520 nm
2. Adjustable pipettes and a repeating pipettor
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable

## INTRODUCTION

### Background

The detection and measurement of free thiols (*i.e.*, free cysteine, glutathione, and cysteine residues on proteins) is one of the essential tasks for investigating biological processes and events in many biological systems. Thiols are extremely efficient antioxidants which are able to protect cellular lipids, proteins, and nucleic acids against peroxidative damage due to their strong reductive capacity and their ability to react with free radicals.<sup>1</sup> Glutathione (GSH) is the most abundant non-protein thiol-containing compound found in the human body. The concentration of GSH ranges from a few micromolar in plasma to several millimolar in tissues such as liver.<sup>2,3</sup> Glutathione is found almost exclusively in its reduced form, since the enzyme that reverts it from its oxidized form, glutathione reductase, is constitutively active and inducible upon oxidative stress.<sup>4</sup> The ratio of reduced glutathione to oxidized glutathione within cells is often used as a measure of cellular toxicity.

Cysteine is a powerful nucleophile and is the most easily oxidized amino acid, forming covalent crosslinks between peptide chains. This unique reactivity of cysteine residues on proteins has provided investigators with sites that can be chemically modified. However, accurately determining the extent of the modification in a complex protein is problematic. The Cayman Thiol Detection Assay has been validated with samples in 4 M guanidine hydrochloride, allowing the thiol content of unfolded proteins to be accurately determined.

### About This Assay

Cayman's Thiol Detection Assay Kit provides a simple, reproducible, and sensitive tool for assaying free thiol content in samples (*i.e.*, plasma, serum, tissue homogenates, cell lysates, and urine). The assay utilizes a proprietary fluorometric detector that reacts with thiol groups to emit a strong fluorescent signal which can be detected using excitation wavelengths between 380-390 nm and emission wavelengths between 510-520 nm.

## Reagent Preparation

### 1. Thiol Assay Buffer (10X) - (Item No. 700341)

The vial contains 5 ml of concentrated Assay Buffer. Dilute 5 ml of Assay Buffer concentrate with 45 ml of HPLC-grade water. This final Buffer (100 mM Potassium Phosphate, pH 7.4, containing 1 mM EDTA) should be used in the assay. This diluted buffer is stable for six months when stored at -20°C.

### 2. Fluorometric Thiol Detector - (Item No. 700006)

Each vial contains 300 µl of detector. Prior to assaying, dilute 50 µl of the supplied Detector with 4.95 ml of diluted Assay Buffer. This is enough solution to assay one plate. This diluted solution is stable for one day at room temperature. Avoid exposure to light when possible. Store undiluted Detector at -20°C.

### 3. Thiol Glutathione Standard - (Item No. 700343)

The vial contains approximately 10 mg of a reduced glutathione standard. Carefully weigh out 1 mg and dissolve it in 3.25 ml of diluted Thiol Assay Buffer. Dilute 10 µl of this solution with 990 µl diluted Thiol Assay Buffer. The concentration of this diluted Standard Solution is 10 µM. The solution is ready to be used to prepare the glutathione standard curve (see page 11). The Glutathione Standard Solution is stable for one day at room temperature.

### 4. Thiol Cysteine Standard - (Item No. 700344)

This vial contains approximately 10 mg of a cysteine standard. Carefully weigh out 1 mg and dissolve it in 3.06 ml of diluted Thiol Assay Buffer. Dilute 5 µl of this solution with 995 µl diluted Thiol Assay Buffer. The concentration of this diluted Standard Solution is 10 µM. The solution is ready to be used to prepare the cysteine standard curve (see page 11). The Cysteine Standard Solution is stable for one day at room temperature.

## Sample Preparation

### Plasma

1. Collect blood using an anticoagulant such as heparin or citrate.
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.
3. Store plasma on ice until assaying or freeze at -80°C. The plasma sample will be stable for one month. Repeated freeze/thaw cycles should be avoided.
4. Plasma should be diluted 1:5 with diluted Assay Buffer before assaying.

