Microplate Assay for GSH/GSSG (Reduced/Oxidized Glutathione)

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INTRODUCTION

Reduced glutathione (GSH) is a tripeptide (γ-glutamylcysteinylglycine) that contains a free thiol group. GSH is a major tissue antioxidant that provides reducing equivalents for the glutathione peroxidase (GPx) catalyzed reduction of lipid hydroperoxides to their corresponding alcohols and hydrogen peroxide to water.

$$H_2O_2 + 2GSH \xrightarrow{GPx} GSSG + 2H_2O$$

In the GPx catalyzed reaction, the formation of a disulfide bond between two GSH molecules gives rise to oxidized glutathione (GSSG). The enzyme glutathione reductase (GR) recycles GSSG to GSH with the simultaneous oxidation of β-nicotinamide adenine dinucleotide phosphate (β-$NADPH_2$).

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+$$

When cells are exposed to increased levels of oxidative stress, GSSG will accumulate and the ratio of GSH to GSSG will decrease. Therefore, the determination of the GSH/GSSG ratio and the quantification of GSSG are useful indicators of oxidative stress in cells and tissues.

PRINCIPLES OF PROCEDURE

The low concentration of GSSG (high GSH/GSSG ratio) in tissues coupled with the need to prevent GSH oxidation during sample preparation, are important considerations for the accurate measurement of GSSG and GSH/GSSG ratios. Guntherberg and Rost (2) first reported the use of N-ethylmaleimide (NEM) reacting with GSH to form a stable complex, therefore removing the GSH prior to the quantification of GSSG in tissues. Unfortunately, NEM inhibits GR. To overcome this problem, Griffith (3) employed 2-vinylpyridine (2-VP) to derivatize GSH. Although 2-VP does not significantly inhibit GR, it reacts relatively slowly with GSH and is not very soluble in aqueous solutions.

Scavenging of Free Thiols: This assay employs a pyridine derivative as a thiol-scavenging reagent thereby overcoming the shortfalls of both prior methods. At the concentration employed in the assay, this derivative reacts quickly with GSH but does not interfere with the GR activity.

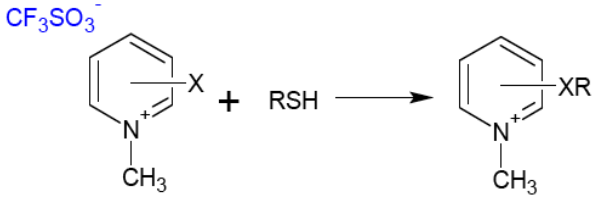

Figure 1: Reaction of the scavanger with a thiol to form a pyridinium salt

Thiol Quantification: The quantitative determination of the total amount of glutathione (GSH + GSSG) employs the enzymatic method first reported by Tietze (1). Briefly, the reaction of GSH with Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid (DTNB)) gives rise to a product that can be quantified

spectrophotometrically at 412 nm. This reaction is used to measure the reduction of GSSG to GSH. The rate of the reaction is proportional to the GSH and GSSG concentration.

MATERIALS PROVIDED

Component	Description	Volume	Storage	Cat no.
Assay Buffer	General buffer used to dilute samples and reagents	100 mL	4°C	GT40a
Standard	10 μM GSSG standard working solution	500 μL	-20°C	GT40b
Scavenger	Thiol scavenger to keep GSSG in its oxidized form	3.0 mL	4°C	GT40c
DTNB	Lyopholized 5,5'-dithiobis-2-nitrobenzoic acid	1 vial	RT	GT40d
5% MPA	Metaphosphoric acid solution used to deproteinate samples	20 mL	4°C	GT40e
Reductase	Recombinant glutathione reductase	35 μL	-20°C	GT40f
NADPH	Lyopholized β–nicotinamide adenine dinucleutide phosphate	1 vial	-20°C	GT40g

MATERIALS NEEDED BUT NOT PROVIDED

1. Microplate reader with 412 or 405 nm filter and kinetic reading capabilities
2. Microcentrifuge capable of 1000 x *g* or greater
3. Polypropylene microcentrifuge tubes
4. Adjustable micropipettes

STORAGE

1. Store the components of this kit at the temperatures specified on the labels.
2. Unopened reagents are stable until the indicated kit expiration date.

