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Spectrophotometric Assay for
Glutathione Reductase

Product No. FR 19
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INTRODUCTION

The Analyte

Glutathione reductase (GR, EC 1.6.4.2) is a ubiquitous enzyme, which catalyzes the reduction of oxidized glutathione (GSSG) to glutathione (GSH). Glutathione reductase is essential for the glutathione redox cycle that maintains adequate levels of reduced cellular GSH. GSH serves as an antioxidant, reacting with free radicals and organic peroxides, in amino acid transport, and as a substrate for the glutathione peroxidases and glutathione S-transferases in the detoxification of organic peroxides and metabolism of xenobiotics, respectively.[1]

This homodimeric enzyme is a member of the family of flavoprotein disulfide oxidoreductases. Each subunit has four domains; beginning at the N-terminus: an FAD-binding domain, an NADPH-binding domain, a central domain, and an interface domain. The active site of GR is at the dimeric interface. Since the GSSG binding site is composed of residues from both subunits, only the dimeric form is active.[2]

$$GSSG + NADPH + H^+ \xrightarrow{\text{Glutathione Reductase}} 2GSH + NADP^+$$

Oxidized glutathione is reduced by a multi-step reaction in which GR is initially reduced by NADPH forming a semiquinone of FAD, a sulfur radical and a thiol. The reduced GR (GR_{red}) reacts with a molecule of GSSG, resulting in a disulfide interchange, which produces a molecule of GSH and the GR_{red}-SG complex. An electron rearrangement in GR_{red}-SG results in a second disulfide interchange, splitting off the second molecule of GSH and restoring the GR to the oxidized form.[3] Table1 lists some properties of glutathione reductase.

Table 1: Properties of Glutathione Reductase

Source	Molecular Weight	Km (μM)		Reference
		NADPH	GSSG	
Human erythrocytes	100,000	8.5	65	4
Horse liver	107,000	8.8	59	5
Calf liver	100,000	21	101	6
Rat liver	100,000	8.2	26	6
Yeast	118,000	3.8	55	3

Principles of the Procedure

This assay is based on the oxidation of NADPH to $NADP^+$ catalyzed by a limiting concentration of glutathione reductase. One GR activity unit is defined as the amount of enzyme catalyzing the reduction of one micromole of GSSG per minute at pH 7.6 and 25°C. As shown in the above reaction, one molecule of NADPH is consumed for each molecule of GSSG reduced. Therefore, the reduction of GSSG is determined indirectly by the measurement of the consumption of NADPH, as demonstrated by a decrease in absorbance at 340 nm (A_{340}) as a function of time.

REAGENTS

Materials Provided (for 100 tests)

·	NADPH	Lypholyzed solution containing NADPH, Tris, manitol. 6 vials
·	GSSG	Oxidized Glutathione in K•PO_4. Buffer containing EDTA. pH 7.5
·	K•PO_4	Potassium Phosphate Buffer, pH 7.5, 20 mL
·	Diluent Buffer	Potassium Buffer containing BSA/EDTA

Materials Required But Not Provided

1. Spectrophotometer, preferably equipped with a 25°C thermostatted cuvette holder and the ability to record the absorbance at 340 ± 2 nm over time.
2. 1 mL semi-micro disposable cuvettes with 1 cm path length.
3. Pipettes, 200 and 400 µL, with disposable tips.
4. Glass or plastic pipette capable of accurately measuring 7 mL.
5. Deionized water.

Warnings and Precautions

Use established laboratory precautions when handling or disposing any chemical contained in this product.
If any of the components come in contact with skin or eyes, rinse immediately with plenty of water. Seek medical advice.

Reagent Storage and Handling

- It is good practice to transfer the desired volume of reagents for an experiment to a clean glass test tube or other vessel and return the stock reagent bottles to 2 to 8°C storage.
- Unopened reagents are stable until the indicated expiration date.

PROCEDURE

Reagent Preparation

NADPH

Reconstitute the desired number of NADPH vials with 7 mL of 25°C deionized water. If capped and protected from direct light, the reconstituted reagent is stable at room temperature for at least 5 hours, and when at stored from 2 to 8°C it is stable for 2 days.

GSSG, K•PO_4 Reagents and Diluent

Bring to 25°C before assay.

Sample Preparation

Thaw GR samples, thoroughly mix, and place on ice. Immediately prior to assay, dilute GR sample in Diluent Buffer to a concentration between 5 and 50 mU/mL. If the sample target concentration is unknown, it may be necessary to make multiple dilutions.

Equipment Preparation

Turn on spectrophotometer, set wavelength to 340 nm, and temperature to 25°C (recommended). Zero the spectrophotometer at 340 nm using deionized water.

Assay

1. Pipette the following into a cuvette:
   - 200 µL sample
   - 400 µL GSSG (or K•PO_4 for sample Blank)
2. Place cuvette in spectrophotometer.

   NOTE: The reaction mixture should be at the desired assay temperature.
3. Add 400 µL NADPH and mix 3 times by drawing and expelling with the pipette. Avoid bubbles by keeping tip below the level of the liquid surface.
4. Record the A_{340} for five minutes.