### Serum

1. Collect blood without using an anticoagulant such as heparin or citrate.
2. Allow blood to clot for 30 minutes at 25°C.
3. 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.
4. Store serum on ice. If not assaying the same day, freeze at -80°C. The sample will be stable for one month. Repeated freeze/thaw cycles should be avoided.
5. Serum should be diluted 1:5 with diluted Assay Buffer before assaying.

### Tissue Homogenate

1. Prior to dissection, rinse the 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.*, 100 mM Tris-HCl, pH 7.8) per gram of tissue.
3. Centrifuge at 10,000 x g for 15 minutes at 4°C.
4. Remove the supernatant and store on ice. If not assaying on the same day, store the sample at -80°C. The sample will be stable for at least one month.

**Cell Lysate**

1. Collect cells (~5 x 10<sup>6</sup>) by centrifugation (*i.e.*, 1,000-2,000 x g for 10 minutes at 4°C). For adherent cells, do not use proteolytic enzymes; rather use a rubber policeman.
2. Homogenize the cell pellet in 0.5-1.0 ml cold buffer (*i.e.*, 100 mM Tris-HCl, pH 7.5, containing 1mM EDTA).
3. Centrifuge at 10,000 x g for 15 minutes at 4°C.
4. Remove the supernatant and store on ice. If not assaying on the same day, freeze the sample at -80°C. The sample should be stable for at least one month.

**Urine**

1. Collect urine in a clean container.
2. If not assaying on the same day, freeze the sample at -80°C. The sample should be stable for at least one month.
3. Urine should be diluted 1:50 or 1:100 with diluted Assay Buffer or HPLC-grade water before assaying.

**Plate Set Up**

There is no specific pattern for using the wells on the plate. However, either a glutathione or cysteine standard curve in duplicate has to be assayed with the samples. We suggest that each sample be assayed at least in duplicate (triplicate recommended) and to record the contents of each well on the template sheet provided on page 22. A typical layout of standards and samples to be measured in duplicate is shown in Figure 1.

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	1	1	9	9	17	17	25	25	33	33
B	S2	S2	2	2	10	10	18	18	26	26	34	34
C	S3	S3	3	3	11	11	19	19	27	27	35	35
D	S4	S4	4	4	12	12	20	20	28	28	36	36
E	S5	S5	5	5	13	13	21	21	29	29	37	37
F	S6	S6	6	6	14	14	22	22	30	30	38	38
G	S7	S7	7	7	15	15	23	23	31	31	39	39
H	S8	S8	8	8	16	16	24	24	32	32	40	40

S1-S8 - Standards A-H  
 1-40 - Samples

Figure 1. Sample plate format

### Pipetting Hints

- It is recommended that a repeating pipettor be used to deliver reagents to the wells. This saves time and helps maintain more precise incubation times.
- 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

- The final volume of the assay is 100  $\mu\text{l}$  in all the wells.
- All reagents must be equilibrated to room temperature before beginning the assay.
- It is not necessary to use all the wells on the plate at one time.
- We recommend assaying samples in triplicate, but it is the user's discretion to do so.
- Twenty-four samples can be assayed in triplicate or forty in duplicate.
- The assay is performed at room temperature.
- Monitor the fluorescence at an excitation wavelength between 380-390 nm and an emission wavelength between 510-520 nm.

### Standard Preparation

*NOTE: Either a glutathione or cysteine standard curve has to be assayed with the samples. It is the user's discretion as to which one is used in the assay:*

Both the glutathione and cysteine standard curves are prepared following the same procedure. Begin by transferring 200  $\mu\text{l}$  of either standard into 800  $\mu\text{l}$  of diluted Assay Buffer. This produces a 2  $\mu\text{M}$  GSH or Cysteine Stock Solution. This stock solution is used in the preparation of the standard curve.