WARNINGS AND PRECAUTIONS

1. Use aseptic technique when opening and dispensing reagents.
2. This kit is designed to work properly as provided and instructed. Additions, deletions or substitutions to the procedure or reagents are not recommended, as they may be detrimental to the assay.

PROCEDURAL NOTES

1. If a plate reader with a 412 nm filter is not available, a 405 nm filter will be adequate.
2. Reagents can be used immediately upon removal from refrigeration.
3. To minimize errors in absorbance measurements due to handling, wipe the exterior bottom of the microplate wells with a lint-free paper towel prior to inserting into the plate reader.

SAMPLE COLLECTION AND STORAGE

The following protocols are for whole blood sample preparation. See **NOTES REGARDING SAMPLES** for additional information regarding the use of whole blood and guidelines for using other sample types.

GSSG Sample:

1. Add 30 μL of Scavenger to a microcentrifuge tube.
2. Carefully add 100 μL of whole blood to the bottom of the centrifuge tube and mix gently.
3. Freeze sample at –70°C; it is stable for at least 30 days.

GSH Sample:

1. Carefully add 50 μL of whole blood to the bottom of a microcentrifuge tube.
2. Freeze the sample at –70°C; it is stable for at least 30 days.

NOTES REGARDING SAMPLES:

Freezing Step: Freezing and thawing will lyse the red blood cells and maximize the concentration of GSSG in the sample.

Frozen Samples: Blood samples that have been frozen without prior treatment with the Scavenger are not ideal for the GSSG assay, although the Scavenger can be added to frozen samples before they thaw. This will result in a reduction of GSSG and an increase in GSH in the sample.

GSH Linearity: Because GSH is present at high concentrations in whole blood, the whole blood sample should be diluted significantly (typically > 1:200) in order to obtain a linear reaction rate.

Sample Stability: Glutathione and oxidized glutathione are relatively stable in intact "resting" cells for up to 24 hr at 4°C. The stability of "elevated" GSSG in the intact red blood cell has not been determined. It is recommended that blood samples be treated with the Scavenger as soon as possible and frozen immediately. Upon disruption of the cell, GSH is rapidly oxidized. Therefore, to minimize the *in vitro* oxidation of GSH to GSSG, the Scavenger should be added to the sample prior to lysis or homogenization.

Tissue Samples: The quantification of the GSH/GSSG ratio in tissue samples has not been thoroughly studied using this specific protocol and may vary depending on tissue or cell culture conditions. The following issues need to be considered when preparing tissue samples for analysis:

1. GSH oxidation occurs rapidly *in vitro* in disrupted tissues. The Scavenger should therefore be added as quickly as possible.
2. GSSG diffusion from the cells during sample preparation may result in underestimating the GSSG concentration. This should not be a major issue except for extensively washed cell preparations.
3. γ-Glutamyltranspeptidase, a membrane bound enzyme expressed at high levels in kidney, pancreas, ciliary body, choroids plexus, intestinal epithelia, bile duct, lymphoid and many tumor cells, metabolizes GSH (5), thereby reducing the levels measured by this assay, with the level of reduction dependent on time, temperature and the sample preparation protocol.

Normal Plasma: The amount of GSSG present in normal resting plasma is at or below the lowest level of detection for the assay.

Urine Samples: The amount of GSSG present in urine is not detectable.

SAMPLE PREPARATION

GSSG Sample:

1. Thaw the GSSG sample (130 µL), mix immediately, and incubate at room temperature for 5-10 minutes.
2. Add 270 µL ice-cold 5% MPA to the tube and vortex briefly. (Dilution Factor = 4)
3. Centrifuge at 1000 x *g* and 4°C for 10 minutes.
4. Add 50 µL of the supernatant to 700 µL Assay Buffer in a new microcentrifuge tube. (Dilution Factor = 15, therefore making the final Dilution Factor = 60)
5. Place the diluted extract on ice until use.

