*Oxis*Research™

A Division of AOXRE LLC

BIOXYTECH® GSH-420™

Quantitative, Colorimetric Determination of Total Glutathione
For Research Use Only. Not For Use In Diagnostic Procedures.
Catalog Number 21023

INTRODUCTION

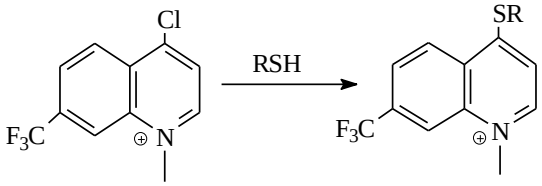
The Analyte

Reduced glutathione (GSH) is an ubiquitous tripeptide (γ-glutamylcysteinylglycine) which functions as a coenzyme, in amino acid transport, in detoxification of xenobiotics and carcinogens, in synthesis of DNA precursors and as an antioxidant.[1] Most GSH in whole blood is found in the erythrocyte.[2]

Principles of the Procedure

The method is based on the formation of a chromophoric thione[3]. The absorbance measured at 420 nm is directly proportional to the GSH concentration. There are three steps to the reaction. First, the sample is buffered and the reducing agent, tris(2-carboxyethyl)phosphine (TCEP)[4], is added to reduce any oxidized glutathione (GSSG) to the reduced state (GSH), equation (1). The chromogen, 4-chloro-1-methyl-7-trifluoromethylquinolinium methylsulfate, is added forming thioethers with all thiols present in the sample, equation (2). Upon addition of base to raise the pH greater than 13, an β-elimination specific to the GSH-thioether results in the chromophoric thione, equation (3).

$$GSSG + TCEP + H_2O \rightarrow 2GSH + TCEP{=}O \quad (1)$$



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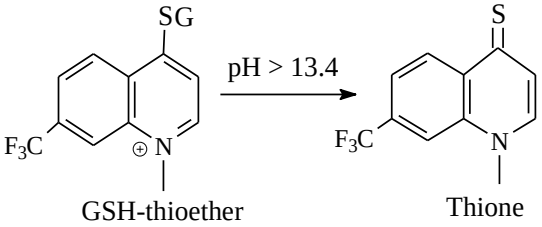

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REAGENTS

Materials Provided (for 100 tests)

- Chromogen — 1-Methyl-4-chloro-7-trifluoromethylquinolinium methylsulfate in HCl, 20 mL.
- Color Developer — NaOH in water, 20 mL.
- Reducing Agent — Tris(2-carboxyethyl)phosphine (TCEP) in HCl, 20 mL.
- Precipitation Reagent — Trichloroacetic acid (TCA) in water, 2 x 25 mL.
- Buffer — Potassium phosphate, Diethylenetriaminepentaacetic acid (DTPA), Lubrol, pH 7.8, 20 mL.

- Calibrators
  Low: 75 μM Glutathione disulfide (150 μM GSH equivalents) in 125 mM potassium phosphate, pH 7.5, 3 mL.
  High: 175 μM Glutathione disulfide (350 μM GSH equivalents) in 125 mM potassium phosphate, pH 7.5, 3 mL.

Materials Required But Not Provided

- Any spectrophotometer set to perform absorbance measurements at 420 nm.
- Test tubes.
- Disposable spectrophotometric cuvettes.
- Microcentrifuge tubes.
- Centrifuge.
- Pipettors with disposable tips.
- Vortex mixer.
- Timer or clock.
- 0.9% NaCl (not required for whole blood samples).

Warnings and Precautions

- For research use only. Not for use in diagnostic procedures. For *in vitro* use only.
- The Color Developer and Precipitation Reagent are corrosive and may cause burns. In case of contact with skin or eyes, rinse immediately with plenty of water. Seek medical advice.
- The Color Developer contains sodium hydroxide, which is harmful by inhalation, contact and if swallowed.
- The Precipitation Reagent contains trichloroacetic acid, which is harmful by inhalation, contact, if absorbed through the skin and if swallowed. Toxic. May cause cancer. Vesicant. Target organs/tissue - central nervous system.

Reagent Storage and Handling

Store the reagents at 2-8°C in the dark. Unopened reagents are stable until expiration date printed on the label. The Precipitation Reagent may degrade with time after being opened. A chloroform odor is a sign of degradation. All other opened reagents will perform until the expiration date if they are properly stored and not contaminated during use. Use a new pipet tip for each sample.