The standard curve is prepared through a series of 1:2 serial dilutions. Set up eight test tubes and label them A-H. Add 300  $\mu\text{l}$  of diluted Assay Buffer to tubes A-H. To tube A, add 300  $\mu\text{l}$  of either the 2  $\mu\text{M}$  GSH or Cysteine Stock Solution and vortex. Next, transfer 300  $\mu\text{l}$  from Tube A to Tube B and vortex. Continue for tubes C-G. Do not add anything except Assay Buffer to Tube H. Tube H is the blank and has no standard added. Diluted standards are stable for one day at room temperature.

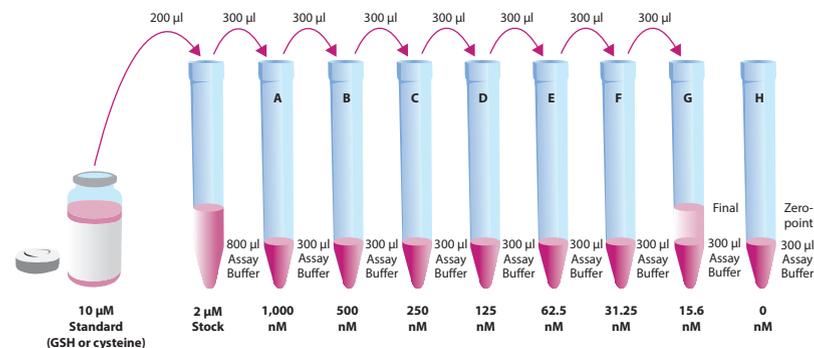


Figure 2. Standard preparation

## Performing the Assay

1. **Standard Wells** - add 50  $\mu$ l of either standard (tubes A-H) per well in the designated wells on the plate (see **Sample plate format**, Figure 1, page 9).
2. **Sample Wells** - add 50  $\mu$ l of sample to at least two wells. To obtain reproducible results, thiol levels should fall within the standard curve. When necessary, samples can be diluted with Assay Buffer or HPLC-grade water to bring the thiol concentration to this level.
3. Add 50  $\mu$ l of diluted Fluorometric Thiol Detector to each well being used.
4. Incubate the plate at room temperature for five minutes.
5. Remove the plate cover and read the plate using an excitation wavelength between 380-390 nm and an emission wavelength between 510-520 nm.

## ANALYSIS

### Calculations

1. Calculate the average fluorescence of each standard and sample.
2. Subtract the average fluorescence of standard H from itself and all other standards and samples. This is the adjusted fluorescence.
3. Plot the adjusted fluorescence of the standards (from step 2 above) as a function of the final concentration of glutathione or cysteine from Figure 2. See Figure 3 and 4 for typical standard curves (pages 14 and 15).
4. Calculate the thiol concentration of the samples using the equation obtained from the linear regression of the standard curve, substituting adjusted fluorescence values for each sample.

Thiol concentration (nM) =

$$\left[ \frac{\text{Adjusted sample fluorescence} - (\text{y-intercept})}{\text{Slope}} \right] \times \text{Sample dilution}$$

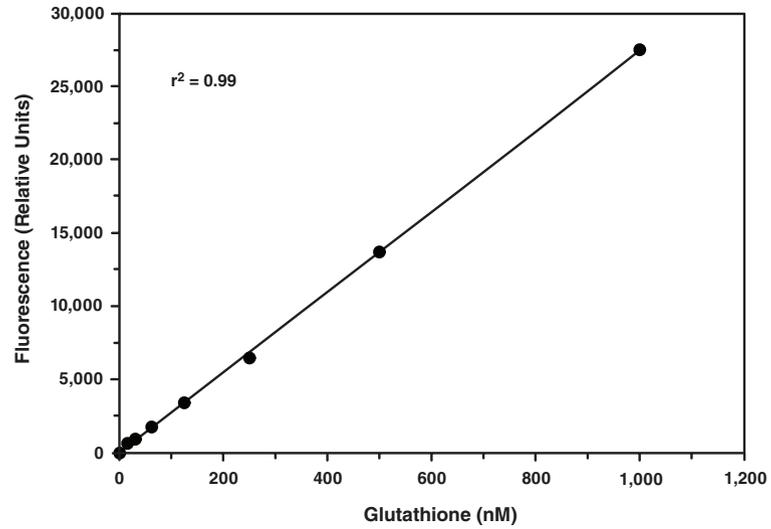
### Performance Characteristics

#### Sensitivity:

The limit of detection for this assay is approximately 15 nM.

