LUMINOS®
INTERACTIVE ASSAY SOLUTIONS

DetectX[a]

Glutathione
Fluorescent Detection Kit
Catalog Number K006-F1

Sample Types Validated:
Whole Blood, Serum, Plasma, Erythrocytes, Urine and Cell Lysates

Developed under an exclusive agreement with Berry and Associates.
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Patent Pending

Please read this insert completely prior to using the product.

FOR RESEARCH USE ONLY
NOT FOR USE IN DIAGNOSTIC PROTOCOLS

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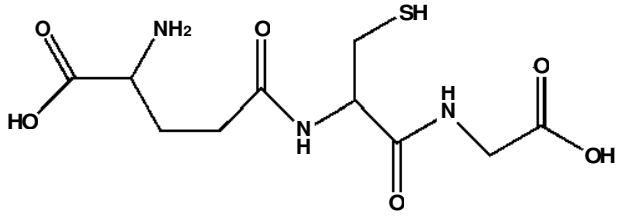
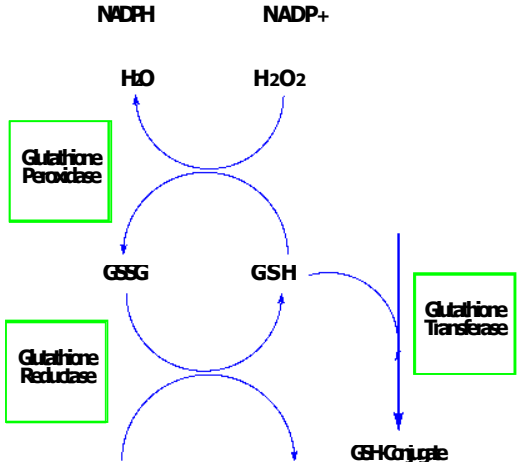
INTRODUCTION

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[1]. 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[2].

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[3].



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 A to $_4$ (LTA $_4$). LTA $_4$ is either hydrated to form LTB $_4$ or it is conjugated GSH by a GST, leukotriene C $_4$ synthase, to form leukotriene C $_4$. LTC $_4$ and its derivative LTD $_4$ are important molecules in bronchial asthma. Leukotriene C$_4$ synthase is therefore an important therapeutic target. It has also been shown that increased expression of

GSTs can lead to drug resistance. Drug resistant MCF-7 breast cancer cells have been shown to have 45 fold more GST activity than normal cells. Expressed GST 3-3 in mammalian cells in vitro increases cisplatin resistance and correlates with clinical findings. Three glutathione adducts of the drug melphalan, used to treat ovarian cancer and multiple myeloma, have been isolated from reactions involving human microsomal GSTs.

1. Meister, A. "On the Discovery of Glutathione." Trends Biochem. Sci. 1988 13(5):185-188.
2. Meister, A. "The Glutathione-Ascorbic Acid Antioxidant Systems in Animals" J. Biol. Chem. 1994 269:9397-9400.
3. Dröge W, et al., "Functions of Glutathione and Glutathione Disulfide in Immunology and Immunopathology" FASEB J., 1994 8:1131 -1138.

THE LUMINOS DetectXTM GLUTATHIONE FLUORESCENT DETECTION KIT

Assay Principle

The DetectXª Glutathione kit is designed to quantitatively measure glutathione (GSH), and oxidized glutathione (GSSG) present in a variety of samples. The kit is unique in that both free and oxidized glutathione are detected in the same well in the microtiter plate. No separation or washing is required. Total glutathione is the sum of GSSG plus GSH. Please read the complete kit insert before performing this assay. A GSH standard is provided to generate a standard curve for the assay and all samples should be read off the standard curve. The kit utilizes a proprietary non-fluorescent molecule, ThioStarª, that will covalently bind to the free thiol group on GSH to yield a highly fluorescent product. After mixing the sample or standard with ThioStar and incubating at room temperature for 15 minutes, the fluorescent product is read at 510 nm in a fluorescent plate reader with excitation at 390 nm. The concentration of the GSH in the sample is calculated, after making a suitable correction for any dilution of the sample, using software available with most fluorescence plate readers.