GSH Sample:

1. Thaw the GSH sample (50 µL) and mix immediately.
2. Add 350 µL ice-cold 5% MPA to the microcentrifuge tube and vortex briefly. (Dilution Factor = 8)
3. Centrifuge at 1000 x *g* and 4°C for 10 minutes.
4. Add 25 µL of the supernatant to 1.5 mL Assay Buffer in a new microcentrifuge tube. (Dilution Factor = 61, therefore making the final Dilution Factor = 488)
5. Place the diluted extract on ice until use.

REAGENT PREPARATION

NOTE: The stability of reagents, once reconstituted, is greatly diminished. The kit should be used in its entirety within 2 hours of reagent reconstitution. During this time reagents should be stored as recommended in the following reconstitution instruction.

1. **NADPH:** Reconstitute contents of the vial with 500 μL Assay Buffer. Add this to 5.5 mL Assay Buffer and vortex. Leave on ice.
2. **Reductase:** Add 30 μL Reductase to 6 mL Assay Buffer. Leave on ice.
3. **DTNB:** Reconstitute contents of the vial with 500 μL Assay Buffer. Add this to 5.5 mL Assay Buffer and vortex. Leave at room temperature.

STANDARD CURVE PREPARATION

Set up for the standard curve by labeling dilution tubes and dispensing the indicated volumes of Assay Buffer and 10 µM GSSG Standard Stock according to Table 1 below.

Table 1: Standard Curve Preparation

Standard	GSH Concentration (µM)	GSSG Concentration (µM)	Assay Buffer (µL)	Volume of 10 µM GSSG Stock (µL)	Final Volume (µL)
S_7	3.0	1.5	850	150	1000
S_6	2.0	1.0	900	100	1000
S_5	1.5	0.75	925	75	1000
S_4	1.0	0.50	950	50	1000
S_3	0.5	0.25	975	25	1000
S_2	0.25	0.125	987.5	12.5	1000
S_1	0.10	0.05	995	5	1000
B_0	0.0	0.0	1000	-	1000

ASSAY PROCEDURE

1. Add 50 μL of standards, samples, or blank to the corresponding wells on the microplate. See Scheme 1 on the following page for a sample plate layout.
2. Add 50 μL DTNB Solution to each well.
3. Add 50 μL Reductase Solution to each well.
4. Mix by tapping the plate or placing the plate on an orbital shaker and incubate at room temperature for 5 minutes.
5. Add 50 μL NADPH Solution to each well.
6. Place the plate in a kinetic plate reader and record the change of absorbance at 412 nm by taking readings every minute for 10 minutes. If a kinetic plate reader is not available, take a reading at zero minutes and another at 10 minutes. A 405 nm filter will also work if a 412 nm is not available.

Scheme I: Sample Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	B_0	B_0	U_1	U_1	U_9	U_9	U_{17}	U_{17}	U_{25}	U_{25}	U_{33}	U_{33}
B	S_1	S_1	U_2	U_2	U_{10}	U_{10}	U_{18}	U_{18}	U_{26}	U_{26}	U_{34}	U_{34}
C	S_2	S_2	U_3	U_3	U_{11}	U_{11}	U_{19}	U_{19}	U_{27}	U_{27}	U_{35}	U_{35}
D	S_3	S_3	U_4	U_4	U_{12}	U_{12}	U_{20}	U_{20}	U_{28}	U_{28}	U_{36}	U_{36}
E	S_4	S_4	U_5	U_5	U_{13}	U_{13}	U_{21}	U_{21}	U_{29}	U_{29}	U_{37}	U_{37}
F	S_5	S_5	U_6	U_6	U_{14}	U_{14}	U_{22}	U_{22}	U_{30}	U_{30}	U_{38}	U_{38}
G	S_6	S_6	U_7	U_7	U_{15}	U_{15}	U_{23}	U_{23}	U_{31}	U_{31}	U_{39}	U_{39}
H	S_7	S_7	U_8	U_8	U_{16}	U_{16}	U_{24}	U_{24}	U_{32}	U_{32}	U_{40}	U_{40}