PROCEDURE

Sample Collection Guidelines

Erythrocyte Lysates:

1. Draw blood into an anticoagulant tube and mix by inversion.
2. Centrifuge at least 500 μL whole blood at 2500 *g* for 5 minutes at 4ºC.
3. Discard plasma supernatant, wash cells 3 times in ice cold 0.9% NaCl.
4. Resuspend the packed cells in 4 volumes of ice cold deionized water, vortex well.
5. Add 100 μL of the lysate to a microcentrifuge tube.
6. Add 300 μL Precipitation Reagent to the tube.
7. Vortex for at least 15 seconds.
8. Centrifuge at 10,000 *g* for 5 minutes at room temperature.
9. Collect the supernatant for assay. If not assayed immediately following precipitation, store on ice for up to 24 hours. For longer storage, supernatant should be frozen at -70°C.

   Note: If not lysed immediately, packed cell pellets may be frozen at -70°C.

Whole Blood:

1. Omit steps 2-4 of the above procedure.
2. At step 5, add 100 μL of whole blood to a microcentrifuge tube.
3. Continue with steps 6-9.

Note: If not assayed immediately, store samples on ice for up to 24 hours. For longer storage, sample should be frozen at -70°C.

Tissue Homogenates:

1. Wash tissue in 0.9% NaCl solution, blot dry.
2. Homogenize tissue in Precipitation Reagent at a ratio of 1 to 20 (w/v).
3. Centrifuge homogenate at 3000 *g* for 10 minutes at 4ºC.
4. Collect upper aqueous layer for assay.

   Note: If not homogenized immediately, wrap tissue in aluminum foil and flash-freeze. Store at -70ºC.

POTENTIALLY INTERFERING FACTORS

Additives or Preservatives to Use

Table 1 shows the anticoagulants that were tested for interference in the GSH-420 Assay. Interference is calculated as the percent change in absorbance measurement. Potassium oxalate is not recommended for use as an anticoagulant.

Table 1: Anticoagulants

Test Compound	Tested Concentration	Percent Interference
Heparin, sodium	40 U/mL	1.2
Potassium Oxalate	15 mg/mL	14.0
Potassium EDTA	8 mg/mL	4.6
Sodium Citrate	10 mg/mL	0.9
Sodium Fluoride	10 mg/mL	-0.6

Sample Substances

Table 2 shows potential interfering whole blood constituents were tested for interference in the assay.

Table 2: Whole blood samples.

Test Compound	Tested Concentration	Percent Interference
Bilirubin	0.2 mg/mL	2.0
Cholesterol	4 mg/mL	4.2
Albumin	60 mg/mL	3.6
Hemoglobin	5 mg/mL	0.6
Triglycerides	10 mg/mL	1.8

Extrinsic Interfering Factors

Table 3 shows selected over-the-counter analgesics were tested for absorbance interference in the assay.

Table 3: Extrinsic Interference Factors

Test Compound	Tested Concentration	Percent Interference
Acetaminophen	200 µg/mL	4.2
Acetylsalicylic Acid	500 µg/mL	0.8
Ibuprofen	400 µg/mL	0.1

Stability of Final Reaction Material

The final reaction mixture is stable at room temperature, sealed, and **in the dark** for at least 6 days. It is preferable to measure absorbance immediately following 30 minutes of incubation time.

Assay

1. Add 200 µL of sample supernatant (or calibrator) to a test tube or spectrophotometric cuvette.
2. Add 200 µL Buffer to the reaction mixture.
3. Add 200 µL Reducing Agent to the reaction mixture, mix well.
4. Add 200 µL Chromogen to the reaction mixture, mix well.
5. Add 200 µL Color Developer to the reaction mixture, mix well.
6. Incubate at room temperature **in the dark** for 30 minutes.
7. Measure the absorbance at 420 nm.

Details of Calibration

A three-point calibration curve, as shown in figure 1, is prepared with each batch of sample assays using water for the zero point calibrator. A high and a low Calibrator is supplied and then added in Step 1 of the Assay Method. The calibrators are comprised of glutathione disulfide, which is oxidized glutathione (GSSG), and during the assay it is reduced to GSH. Two moles of GSH result from each mole of GSSG.