Calculations

1. Using the linear portion of the curve, calculate the rate of decrease in the A_{340} per minute.
2. Calculate the net rate for the sample by subtracting the rate obtained for the Blank, where K•PO_4 was used instead of GSSG.
3. The molar extinction coefficient, ε, for NADPH is 6220 $M^{-1}cm^{-1}$.

Given: ε = 6220 $M^{-1}cm^{-1}$ or if the pathlength = 1 cm;
ε = 6.22 x 10^{-3} (nmol/mL), and;
mU = nmol/min;
Then: mU/mL = (A_{340}/min)/ε, or;

$$\text{mU/mL} = \frac{A_{340}/\text{min}}{6.22 \times 10^{-3}\ \text{mL/nmol}}$$

4. Correct for the sample dilution in the assay by multiplying the mU/mL by 5 (*i.e.*, 200 µL was diluted to 1000 µL).
5. Correct for the sample dilution performed prior to assay, as necessary.
6.

Example

A sample was diluted 1/25 prior to assay and the data collected is shown below in table 2.

Sample Rate, A_{340}/min	= Average $\Delta A_{340}/\Delta t$	= 0.0523 A_{340}/min
Blank Rate (data not shown)	= Average $\Delta A_{340}/\Delta t$	= 0.0005 A_{340}/min
Net Rate, A_{340}/min	= Sample Rate – Blank Rate	= 0.0518 A_{340}/min
GR Activity	= 0.0518/6.22 x 10^{-3}	= 8.33 mU/mL
Assay Dilution Correction,1/5	= 5 x 8.33	= 41.6 mU/mL
Sample Dilution Correction, 1/25	= 25 x 41.6	= 1.04 x 10^{-3} mU/mL

PERFORMANCE CHARACTERISTICS

Linearity

Shown below in figures 1 and 2 are the linearity data for the GR-340 assay.

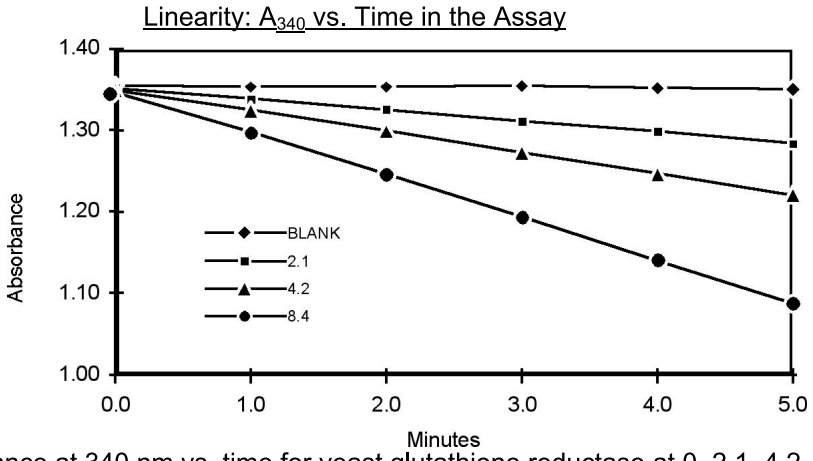

Figure 1. Absorbance at 340 nm vs. time for yeast glutathione reductase at 0. 2.1, 4.2, and 8.4 mU/mL.

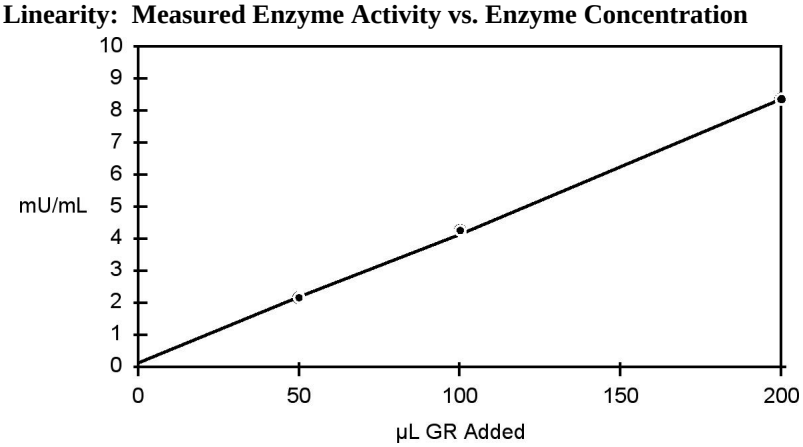

Figure 2. Linearity of GR activity observed with this assay as a function of the concentration of GR.

Total Precision Estimate

The precision of this assay was determined by measuring the activity of 3 samples at different GR concentrations twice per day over a 10-day period. A new vial of NADPH was reconstituted each day and a new vial of the GR Control was thawed just before each run. Glutathione reductase reaction rates were calculated by linear regression of A_{340} on time over a 0.5 to 4 minute interval. The results are shown in Table 2.