CALCULATIONS

1. Rate Determination (Mean V):

The change in absorbance, or optical density (OD), is a linear function of the GSH concentration in the reaction mixture. It is described by the following linear regression equation (in the form of y=mx+b) in which the slope of the regression equation is equal to the rate:

OD = Slope x Minutes + Intercept

Many plate readers can calculate the rate for you. The y-intercepts for these rate curves should not be used because they are dependent on the DTNB background and the time interval between the addition of the NADPH (start of the reaction) and the first recorded measurement. Examples are provided below for the determination of total GSH (GSH + GSSG in the absence of the thiol scavenger) and GSSG (determined with use of the thiol scavenger) by linear regression analysis using the equation given above.

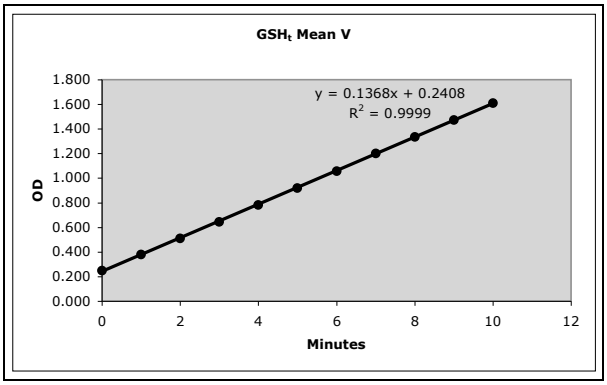

Figure 5: Reaction rate for an untreated sample. The rate is proportional to the concentration of GSH_t.

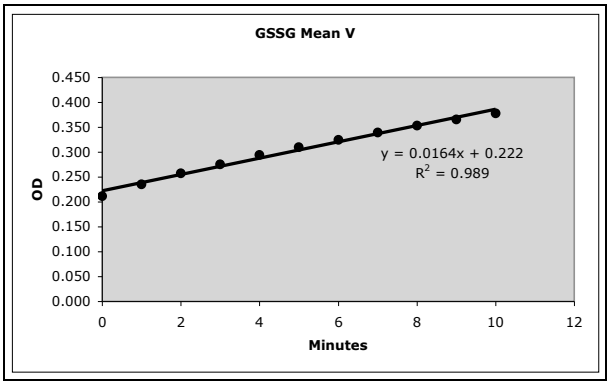

Figure 6: Reaction rate for a M4VP treated sample. The rate is proportional to the concentration of GSSG.

GSH_t: OD = 0.1368 x Minutes + 0.2408. Therefore, the rate for the GSH_t sample is 0.1368.
GSSG: OD = 0.0164 x Minutes + 0.222. Therefore, the rate for the GSSG sample is 0.0164.

2. Calibration Curves:

The GSH/GSSG assay uses an eight-point standard curve for both GSH_t and GSSG determinations. For each concentration of GSH, the Net Rate is the difference between the sample or standard rate and the blank (B_0) rate.

Table 2: Sample Standard Curve

Standard	μM GSH	μM GSSG	Rate	Net Rate
B_0	0.00	0.00	0.0128	0
S_1	0.10	0.05	0.0176	0.0048
S_2	0.25	0.125	0.0259	0.0131
S_3	0.50	0.25	0.0407	0.0279
S_4	1.0	0.50	0.0665	0.0537
S_5	1.5	0.75	0.0928	0.0800
S_6	2.0	1.0	0.1157	0.1029
S_7	3.0	1.50	0.1635	0.1507

Because the concentration of GSSG is much lower in the reaction mixture compared to GSH_t it is recommended that selected data ranges from the calibration curve be plotted separately. For GSH_t, perform linear regression on a three-point curve using the 0, 0.50, 0.75, 1.0, and 1.50 μM GSSG (0, 1.0, 1.5, 2.0, and 3.0 μM GSH) data points (Figure 7). In the case of GSSG, use the 0, 0.05, 0.125, and 0.25 μM GSSG data points (Figure 8). Please see next page for figures.