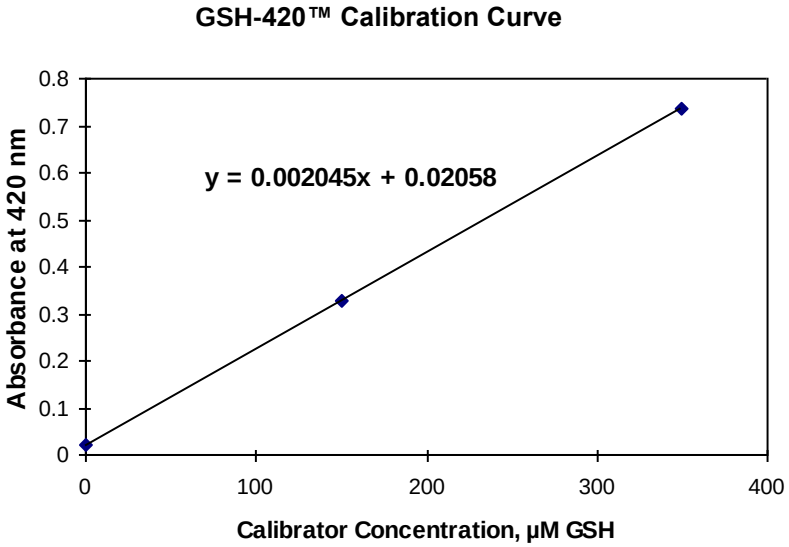

Figure 1: Calibration Curve

Quality Control/Standardization Procedures and Materials

- Frozen (-70°C) pools of erythrocyte lysate or similar preparation may be monitored with appropriate statistical methods to establish control values and acceptable ranges.[5] Control samples are included with calibration and sample assays. Results outside the established acceptable ranges require remediation and/or repetition.[6]
- Use normalization based on the hemoglobin content, if desired and strongly recommended for erythrocyte lysates.

Calculations

The absorbance at 420 nm is linearly proportional to the concentration of GSH. A calibration curve is established by linear regression of the absorbance *versus* concentration for the three Calibrators including zero. The equation of the line, $A_{420} = m[GSH] + b$, can be rearranged to solve for concentration of samples in the assay:

$$[GSH] = \rho(A_{420} - b)/m$$

Where: [GSH] is the GSH concentration in the sample.
ρ is the dilution factor of the original sample prior to its addition to the assay.
A_{420} is the sample absorbance a 420 nm.
b is the intercept from the linear regression.
m is the slope from the linear regression.

Sample Calculation

The following average absorbances were measured for a calibration curve (shown above) and a whole blood sample:

Sample:	0 μM Calibrator	150 μM Calibrator	350 μM Calibrator	Patient Sample
Absorbance:	0.0208	0.3269	0.7365	0.3324

The intercept of the calibration curve is 0.02058, and the slope is 0.002045. Using 0.3324 for absorbance and a dilution factor of 4 (100 μL blood + 300 μL Precipitation Reagent) and solving for the concentration of the sample gives:

$$[GSH] = \rho (A_{420} - b)/m$$
$$[GSH] = 4(0.3324 - 0.02056)/0.002045$$
$$[GSH] = 610\ \mu M \text{ (original sample)}$$

PERFORMANCE CHARACTERISTICS

Specificity

Potentially interfering glutathione analogues were measured in the assay, and the results are listed in table 4 below. To correct for concentration, interference is calculated from the apparent molar extinction coefficient (A_{420}/M) for each analogue as a percentage relative to that of glutathione. Percent Interference = (absorbance per mole test substance / absorbance per mole glutathione) x 100.

Table 4: GSH Analogue Interference

Test Analogue	Concentration, μM	% Interference
Cysteine	82	23.8
N-Acetylcysteine	80	2.0
Captopril	80	-0.6
Cysteinylglycine	80	24.2
Dithiotreitol	80	4.1
L-Ergothioneine	80	10.3
Homocysteine	80	2.5
Mercaptosuccinic Acid	80	-0.5
Mercaptopropionlglycine	80	-0.9
Penicillamine	80	5.4
Lysine	80	0.0

Precision

For precision studies, three concentrations of GSSG were prepared in 5% TCA and a human red blood cell lysate was collected. All samples were stored at -70°C in single use aliquots. In addition, one concentration of GSSG was prepared in phosphate buffer and stored at 4°C. All samples were assayed in duplicate twice per day on each of thirteen days for N = 26 runs and N = 52 replicates. Precision was calculated per NCCLS guidelines.[9] Table 5 shows this data.

