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Northwest
Life Science Specialties, LLC

Premier Products for Superior Life Science Research

NWLSS™ Glutathione Reductase Activity (Microplate Assay)

Product NWK-GR01
For Research Use Only

Simple assay kit for measurement of Glutathione Reductase (GR) enzyme activity in tissue homogenates and cell lysates.

nwkgr01rev092507

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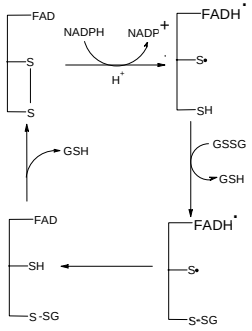
Introduction:

Glutathione Reductase (GR, EC 1.6.4.2) is a ubiquitous 100-120 kD dimeric flavoprotein that catalyzes the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) using β-nicotinamide dinucleotide phosphate (NADPH) as the hydrogen donor.[1]



The active site for GR contains a flavin adenine dinucleotide (FAD) and a disulfide. In the presence of NADPH, there is a two electron reduction of GR to produce a semiquinone of FAD, a sulfur radical and a thiol.[2]

A measurable fraction of GR derived from various sources is often found as the inactive apoenzyme. Dietary supplementation of riboflavin or thyroxin has been shown to activate GR. The *in vitro* addition of FAD is the basis of assessing riboflavin deficiency.[3,4,5,6]

Purified GR tends to form aggregates in the absence of thiols and these aggregates retain full enzymatic activity.[7] Purified enzyme is reversibly inactivated by NADPH, β-nicotinamide adenine dinucleotide (NADH), GSH, dithionite or borohydride. This inactivation likely requires the presence of divalent cations such as Zn^{++} and Cd^{++}. The GR enzyme is fully protected from inactivation by ethylenediamine tetraacetic acid (EDTA). Inactivated GR is activated by GSSG, $NADP^+$ and NAD^+. *In vivo*, GR activity is regulated through a redox interconversion mechanism mediated by GSSG regulation of the NADPH generating pathways.[8]

Intended Use:

The NWLSS™ Glutathione Reductase (GR) Activity Assay is intended for the quantification of GR enzyme activity in biological samples such as tissue homogenates and cell lysates.

Test Principle:
The most widely used procedure to measure GR is to monitor the oxidation of NADPH as a decrease in absorbance at 340 nm as shown in Figure 1.[9]

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

Figure1. Reduction of GSSG by NADPH catalyzed by GR.

For each mole of GSSG reduced, one mole of NADPH is oxidized resulting in loss of absorbance at 340 nm. One Unit GR activity is defined as the amount of enzyme that will reduce 1 µmole GSSG per minute at pH 7.6 and 25°C.

General Specifications:

Format:	2 X 96 well Microplate
Number of tests:	198
Specificity:	Glutathione Reductase Activity
Sensitivity:	LLD = 1.5 mU/mL

Kit Contents:

Assay Buffer:		1 X 125 mL
GR Calibrator	(1-1.5 units per mL)	1 mL
GSSG Reagent		1 X 11 mL
NADPH Reagent		2 X 5mg
NADPH Diluent		2 X 6 mL
Microplates		2 X 96 Well

Required Materials Not Provided:
Adjustable pipettes capable of transferring 10 µL to 1000 µL volumes.

Required Instrumentation:
96 well microplate reader with 340 nm capability.

Statement of Limited Warranty:
Northwest Life Science Specialties, LLC (NWLSS) makes no guarantee of any kind, expressed or implied, that extends beyond the description of the material in this kit, except that they will meet our specifications at the time of delivery. Customer's remedy and NWLSS' sole liability is limited to, at NWLSS' option, refund of the purchase price, or the replacement of material not meeting our specification. By acceptance of our product, customer assumes all liability and will indemnify and hold NWLSS harmless for the consequence of this product's use or misuse by the customer, its employees, or others. Refund or replacement is conditioned of customer notifying NWLSS within twenty-one (21) days of the receipt of product. Failure to give notice within 21 days shall constitute a waiver by the customer of all claims hereunder with respect to said product.

Notes:

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References (continue):
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6. Horwitz, J. (1987) Glutathione reductase in human lens epithelium: FAD-induced in vitro activation, Current Eye Research **6(10)**, 1249-1256.

7. Worthington, D.R., and Rosenmeyer, M.A., (1975) Glutathione Reductase from Human Erythrocytes: Molecular Weight, Subunit Compositio0n and Aggregation Properties, *European Journal of Biochemistry* **60**, 459-466.**References (continued):**
8. García-Alfonso, C., et. al., (1993) Regulation of Horse-Liver Glutathione Reductase, *International Journal of Biochemistry* **25:4**, 513-520.