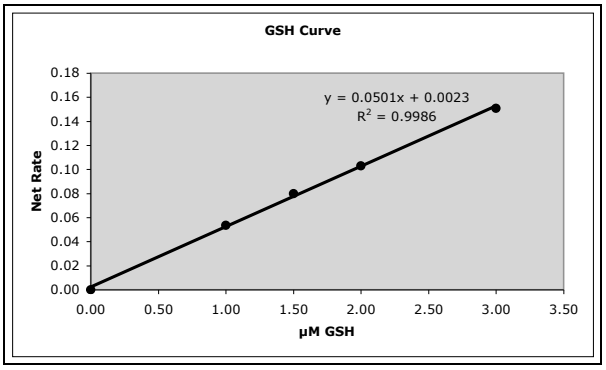

Figure 7: A five-point calibration curve of Net Rate vs. μM GSH used to determine the concentration of GSH_t.

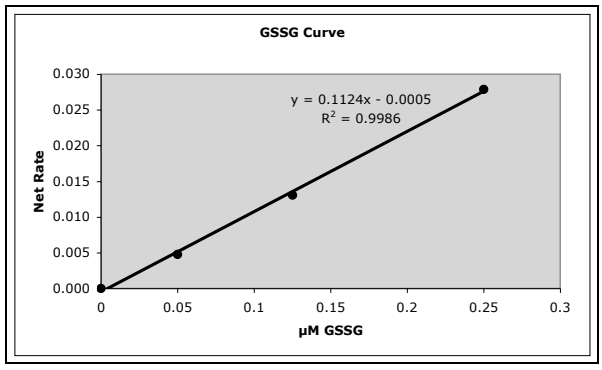

Figure 8: A four-point calibration curve of Net Rate vs. μM GSSG used to determine the concentration of GSSG.

3. GSH_t and GSSG Concentrations:

The general form of the regression equation describing the calibration curve is:

Net Rate = Slope x GSH + Intercept

Therefore, to calculate the total GSH (GSH_t) or GSSG concentration from the GSH calibration curve:

$$\text{GSH} = \frac{\text{Net Rate} - \text{Intercept}}{\text{Slope}} \bullet \text{Dilution Factor}$$

For example, in the experiment depicted in Figure 5, the net rate of change for the GSH_t sample is 0.1368 – 0.0128 or 0.124. Using the calibration curve shown in Figure 7, the GSH_t can be calculated as follows:

$$\text{GSH}_t = \frac{0.124 - 0.0023}{0.0501} \bullet 488 = 1185.4\ \mu\text{M}$$

Similarly, from the rate of change for the GSSG sample in Figure 6 is 0.0164 – 0.0128 or 0.0036. Using the calibration curve shown in Figure 8, the GSSG concentration can be calculated as shown below. Note the dilution factor correction that accounts for the conversion of GSH to GSSG; 60-fold dilution divided by 2 GSH molecules per GSSG molecule.

$$\text{GSSG} = \frac{0.0036 + 0.0005}{0.1124} \bullet 30 = 1.094\ \mu\text{M}$$

4. GSH/GSSG Ratio:

The GSH/GSSG Ratio is calculated by dividing the difference between the GSH_t and GSSG concentrations by the concentration of GSSG.

$$\text{Ratio} = \frac{GSH_t - 2GSSG}{GSSG}$$

The GSH/GSSG ratio for the sample data is:

$$\text{Ratio} = \frac{1185.4 - 2(1.094)}{1.094} = 1081.5$$

REFERENCES

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