Table 2: Precision Estimates

n=20	Low	Medium	High
Mean mU/mL	2.36	4.60	9.12
SD Total	0.22	0.40	0.72
CV Total	9.2%	8.6%	7.8%

Assay Range

Glutathione reductase samples should be diluted to provide a minimum net rate of 0.0050 A_{340} per minute (10x the typical blank rate) and a maximum of 0.0625 A_{340}/min. This corresponds to a final concentration in the assay of approximately 0.8 to 10.0 mU/mL. Lower concentrations may not provide sufficient ΔA_{340} in a five-minute interval and excess GR may cause the rate to be non-linear.

Control of Assay Performance

The following parameters can serve as indicators of proper assay performance:

- The plot of A_{340} vs. time is linear over a five-minute interval at GR concentrations up to 10 mU/mL of the reaction mixture.
- The Blank rate for diluent alone should be less than 0.0006 A_{340}/min.
- The A_{340} of a diluent Blank is 1.35 ± 0.10.
- Recovery of the stated values for other known GR activity.

NOTES

Interference

- Substances in tissue homogenates that consume NADPH can cause an over-estimation of GR activity. This interference can be assessed by substituting the $K•PO_4$ for GSSG. This sample Blank rate is then subtracted from the sample "GSSG" rate.
- Oxidized glutathione in the sample can interfere with the determination of the sample Blank. If the presence of a significant amount of GSSG is suspected, the sample should be passed through an appropriate gel-filtration column prior to measuring the GR activity.
- Glutathione reductase activity is pH dependent; therefore, the final pH of the sample should be 7.4 - 7.6. Use sample preparation buffers that will maintain the pH of the reaction mixture.[4]

Sample Processing Guidelines

The sample preparation guidelines described are not exclusive. They are intended to assist the researcher with the preparation of biological samples for this assay.

- Purified glutathione reductase is reported to be stable at 4°C; however, it is recommended that biological samples be stored at –70°C, and kept on ice during analysis as a precaution against inactivation. The reduced form of the enzyme can be inactivated by metal cations (Zn^{+2}, Cd^{+2}); however, the presence of EDTA fully protects glutathione reductase.[7] Reduced GR is also susceptible to aggregation due to the formation of intermolecular disulfide bridges.[4]
- Compounds reported to reversibly inactivate GR include NADPH, NADH, GSH, sodium sulfate, ammonium sulfate (>100 mM), and ferricyanide. In general, compounds such as GSSG and $NADP^+$ reactivate and protect glutathione reductase.[8] Other compounds, commonly present in enzyme preparations that are reported to have little affect on GR include: urea (600 mM), polyvinylpyrrolidone-10 (0.1%), potassium chloride (100 mM), Triton X-100 (0.1%), and ammonium sulfate (<100 mM).[9]
- Erythrocyte GR has been reported to contain a significant proportion of enzyme that lacks FAD (apoenzyme).[10] The GR-340™ Reagent Set does not include FAD; therefore, investigators can supplement the sample or reaction mixture to one µM FAD to assess the relative concentration of the apoenzyme.

Purified GR. The activity can be measured without additional sample preparation following an appropriate dilution in GR-340™ assay diluent or similar buffer. It is highly recommended that the buffer contain 1 mM EDTA and a suitable carrier protein, such as 1 mg/mL bovine serum albumin, to prevent loss of enzyme activity.

Erythrocyte GR. The activity is determined from erythrocyte lysates. Collect blood in EDTA or heparin as an anticoagulant. Following removal of the plasma and buffy coat, wash the erythrocytes twice in cold saline. Lyse the cells in four packed-cell volumes of cold deionized water and centrifuge (*e.g.*, 8500 x *g* for 10 minutes at 2 – 8°C) to remove the red cell stroma. Place lysate on ice. If not assayed promptly, store at -70°C. See the **Limitations** section concerning the absorption of hemoglobin at 340 nm.

Whole Tissue GR. The activity is measured in cell free homogenates. Briefly, perfuse and/or wash the tissue to remove erythrocytes with cold isotonic saline containing 1 mM EDTA. Mince the tissue in a cold buffer, such as 50 mM potassium phosphate pH 7.5 containing 1 mM EDTA, and homogenize. Centrifuge the homogenate at an average force of 8,500 x *g* and 4°C for 10 minutes. Collect the supernatant and place on ice. If not assayed promptly, store at –70°C. If the tissue has not been tested previously, add 0.1 - 1.0 mg protein to the reaction mixture for a trial assay.

Limitations

The NADPH, under the standard assay conditions, has an absorbance of 1.35 ± 0.10 at 340 nm. Because the absorbance from the NADPH and the intrinsic absorbance of the sample (at 340 nm) are additive, samples with high intrinsic absorbance at 340 nm may exceed the absorbance maximum of the spectrophotometer. Therefore, samples with high A_{340} should be appropriately diluted with diluent prior to assay. In particular, due to the absorption of hemoglobin at 340 nm, erythrocyte lysate samples are limited to 1 mg hemoglobin per assay volume (1 mL).

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