9. Carlberg, I. and Mannervik, B. (1985) Glutathione Reductase in Methods in Enzymology Vol **113**, 484-490.

10. Smith I.K., et. al. (1988) Assay of Glutathione Reductase in Crude Tissue Homogenates Using 5,5'-Dithiobis(2-nitrobenzoic Acid), *Analytical Biochemistry* **175**, 408-413.

11. Tietz Textbook of Clinical Chemistry, 2nd edition Carl A Burtis and Edward R Ashwood, eds., W. B. Saunders, Philidelphia, 1994 page 2190-2192.

12. Corrocher, R., et. al., (1980) Glutathione-peroxidase and glutathione-reductase activities of normal and pathological liver: relationship with age. *Scandinavian Journal of* Gastroenterology 15(7), 781-786.

13. Delides, A., et. Al., (1976) An optimized semi-automatic rate method for serum glutathione reductase activity and its application to patients with malignant disease. *Journal of Clinical Pathology* ***29,*** *73-77.*

14. Anderson, D.J., (1989) Determination of the Lower Limit of Detection, *Clinical Chemistry* **35**, 2152-2153.

Warnings, Limitations, Precautions:

Possible Interference

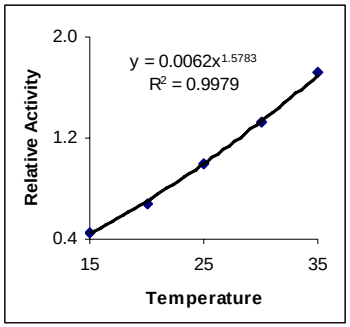
Samples containing substances that have significant absorbance at 340 nm will interfere with the assay by causing the initial absorbance to exceed the upper limit of the instrument. As an example, hemoglobin should not be present at concentrations greater than 1 mg/mL.

Ammonium sulfate ($(NH_4)_2SO_4$) inhibits GR activity at concentrations greater than 100 mM.

Polyvinylpyrrolidone -10 (0.1%), potassium chloride (100 mM), urea (600 mM), and Triton X-100 (0.1%) have no effect on GR activity under standard assay conditions. [10]

Temperature Dependence

Glutathione reductase activity is temperature dependent. In the event that the NADPH extinction coefficient is used in analyzing data and instrument lacks adequate temperature control, the temperature correction table below can be used.



°C	mRate	%
15	69.8	46
20	103.1	68
25	152.8	100
30	203.6	133
35	262.2	172

Figure 3. Temperature dependence was determined by measuring the rate of a GR sample between 15 and 35 °C in 5 °C increments in a thermostatically controlled cuvette

The temperature correction factor can be calculated as:

$$\sigma = 0.0062T^{1.578}$$

Where σ = correction factor and T = Temperature in °C.

Handling

Use good laboratory practices including adequate gloves and eye protection while handling and discarding reagents. Should the reagent and/or test sample come into contact with skin and/or mucous membrane, rinse quickly with a large amount of water.

Storage Instructions:

Upon receipt, store the reagents at 2-8ºC. This kit is stable if unopened for 12 months from the date of manufacture. Do not use components beyond the expiration date printed on the kit box label.

Opened reagents must be stored at 2-8ºC and should be used in a timeframe according to specific reagent guidelines.

Assay Preparation:

Instrument Setup

Wavelength: 340 nm

Mode: Kinetic, Read at ≤ 30 sec. intervals

Reagent Preparation:

Assay Buffer

Supplied ready to use

GSSG Reagent

Supplied ready to use

NADPH

Reconstitute each bottle NADPH with 1 bottle NADPH Diluent. Stored cold and in the original bottle, the reconstituted NADPH is stable for up to 14 days. The consequence of NADPH degradation in solution is the reduction of the assay range.

GR Calibrator

The GR Calibrator is supplied as a solution of GR in Assay Buffer at 1-1.5 units per mL with the assigned GR activity provided on the vial label. To obtain a calibration curve, the Calibrator should be diluted with **Assay Buffer** targeting 20-25 mU GR activity/mL reaction mixture. For highest standard.

Example for Calibrator = 1440 mU/mL:
Add 78 µL **GR Calibrator** as supplied to 1500 µL **Assay Buffer** to create a **75 mU/mL Working Stock** then additionally dilute as follows:

Diluted GR (from 50 mU/mL stock) (µL)	Assay Buffer (µL)	Diluted Calibrator Activity (mU/mL)	Calibrator Activity In The Reaction Mixture (mU/mL)
0	1500	0	0
100	1400	5	4
200	1300	10	8
300	1200	15	12
500	1000	25	20

Performance Characteristics (continued):

Dynamic Range

Under standard conditions, the oxidation of NADPH is the limiting reaction in determining the upper range of the assay. Samples that exceed the upper limit of the assay can exhaust the available NADPH and tend toward non-linearity. Therefore, the dynamic range for the Microplate Method is 1.5 – 20.0 mU/mL in the Rxm or 7.5 – 200 mU/mL in the diluted sample applied to the plate.