Free glutathione, GSH, is read first after 15 minutes, followed by addition of a reaction mixture that converts all the oxidized glutathione, GSSG, into free GSH, which then reacts with the excess ThioStarª to yield the signal related to Total GSH content. The total concentration of GSH generated in the sample is calculated from the generated signal. We have provided a 96 well plate for measurement but thi s assay is adaptable for higher density plate formats. The end user should ensure that their HTS black plate is suitable for use with these reagents prior to running samples.

REACTION OVERVIEW

1. **Sample or standard added to well.**
2. **ThioStarTM Reagent added and fluorescent signal read after 15 minutes. Calculate Free GSH concentration.**
3. **Add Reaction Mixture to same wells.**
4. **Incubate for 15 minutes and read fluorescent signal. Calculate Total GSH concentration.**

Measure GSH and Total GSH in 30 minutes in the same sample in one well.

Supplied Components

Black Half Area Microtiter Plate 96 well Catalog Number X023-1EA

Glutathione Standard 100 µL Catalog Number C018-100UL
Glutathione at 250 µM in a special stabilizing solution.

ThioStar [a] Detection Reagent 2 vials Catalog Number C021-1EA
ThioStar thiol detection substrate stored in a desiccator. Reconstitute with dry DMSO.

Dry DMSO 4 mL Catalog Number X022-4ML
Dry Dimethyl sulfoxide solvent over molecular sieves. **May be stored at room temperature.**

Assay Buffer Concentrate 50 mL Catalog Number X029-50ML
A 2x concentrated phosphate buffer containing detergents and stabilizers.

NADPH 1 vial Catalog Number X031-1EA
Reduced ß -nicotinamide adenine dinucleotide 2'-phosphate freeze dried with stabilizers.

Glutathione Reductase 40 µL Catalog Number C019-40UL
Glutathione Reductase as a stable solution.

Oxidized Glutathione Control 10 µL Catalog Number C020-10UL
Oxidized Glutathione (GSSG) in a special stabilizing solution.

Storage Instructions

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

Other Materials Required

Aqueous sulfosalicylic acid (SSA) solution at 5% for treating samples to remove protein. We recommend Sigma-Aldrich Catalog Number S2130.

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

Software for converting raw relative fluorescent unit (FLU) 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.

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 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.

Reconstituted ThioStarTM Detection Reagent should be stored at 4°C in the desiccator. Allow to warm to room temperature prior to opening.

ThioStar will react with strong nucleophiles. Buffers containing the preservatives sodium azide, Proclin and Kathon will react with the substrate.

Sample Types

This assay has been validated for human whole blood, serum, EDTA and heparin plasma, urine, and isolated erythrocytes. Most cell lysates should also be compatible. Samples containing visible particulate should be centrifuged prior to using.

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.

Sample Preparation

General Information

Only fresh samples should be used and all processed samples should be kept on ice. All samples need to be deproteinized using 5% SSA. The supernatants are then further diluted with Assay Buffer to 1% SSA for measurement in the assay. Any samples requiring larger dilutions or with GSH concentrations outside the stan dard curve range should be diluted further with 1% SSA Standard Diluent to obtain readings within the standard curve.

Use all samples within 2 hours of dilution.

Whole Blood

Fresh EDTA or heparin blood is deproteinized by mixing equal volumes of sample and cold 5% SSA. After incubation for 10 minutes at 4°C samples are centrifuged at 14,000 rpm for 10 minutes at 4°C and the supernatants collected. Samples can be stored in aliquots at -80°C or analyzed immediately. For most whole blood samples, a final dilution of ≥ 1:100 is recommended.

Sample Preparation Continued

Serum & Plasma

Serum from whole blood or plasma from fresh EDTA or heparin whole blood is separated by centrifugation at 600 x g for 10 minutes and transferred to fresh tubes. The fresh serum or plasma is deproteinized like whole blood then measured at a recommended final dilution of ≥ 1:5.

Erythrocytes (RBCs)

Plasma or serum are separated from whole blood and the white cell layer is discarded. The remaining RBCs are suspended and gently washed twice with three volumes of PBS, separating the cells by centrifugation at 600 x g for 10 minutes. To lyse and deproteinize the RBCs, 1mL cold 5% SSA is added to 250µL RBCs, vortexed and incubated for 10 minutes at 4°C. Samples ar e spun and treated like whole blood. For most erythrocyte lysates, a final dilution of ≥ 1:40 is recommended.

