

TREVIGEN® Instructions

For Research Use Only. Not For Use In Diagnostic Procedures

HT Glutathione Reductase Assay Kit

Cat# 7513-500-K

**High throughput (HT) assay for the
analysis of Glutathione Reductase
in cell and tissue extracts.**

**Sufficient reagents for 480 tests
with five 96-well plates.**

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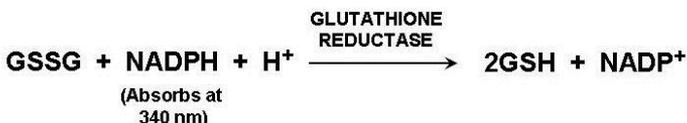
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I. Introduction

Glutathione reductase (GR), a homodimeric flavoprotein disulfide oxidoreductase, plays an indirect but essential role in the prevention of oxidative damage within the cell by helping to maintain appropriate levels of intracellular reduced glutathione (GSH). Reduced glutathione is essential for maintaining the normal structure of red blood cells and for keeping hemoglobin in the ferrous state [1]. Glutathione reductase together with its co-factor, NADPH, catalyzes the reduction of oxidized glutathione (glutathione disulfide, GSSG) to glutathione (**Figure 1**). GSH is also a reactant for glutathione peroxidase, which converts hydrogen peroxide (H₂O₂) into water. Trevigen's Glutathione Reductase Assay Kit is a spectrophotometric assay in which the oxidation of NADPH to NADP⁺ (**Figure 1**) is monitored by the decrease in absorbance at 340 nm. This rate of decrease in absorbance at 340 nm is directly proportional to the glutathione reductase activity in the sample because the enzyme is present at rate limiting concentrations [2-4]. The unit definition for glutathione reductase activity may be expressed in terms of the oxidation of NADPH or the reduction of GSSG since their molar ratio is 1:1. One unit of glutathione reductase oxidizes 1 μmol of NADPH per minute at 25 °C, pH 7.5. The kit provides the user with all the reagents and plates to easily and rapidly assay for glutathione reductase in cell and tissue extracts.

Figure 1. Reduction of glutathione disulfide (GSSG) by glutathione reductase and NADPH



II. Precautions and Limitations

1. For Research Use Only. Not for use in diagnostic procedures.
2. The physical, chemical, and toxicological properties of the chemicals and reagents contained in the GR Assay Kit may not yet have been fully investigated. Therefore, Trevigen recommends the use of gloves, lab coats, and eye protection while using any of these chemical reagents. Trevigen assumes no liability for damage resulting from handling or contact with these products. MSDS are available on request.

III. Materials Supplied

<u>Catalog Number</u>	<u>Component</u>	<u>Amount Provided</u>	<u>Storage Temperature</u>
7513-500-01	GR Standard, 1 unit/ml	1 ml	-20°C
7513-500-02	10X GR Buffer	20 ml	4°C
7510-100-03	NADPH	5 vials	-20°C
7513-500-03	GSSG	3 ml	4°C
7501-500-05	20% Triton® X-100	1 ml	4°C
7512-100-06	96-well plates	5	RT

IV. Materials/Equipment Required But Not Supplied

Reagents:

1. Cells/tissue to be tested
2. PBS (Trevigen # 4870-500-6)
3. Distilled water
4. Protease inhibitors (optional) such as phenylmethylsulfonyl fluoride (PMSF)
5. Reagents to determine protein concentration
6. Ficoll-Hypaque™ (erythrocyte, lymphocyte and monocyte preparations)

Disposables:

1. 1 - 200 µl and 100 - 1000 µl pipette tips
2. 0.5 and 1.5 ml microtubes
3. 15 ml conical (adherent and suspension cell preparation)
4. 50 ml conical (tissue preparation)

Equipment:

1. Micropipettes
2. Multichannel pipettor 1 - 50 µl and 50 µl – 200 µl
3. 96-well plate reader with filters for measuring absorbance at 340 nm (capable of taking readings every minute for ten minutes and exporting data to an Excel spreadsheet)
4. Centrifuge for processing samples

V. Reagent Preparation

1. 1X GR Buffer

Dilute the 10X GR Buffer (Cat# 7513-500-02) to **1X (1:10)** with dH₂O. The **1X GR Buffer** is used to prepare dilutions of GR Standard and to prepare the NADPH reagent. The 10X GR Buffer is used directly to prepare 1X Cell Extraction Buffer and Master Mix.

2. GR Standard (Enzyme)

One unit of glutathione reductase oxidizes 1 µmol of NADPH per minute at 25 °C, pH 7.5. The kit contains 500 µl of GR Standard at a concentration of 1 unit/ml. The enzyme should be diluted appropriately with **1X GR Buffer** just before use. **Note: Diluted enzyme should be used immediately and any remainder discarded. Store the stock GR reagent at -20°C.**

3. 1X Cell Extraction Buffer

Prepare sufficient amount of Cell Extraction Buffer. Preparation for 10 ml is as follows:

10X GR Buffer (Cat# 7501-500-02)	1.0 ml
20% (v/v) Triton® X-100 (Cat# 7501-500-05)	0.2 ml
dH ₂ O	8.8 ml
200 mM PMSF (optional)	(10 µl)

4. NADPH

The kit contains 5 vials of lyophilized NADPH (Cat# 7510-100-03), each sufficient for 100 tests. Add 0.5 ml of 1X GR Buffer to dissolve the contents of the vial. Transfer the solution to a 15 ml conical tube. Wash the vial two more times with 0.5 ml of 1X GR Buffer and transfer to the 15 ml conical tube. Add 1X GR Buffer to the 15 ml conical tube to a final volume of 6.5 ml. Note: Reconstituted NADPH is temperature sensitive; it should be stored on ice and be used within 4 hours after preparation.

