BIOXYTECH®

Quantitative Glutathione Microplate (Q. GSH-MP™) Assay Kit

Catalog Number A21038 (AOXRE Catalog Number 21038)
For Research Use Only.
Store at 2 to 8°C.

INTRODUCTION

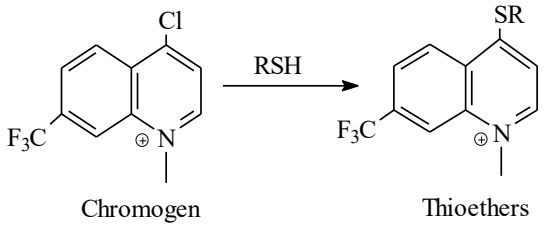
The Analyte

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] GSH, as a single agent, is found to affect DNA damage and repair, redox regulation and multiple cell signaling pathways.[2] Most GSH in whole blood is found in the erythrocyte.[3] Recent studies on GSH in erythrocytes have provided us detailed information of cell membrane and its structural organization that may help in revealing the aging and age associated changes.[4]

Principles of the Procedure

The method is based on the formation of a chromophoric thione[5]. The absorbance measured at 405 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)[6], 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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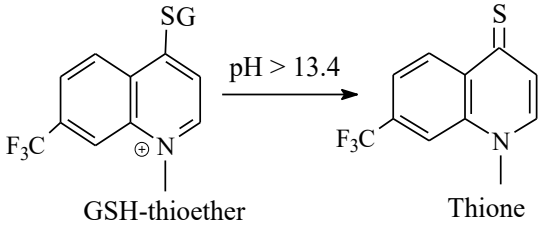

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REAGENTS

Materials Provided (for 96 tests)

- Chromogen — 1-Methyl-4-chloro-7-trifluoromethylquinolinium methylsulfate in HCl, 1 X 4 mL.
- Color Developer — NaOH in water, 1 X 4 mL.
- Reducing Agent — Tris(2-carboxyethyl)phosphine (TCEP) in HCl, 1 X 4 mL.
- Precipitation Reagent — Trichloroacetic acid (TCA) in water, 1 X 10 mL.
- Buffer — Potassium phosphate, Diethylenetriaminepentaacetic acid (DTPA), Lubrol, pH 7.8, 1 X 4 mL.
- Calibrators — Low: 75 μM Glutathione disulfide (150 μM GSH equivalents) in 125 mM potassium phosphate, pH 7.5, 1 X 0.6 mL.
  High: 175 μM Glutathione disulfide (350 μM GSH equivalents) in 125 mM potassium phosphate, pH 7.5, 1 X 0.6 mL.
- Microplate — 96-well Microplate, 1 X plate

Materials Required But Not Provided

- Any spectrophotometer plate reader with a 405 nm filter (filters between 400 nm or 410 nm are also acceptable).
- Microcentrifuge tubes.
- Centrifuge.
- Pipettors with disposable tips.
- Shake 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 100 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 20 μL of the lysate to a microcentrifuge tube.
6. Add 60 μL Precipitation Reagent to the tube.

7. Shake 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 20 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-405 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 40 µL of sample supernatant (or calibrator) to a microplate well.
2. Add 40 µL Buffer to the reaction mixture.
3. Add 40 µL Reducing Agent to the reaction mixture, mix well.
4. Add 40 µL Chromogen to the reaction mixture, mix well.
5. Add 40 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 405 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.

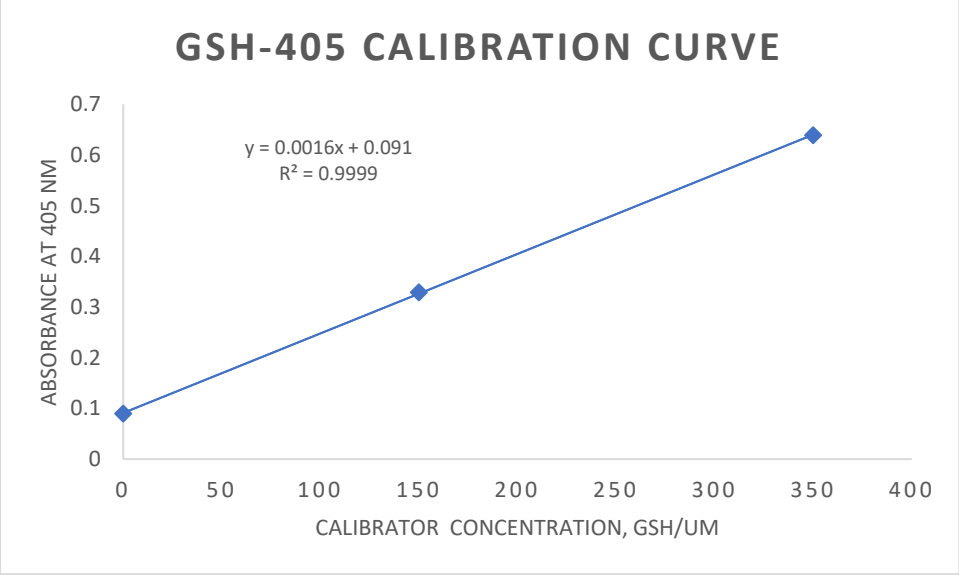

Figure 1: Calibration Curve

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.[7] Control samples are included with calibration and sample assays. Results outside the established acceptable ranges require remediation and/or repetition.[8]

- Use normalization based on the hemoglobin content, if desired and strongly recommended for erythrocyte lysates.

Calculations

The absorbance at 405 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_{405} is the sample absorbance a 405 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:	0.0895	0.329	0.639	0.352

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:

$$[GSH] = \rho\,(A_{420} - b)/m$$
$$[GSH] = 4(0.352 - 0.091)/0.0016$$
$$[GSH] = 652.5\ \mu M \text{ (original sample)}$$

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.

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