Urine

Fresh urine is deproteinized like whole blood then measured at a recommended final dilution of ≥ 1:5.

Cell lysates

Washed cell pellets are resuspended at 1 -10x10^6cells/mL in cold 5% SSA (we used Jurkats at 5x10^6 cells/mL) and are lysed by vigorous vortexing, freeze/thaw cycling or other suitable disruption method. Resulting lysates are deproteinized like whole blood then measured at a recommended final dilution of ≥ 1:20. The protocol might require adjustment for other cell types.

Use all samples within 2 hours of dilution.

Assay Buffer Preparation

Prepare the Assay Buffer by diluting the 2x Assay Buffer Concentrate 1:2 with deionized water. It is stable for up to 3 months when stored at 4°C.

Standard Diluent

Prepare the Standard Diluent by diluting your 5% SSA 1:5 with the prepared Assay Buffer. This should be prepared fresh, prior to use.

Standard Preparation

GSH Standards are prepared by labeling eight test tubes as #1 through #8. Briefly spin vial of standard in a microcentrifuge to ensure contents are at bottom of vial. Pipet 450 µL of Standard Diluent into tube #1 and 250 µL into tubes #2 to #8. Carefully add 50 µL of the Glutathione Standard to tube #1 and vortex completely. Take 250 µL of the GSH solution in tube #1 and add it to tube #2 and vortex completely. Repeat this for tubes #3 through #7. The concentration of GSH in tubes 1 through 8 will be 25, 12.5, 6.25, 3.125, 1.56, 0.781, 0.391 and 0.195 µM.

Use all Standards within 1 hour of preparation.

Control Preparation

Prepare the Oxidized Glutathione Control by adding 5 µL of control to 245 µL of Standard Diluent. **Use within 2 hours.**

ThioStar[a] Detection Reagent

Remove the vial of ThioStar Reagent from the desiccator and add 1.5 mL of the provided DMSO to the vial. Vortex thoroughly. Store any unused reconstituted Detection Reagent at 4°C in the desiccator and use within 2 weeks.

Reaction Mixture

Prepare the Reaction Mixture by adding 1 mL of the Assay Buffer to the NADPH vial and vortex. Transfer the contents of the vial to a tube. Add 1 mL of Assay Buffer to the vial and transfer to the NADPH tube. Repeat this to transfer all of the NADPH to the tube for a total volume of 3 mL. Add 40 µL of the Glutathione Reductase to the tube and vortex. Store any unused Reaction Mixture at 4°C for no more than 7 days.

Assay Protocol

Allow the kit reagents to come to room temperature for 30 minutes. The recommended format is 15 minutes for Free or Total GSH at room temperature. We recommend that all standards 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.

Assay Protocol - Free and Total GSH

1. Use the plate layout sheet on page 20 to aid in proper sample and standard identification.

2. Pipet 50 µL of treated samples, standards or control into wells in the plate.

3. Add 25 µL of the ThioStar[a] Reagent to each well using a repeater or multichannel pipet.

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 in a plate reader capable of reading the fluorescent emission at 510 nm with excitation at 370-410 nm. Please contact your plate reader manufacturer for suitable filter sets. This data will be used to determine Free GSH concentration.

7. Add 25 µL of the Reaction Mixture to each of the wells using a repeater or multichannel 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. This data will be used to determine Total GSH concentration.

Total GSH Content Only

Total GSH content can be determined directly by leaving out steps 4, 5 and 6.

Calculation of Results

Average the duplicate FLU readings for each standard and sample. Create a standard curve by reducing the data using the 4PLC fitting routine on the plate reader, after subtracting the mean FLUs for the zero standard. 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 step 6 on page 11 utilizing the curve fitting routine supplied with the plate reader.

Total glutathione concentrations of the samples are calculated from the data obtained using from step 10 on page 11 utilizing the curve fitting routine supplied with the plate reader. Ensure that the Reaction Mixture is added to all the wells used, including the standard and control wells . This ensures that the same volume is in both the standard, control and samples wells.