Table 5: Assay Precision

Sample	Low	Medium	High	Blood	4°C
Mean	124.3	248.6	478.6	687.7	475.1
Intra SD	2.8	3.5	7.9	18.0	3.8
Intra %CV	2.3	1.4	1.6	2.6	0.8
Inter SD	7.2	8.6	16.8	40.0	16.4
Inter %CV	5.8	3.5	3.5	5.8	3.5
Total SD	7.5	9.0	17.7	42.0	16.6
Total %CV	6.0	3.6	3.7	6.1	3.5

Sensitivity

The lower limit of detection is defined as the lowest concentration that can be distinguished from zero at a 95% confidence level. It was calculated as the concentration equivalent to the absorbance at 3.29 standard deviations from the absorbance at zero concentration.[10] The LLD is 9 μM in the sample added to the assay.

Accuracy

Four experimental methods of demonstrating accuracy were performed with the GSH-420™ analytical method.

Gravimetric Recovery Method:

The three GSSG samples, as shown in table 6, were prepared in 5% TCA to have actual gravimetric concentrations of 124.4, 248.7, and 496.0 μM GSH equivalents. The average percent recovery is 98.8%, N = 52.

Table 6: Gravimetric Recovery

Sample	Low	Medium	High
Gravimetric	124.4	248.7	496.0
Measured	124.3	248.6	478.6
Percent Recovery	99.9%	100.0%	96.5%

Addition Recovery Method*:*

Three freshly drawn human whole blood samples were spiked with two known concentrations of GSSG. The samples were subjected to the sample preparation procedure and measured in the GSH-420™ Assay. Percent recovery of added glutathione was calculated. The results are shown in table 7.

Table 7: Addition Recovery

Sample	A	B	C
Neat	602	793	825
264 μM Added	890	1035	1058
Percent Recovery	109 %	92 %	88 %
529 μM Added	1099	1289	1309
Percent Recovery	94 %	94 %	92 %

Linearity on Dilution

Extracted whole blood samples were volumetrically diluted with 5% TCA and assayed. The measured concentration was plotted versus the dilution of the original extract sample. Representative dilution plots are presented in figure 2.

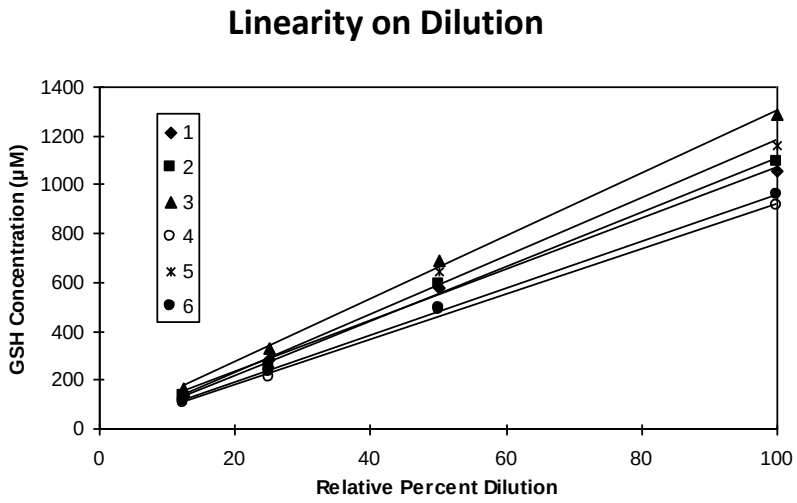

Figure 2: Linearity

Correlation with Reference Assay

The DTNB-Enzyme (Glutathione Reductase) Recycling Assay[7,8] was utilized as the reference assay (Enzyme Assay). Unscreened human whole blood samples (N=115) were measured in both the GSH-420™ Assay and the Enzyme Assay. The correlation of the GSH-420™ Assay to the Enzyme Assay was determined by linear regression and is shown in Figure 3. The correlation coefficient (R) obtained is 0.9875.