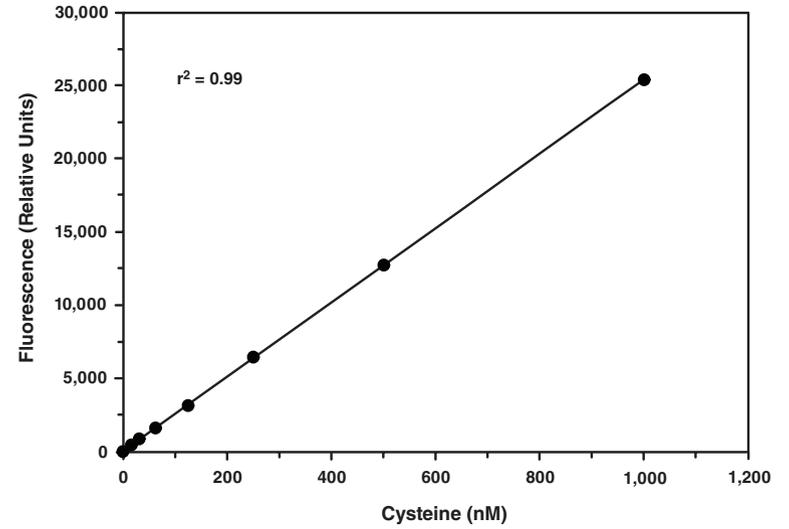
#### Precision:

When a series of 80 human urine measurements were performed on the same day under the same experimental conditions, the intra-assay coefficient of variation was 4%. When a series of 10 human urine samples were performed on five different days under the same experimental conditions, the inter-assay coefficient of variation was 5.6%.



**Figure 3. Glutathione standard curve**

*NOTE: The actual slope will vary depending on gain settings and equipment variances.*



**Figure 4. Cysteine standard curve**

*NOTE: The actual slope will vary depending on gain settings and equipment variances.*

## Comparison to Ellman's Reagent

The following graphs show the response of Ellman's Reagent and the Thiol Fluorometric Detector at various glutathione concentrations. As shown in Figures 5 and 6, the Thiol Fluorometric Detector is 400-fold more sensitive than the colorimetric detector.