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

$$\underline{\text{GSSG} = (\text{Total GSH} - \text{Free GSH})}$$
$$2$$

Typical Data - Free GSH

Sample	Mean FLU	Net FLU	GSH Concentration µM
Zero	469	0	0
Std 1	45,833	45,364	25
Std 2	22,968	22,499	12.5
Std 3	11,963	11,494	6.25
Std 4	6,233	5,764	3.125
Std 5	3,465	2,996	1.56
Std 6	1,993	1,524	0.781
Std 7	1,241	772	0.391
Std 8	801	332	0.195
Sample 1	2,870	2,401	1.29
Sample 2	18,225	17,756	9.78

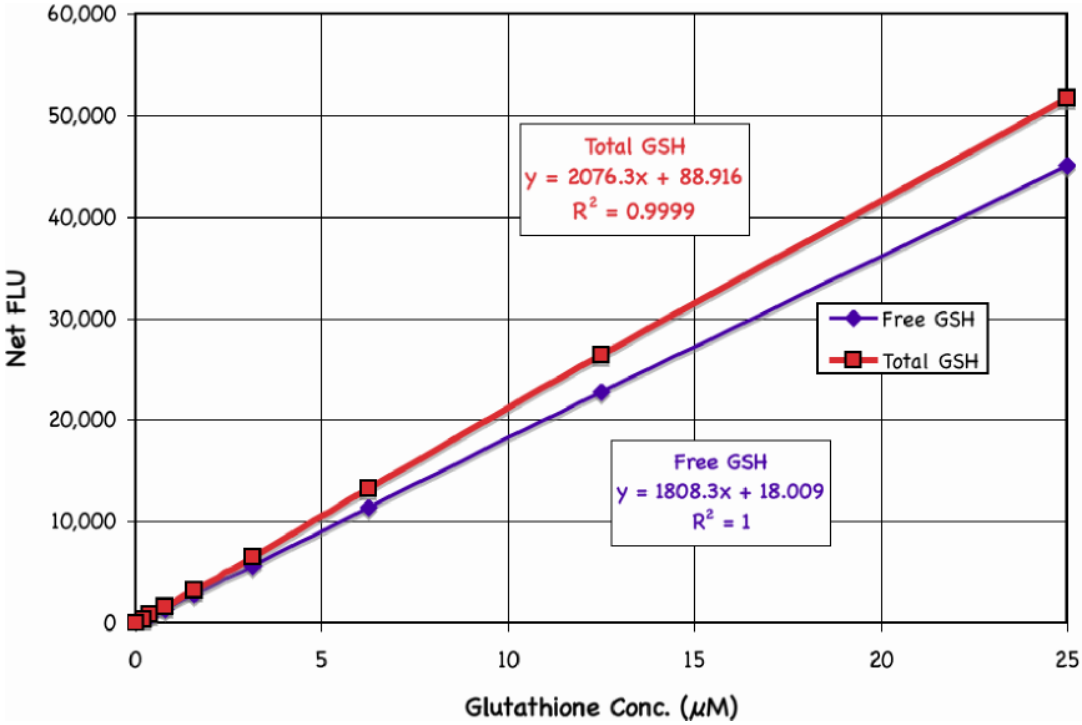
Always run your own standard curve for calculation of results.
Do not use this data.

Typical Data - Total GSH

Sample	Mean FLU	Mean Net FLU	GSH Concentration µM
Zero	1,086	0	0
Std 1	54,339	53,253	25
Std 2	27,953	26,867	12.5
Std 3	14,583	13,497	6.25
Std 4	8,028	6,942	3.125
Std 5	4,775	3,689	1.56
Std 6	2,942	1,856	0.781
Std 7	2,047	961	0.391
Std 8	1,600	514	0.195
Sample 1	6,238	5,152	2.31
Sample 2	27,147	26,061	12.12

Always run your own standard curve for calculation of results.
Do not use this data.

Typical Standard Curves



Always run your own standard curves for calculation of results. Do not use these data.

Sensitivity and Limit of Detection

Sensitivity was calculated by comparing the FLUs for twenty wells run for each of the zero and standard #8. The detection limit was determined at two (2) standard deviations from the zero along the standard curve.

Sensitivity was determined as 45 nM in the Free GSH and 48 nM in the Total GSH assays.