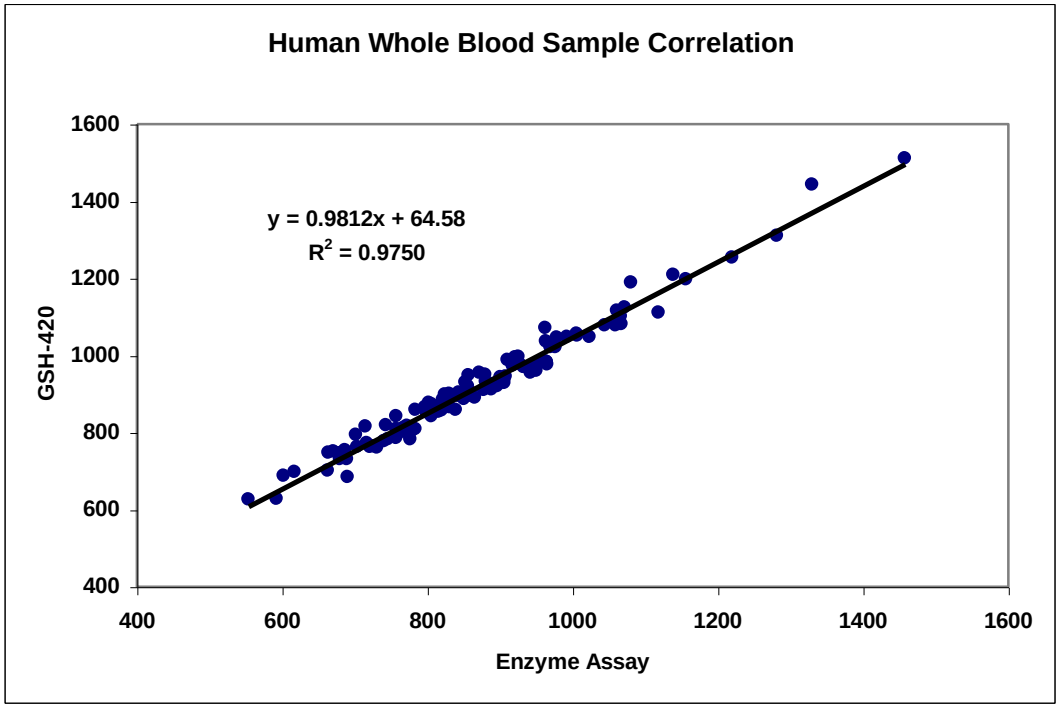

Figure 3: Correlation of GSH-420 Assay to the Enzyme Assay

Assay Range

In order to establish an assay range, samples of increasing GSH concentration were assayed. The highest concentration that maintains linearity is considered the upper limit to the range. The LLD is used for the lower limit. The dose response departs from linearity above 400 µM GSH. This is equivalent to

1600 μM GSH in the pre-extracted whole blood sample. The assay range is 9 - 400 μM in the extracted sample.

Table 8: Assay Linearity Range

Original Sample	Extracted Sample	Assay Cuvette
36 – 1600 μM GSH	9 – 400 μM GSH	1.8 – 80 μM GSH

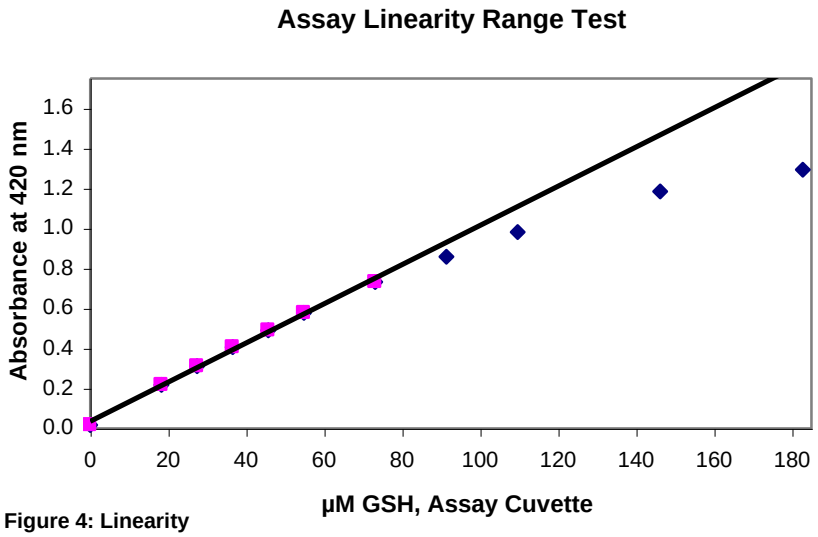

Figure 4: Linearity

NOTES

Limitations

- The chromophore formed in this assay is photosensitive. The reaction mixture must be incubated in the dark. There is a 5% loss in absorbance when exposed to light for 30 minutes and 70% loss after 24 hours.
- Whole blood levels may be higher than the corresponding erythrocyte lysate levels due to plasma interferents.

Expected Values

In laboratory experiments with human blood samples (N = 118), the average GSH concentration in the GSH-420™ Assay was 902 μM (±146), with a minimum of 568 and maximum of 1511.

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