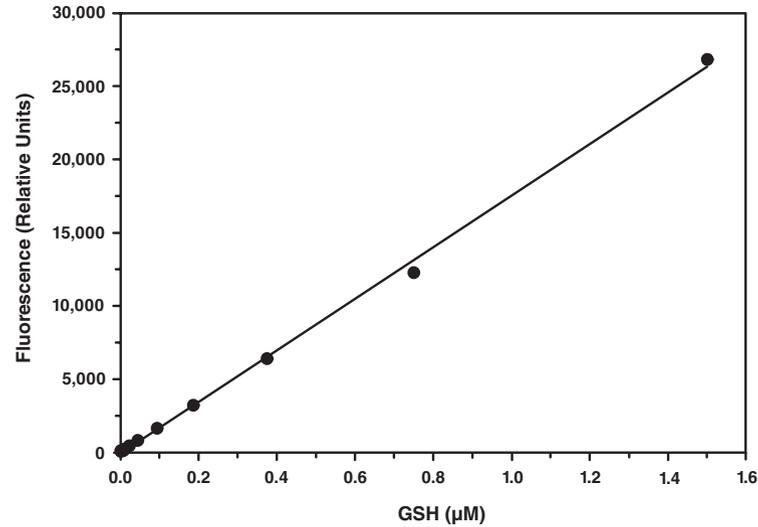


Figure 5. Glutathione detected by Thiol Fluorometric Detector

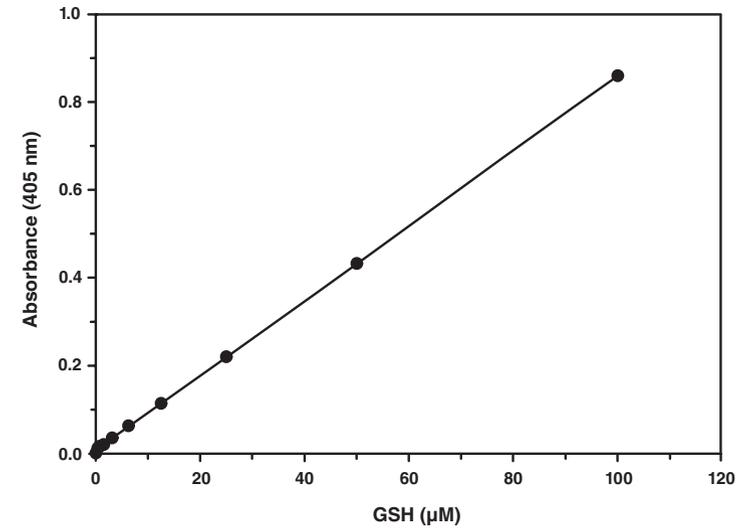


Figure 6. Glutathione detected by Ellman's Reagent

## RESOURCES

### Interferences

The following reagents were tested in the assay for interference in the assay:

	Reagent	Will Interfere (Yes or No)
Buffers	Tris	No
	HEPES	No
	MES	No
	Phosphate	No
Detergents	Polysorbate 20 (0.1%)	No
	Polysorbate 20 (1%)	No
	Triton X-100 (0.1%)	No
	Triton X-100 (1%)	No
Protease Inhibitors/ Chelators/ Enzymes	EDTA (1 mM)	No
	EGTA (1 mM)	No
	Trypsin (10 µg/ml)	No
	PMSF (200 µM)	No
	Leupeptin (10 µg/ml)	No
	Antipain (10 µg/ml)	No
	Chymostatin (10 µg/ml)	No
Solvents	Ethanol (5%)	No
	Methanol (5%)	No
	Dimethylsulfoxide (5%)	No

### Interferences cont.

	Reagent	Will Interfere (Yes or No)
Others	BSA (0.1%)	Yes
	Glycerol (10%)	No
	Guanidine HCl (4 M)	No
	β-Mercaptoethanol	Yes
	TCEP	Yes
	DTT	Yes
	Sodium Azide	Yes

## 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 fluorescence was detected in sample wells	Sample was too dilute	Re-assay the sample using a lower dilution
Fluorometer exhibited 'MAX' values for the wells	The GAIN setting is too high	Reduce the GAIN and re-read; set GAIN to optimal if possible
Fluorescence in the sample wells are above the last standard	Sample is too concentrated	Dilute the sample with Assay Buffer or HPLC-grade water and re-assay; assaying a few dilutions of each sample will help avoid this problem

## References

1. Wlodek, L. Beneficial and harmful effects of thiols. *Polish Journal of Pharmacology* **54**, 215-223 (2002).
2. Lash, L.H. and Jones, D.P. Distribution of oxidized and reduced forms of glutathione and cysteine in rat plasma. *Arch. Biochem. Biophys.* **240**, 583-592 (1985).
3. Wendel, A. and Cikryt, P. The level and half-life of glutathione in human plasma. *FEBS Lett.* **120**, 209-211 (1980).
4. Dickenson, D., Forman, H. Cellular glutathione and thiols metabolism. *Biochemical Pharmacology* **64**, 1019-1026 (2002).

## NOTES

12								
11								
10								
9								
8								
7								
6								
5								
4								
3								
2								
1								
	A	B	C	D	E	F	G	H

### 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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