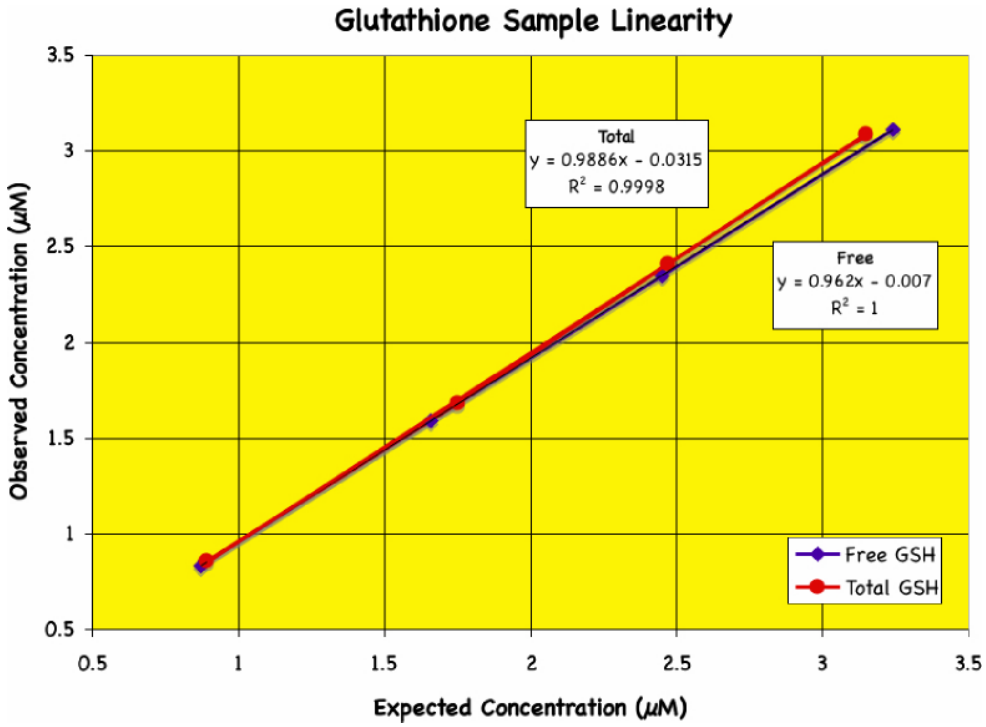
The Limit of Detection was determined in a similar manner by comparing the FLUs for twenty wells run for each of the zero and a low concentration human serum sample.

The Limit of Detection was determined as 38 nM in the Free GSH and 42 nM in the Total GSH assays.

Linearity

Linearity was determined by taking Jukat cell lysates at 25 x 10^6 cells/mL and one at 1.28 x 10^6 cells/mL, diluted 1:20, and mixed in the ratios given below. The measured concentrations were compared to the expected values based on the ratios used.

High Cell Count	Low Cell Count	Expected Conc. (µM)		Observed Conc. (µM)		% Recovery	
		Free	Total	Free	Total	Free	Total
100%	0%	4.02	3.87	---	---	---	---
80%	20%	3.24	3.11	3.15	3.09	97.3	99.5
60%	40%	2.45	2.35	2.47	2.41	100.8	102.5
40%	60%	1.66	1.59	1.75	1.68	105.6	105.7
20%	80%	0.87	0.83	0.89	0.86	102.4	104.1
0%	100%	0.08	0.07	---	---	- - -	---
				Mean Recovery		101.5 %	102.9 %



Intra Assay Precision

Two each of SSA treated human urine and whole blood samples were diluted in 1% SSA Standard Diluent and run in replicates of n=20 in an assay. The mean and standard deviation of the calculated GSH concentrations were:

Sample	GSH Conc. (µM)		Standard Deviation		%CV	
	Free	Total	Free	Total	Free	Total
1	1.27	2.30	0.051	0.107	4.0	4.7
2	2.00	3.80	0.063	0.178	3.1	4.7
3	8.33	9.77	0.381	0.261	4.6	2.7
4	3.89	4.45	0.115	0.101	3.0	2.3