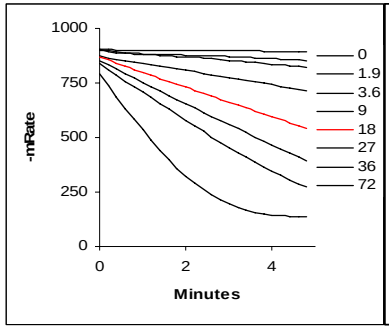


Figure 7: The change in A_{340} absorbance over a five minute interval with increasing GR activity was monitored.

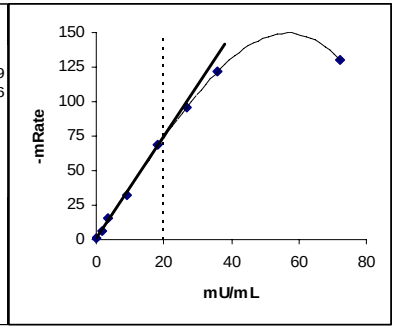


Figure 8: The rate of change in A340 nm absorbance is linearly related to GR activity.

References:

1. Massey, V. and Williams, C.H. (1965) On the Reaction Mechanism of Yeast Glutathione Reductase, *Journal of Biological Chemistry* **240**:11, 4470-4480

2. Worthington, D.R, and Rosenmeyer, M.A., (1976) Glutathione Reductase from Human Erythrocytes: catalytic Properties and Aggragation, *European Journal of Biochemistry* **67**, 231-238.

3. Beutler, E. (1969) Effect of Flavin Compounds on the Glutathione Reductase Activity: In Vivo and In Vitro Studies, Journal Clinical Investigation **48**, 1957-1966.

4. Prentice, A.M. and Bates, C.J. (1981) A biochemical evaluation of the erythrocyte glutathione reductase (EC 1.6.4.2) test for riboflavin status. 2. Dose-response relationships in chronic marginal deficiency, British Journal of Nutrition **45(1)**, 53-65.

Data Analysis (continued):

$$\text{GR Activity} = \frac{(\Delta A_{340})(V_t)(\text{dilution})}{(0.00463\ \mu M)(V_s)} = \text{mU/mL}$$

$$\text{GR Activity} = \frac{(21.33 - 0.550)(250)(60)}{(4.63)(150)} = 449\ \text{mU/mL}$$

Temperature correction
The temperature correction factor is calculated from: $\sigma = 0.0062T^{1.578}$ (**Temperature Dependency**, page 5).

$$\sigma = (0.0062)27.5^{1.578} = 1.158$$

$$\text{GR Activity} = \text{mU/mL}\ (\sigma) = 520\ \text{mU/mL}$$

Normalization
The Hb concentration for the lysate is 90 mg/mL. Therefore, normalization to Hb is calculated as…

$$\text{GR Activity} = \frac{\text{mU GR per mL}}{\text{mg Hb per mL}} = \frac{520}{90} = 5.8\ \text{mU/mL}$$

Performance Characteristics:
Sesitivity
Lower Limit of Detection (LLD)[14] = 1.5 mU/mL Reaction Mix
7.5 mU/mL Diluted Sample

Linearity
Over the 0-1 minute interval, there is an increase in the observed A_{340} that stabilizes after the first minute. The standard method rate curves are then linear to 5 minutes to provide for delays in the start of the A_{340} measurements.

Sample Handling/Preparation:
General guidelines are provided below for various sample types.

Whole Blood:
Prior to assay, dilute the whole blood sample 1:4 cold water (e.g., 50 µL blood in 200 µL water), mix well and place on ice for 5 minutes to lyse the erythrocytes. Store lysates on ice if assayed the same day; otherwise, store at less than -30°C until assay. Just before assay, thaw and re-mix lysate if stored frozen, centrifuge and dilute to 5 mg Hb/mL in Assay Buffer.

Erythrocyte Lysate:
Hemoglobin contributes significantly to the absorbance at 340 nm. The concentration of Hb in the assay reaction mixture should not exceed 1 mg/mL.

Whole blood is washed 2-3 times in cold 0.15 M sodium chloride (NaCl). To the packed erythrocytes, add 2-4 volumes of cold deionized water and mix. Place in ice for 5 minutes to complete erythrocyte lysis. Store lysate on ice if assayed the same day; otherwise, store at less than -30°C until assay. Just before assay, thaw, re-mix lysate, centrifuge and dilute to approximately 5 mg Hb/mL plate in Assay Buffer.

Tissue
Glutathione reductase activity varies with tissue type. The relative GR activity for several murine tissue types is shown in **Table 4**.[11] The user needs to be aware that the appropriate dilution for the given tissue should be established experimentally. The following guidelines may be useful.

High	Moderate	Low	Absent
Liver	Erythrocyte	Skeletal Muscle	Plasma
Kidney	Heart		
	Lung		
	Lens		

For example, perfuse tissue (e.g. liver ~ 40 mU/mg protein[12]) to remove erythrocytes. Homogenize 1 part tissue in 9 parts cold buffer (PBS or 50-100 mM phosphate containing 1-2 mM EDTA, pH 7.0-7.6). Remove debris by centrifugation. Store clarified homogenates on ice if assayed the same day; otherwise, store at less than -30°C until assay. Just before assay, re-mix samples and dilute 1:40 in Assay Buffer.

Plasma
Although it has been reported that patients with various cancers can exhibit elevated GR levels[13], normal blood plasma or serum typically contains no measurable amounts of GR. Additionally, investigators seeking to measure GR in plasma or serum are cautioned that even slight hemolysis during sample preparation can contribute greatly to measurable GR activity.

Assay Protocol:

1. Prepare samples and calibrators

2. Add 150 µL diluted Sample or Calibrator to wells

3. Add 50 µL GSSG to well

4. Shake gently to mix

5. Pause 1 minute

6. Add 50 µL NADPH to each well

7. Shake gently to mix

8. Pause 1 minute

9. Measure the A_{340} nm in 30 second or less intervals for 3 minutes , 25°C

10. Calculate GR activity

Data Analysis:

The GR Activity can be calculated using the regression parameters of the calibration curve or by using the GR unit definition (page 3). However, since the GR Unit Definition requires strict temperature control, using a calibration curve is advisable. The calibration method is described below.

Determine the slope and intercept of the calibration curve using an appropriate linear regression application. There is no need to subtract a blank value. Example data is shown below in figures 4 and 5. Calibrator values may vary according to lot number received.

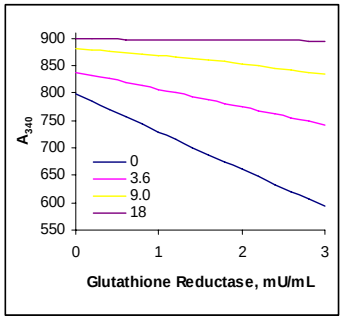


Figure 4: The change in A_{340} absorbance over a three minute interval with increasing GR activity was monitored.

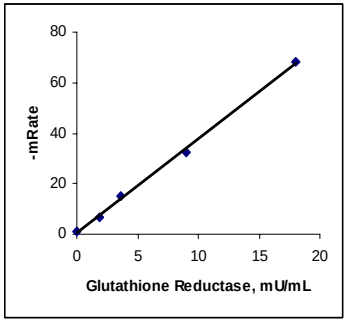


Figure 5: The rate of change in A_{340} nm absorbance is linearly related to GR activity.

Data Analysis (continued):

For each sample, calculate the GR activity using the equation below. *Note that the reaction rates in our calculation have been multiplied by -1000 to provide a more convenient number (mRate).*

$$\text{GR Activity} = \frac{(\text{-mrate - Intercept})(V_t)(\text{dilution})}{(\text{Slope})(V_s)} = \text{mU/mL}$$

Example Calculation:

For example, an RBC lysate was prepared as described previously and assayed with GR calibrators using the standard microplate method. Linear regression parameters were determined using Microsoft Excel slope and intercept functions.

Results

Slope = 3.725
Intercept = .5486
V_t = 250 µL
V_s = 150 µL
Dilution of lysate = 1/60
mRate of sample = 21.33 ΔA_{340}/min

From **the above equation**, the sample GR Activity is ...

$$\text{GR Activity} = \frac{(21.33 - 0.5486)(250(60)}{3.725(150)} = 558 \text{ mU/mL}$$

The Hb concentration for the lysate is 90 mg/mL. Therefore, normalization to Hb is ...

$$\text{GR Activity} = \frac{\text{mU GR per mL}}{\text{mg Hb per mL}} = \frac{558}{90} = 6.2 \text{ mU/mg Hb}$$

Calculation using unit definition.

The GR Activity can be determined using the GR unit definition. However, the GR reaction rate is temperature dependent and requires a thermostatically control plate holder set to 25 °C or the appropriate temperature correction as shown in **Temperature Dependency,** page 5.

Results

Temperature = 27.5°C
V_t = 250 µL
V_s = 50 µL
Dilution of lysate = 1/20.
mRate of sample = 21.33 ΔA_{340}/min
mRate of Blank = 0.550 ΔA_{340}/min