Inter Assay Precision

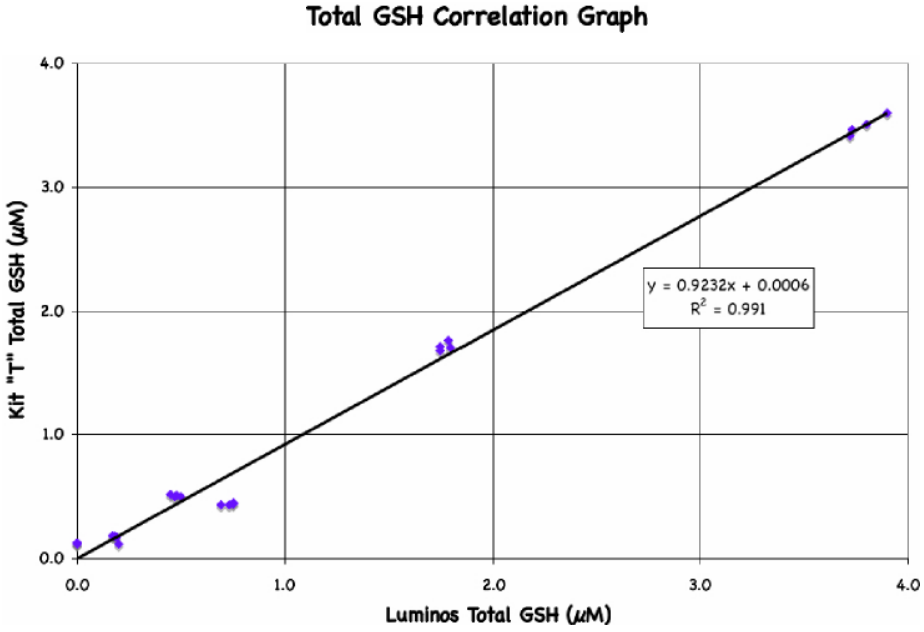
Two each of SSA treated human urine and blood samples were diluted in 1% SSA Standard Diluent and run in duplicates in 20 assays run over multiple days by two operators. The mean and standard deviation of the calculated GSH concentrations were:

Sample	GSH Conc. (µM)		Standard Deviation		%CV	
	Free	Total	Free	Total	Free	Total
1	1.30	2.40	0.112	0.198	8.6	8.3
2	1.83	3.57	0.269	0.356	14.7	10.0
3	9.38	11.67	0.564	0.699	6.0	6.0
4	4.89	5.89	0.354	0.474	7.2	8.0

Kit Correlation Data

We purchased and compared a popular colorimetric total glutathione assay kit (kit "T") that uses Ellman's reagent to detect free glutathione in the sample. Initial experiments used random human urine samples that were processed as described in each kit insert. With kit "T", the values obtained for urine after the recommended treatment with 4 volumes of 5% metaphosphoric acid and subsequent 10 fold dilution with assay buffer put all the values well below the lowest standard. However, the urine samples run in the Luminos kit gave Total GSH values between 0.63 and 4.04 µM.

We also took a Jurkat cell pellet and processed the cells either through the 5% metaphosphoric acid treatment for the kit "T" Ellman's based test or as described on page 9 for the Luminos kit. Cell samples ranged from 25 to 0.78 x 10^6 cell/mL. Twenty-four samples were run according to manufacturers directions for both kits and the correlation of these samples is shown below.



Many of the cell lysate values for the Ellman's based kit, kit "T", read either below the lowest standard (0.25 µM) or above the highest one (2 µM). This data was calculated via extrapolation from the kinetic method required by kit "T". The lysate values for the Luminos kit were calculated directly from the endpoint standard curve.

LIMITED WARRANTY

Luminos LLC warrants that at the time of shipment this product is free from defects in materials and workmanship. This warranty is in lieu of any other warranty expressed or implied, including but not limited to, any implied warranty of merchantability or fitness for a particular purpose.

We must be notified of any breach of this warranty within 48 hours of receipt of the product. No claim shall be honored if we are not notified within this time period, or if the product has been stored in any way other than outlined in this publication. The sole and exclusive remedy of the customer for any liability based upon this warranty is limited to the replacement of the product, or refund of the invoice price of the goods.

PLATE LAYOUT

H	G	F	E	D	C	B	A	
								1
								2
								3
								4
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