5. Master Mix

Prepare sufficient amount of Master Mix (100 µl per well):
Preparation for 100 µl is as follows:

10X GR Buffer (Cat# 7513-500-02)	10 µl
GSSG Reagent (Cat# 7513-500-03)	4 µl
dH ₂ O	86 µl

VI. Preparation of Cell and Tissue Extracts

Choose the appropriate protocol in Section A to Process Sample before proceeding to Section B: Preparation of Cytosolic Extract. Please note that samples should be kept on ice to maintain enzyme activity.

Section A. Processing Samples

Suspension cells:

1. Centrifuge 2 to 6×10^6 suspension cells at $250 \times g$ for 10 minutes at 4°C . Discard the supernatant.
2. Suspend the cell pellet in 1 ml of ice-cold 1X PBS and transfer to a 1.5 ml microtube on ice. Centrifuge, discard supernatant, and place on ice.
3. Proceed to Section B Preparation of Cytosolic Extracts

Adherent cells:

1. Wash 2 to 6×10^6 adherent cells with 1X PBS. Adherent cells may be harvested by gentle trypsinization.
2. Transfer to a 15 ml tube on ice. Centrifuge at $250 \times g$ for 10 minutes at 4°C and discard the supernatant.
3. Suspend the cell pellet in 1 ml of ice-cold 1X PBS and transfer to 1.5 ml microtube on ice. Centrifuge, discard supernatant, and place on ice.
4. Proceed to Section B. Preparation of Cytosolic Extracts.

Erythrocytes:

1. Dilute anticoagulated blood with an equal volume of PBS. Layer over Ficoll-Hypaque™ or similar reagent and centrifuge at $800 \times g$ for 25 min at 12°C with the BRAKE OFF in a swinging bucket rotor.
2. Collect the mononuclear cells (lymphocytes and monocytes) at the interphase and transfer to another tube.
3. Remove the remaining liquid from above the red blood cell pellet. Wash the pellet with 10 cell volumes of PBS.
4. Determine the packed cell volume and add 10 cell volumes of cold dH_2O . Mix well and incubate on ice for 10-15 minutes to lyse the red blood cells. Lysis occurs when the opaque solution changes to a brilliant clear red solution, indicating the release of hemoglobin.
5. Centrifuge at $10,000 \times g$ for 10 minutes at 4°C to remove cell debris. Transfer the supernatant to a fresh tube and store on ice.

Lymphocytes and Monocytes:

1. Dilute anticoagulated blood with an equal volume of PBS. Layer over Ficoll-Hypaque™ or similar reagent and centrifuge at $800 \times g$ for 25 min at 12°C with the BRAKE OFF in a swinging bucket rotor.
2. Collect the mononuclear cells (lymphocytes and monocytes) at the interphase and transfer to another tube.
3. Dilute the blood mononuclear cells with 5 volumes of PBS and centrifuge at $400 \times g$ for 10 minutes at 4°C . Discard the supernatant
4. Suspend the cell pellet in 1 ml of ice-cold 1X PBS and transfer to a prechilled 1.5 ml microtube. Centrifuge, discard supernatant, and place on ice.
5. Proceed to Section B. Preparation of Cytosolic Extracts.

Tissue

1. Remove tissue and place in cold PBS in a 50 ml conical tube. Repeatedly wash the tissue with PBS to remove blood clots and other debris.
2. Transfer the tissue to a Petri dish on ice and mince the tissue to small pieces with surgical scissors.
3. Transfer the tissue pieces to a clean stainless steel sieve. Place the sieve with the tissue pieces in a Petri dish which contains about 20 ml of cold 1X PBS.

4. Create a single cell suspension of the tissue as follows: Using a pestle or a round bottom tube, grind the tissue pieces thoroughly until the bulk of the tissue passes through the sieve.
5. Transfer the PBS containing the single cell suspension to a 50 ml conical tube. Fill with cold PBS and mix by inverting the tube several times. Let the tube stand on ice for 1 minute to allow large aggregates of tissue to settle out of solution.
6. Carefully transfer the supernatant containing the single cell suspension to a clean 50 ml conical centrifuge tube. Centrifuge at 400 x g for 10 minutes at 4°C. Discard the supernatant. Suspend the cell pellet in 1 ml of ice-cold **1X** PBS and transfer to a prechilled 1.5 ml microtube on ice. Centrifuge, discard the supernatant, and place on ice.
7. Proceed to Section B: Preparation of Cytosolic Extracts.

Section B. Preparation of Cytosolic Extracts from Cells and Tissue

1. Measure the approximate volume of the cell pellets prepared above (except for erythrocytes) and suspend the cells in 5-10 volumes of cold 1X Cell Extraction Buffer. Incubate the cell suspensions on ice, with periodic vortexing, for 30 minutes.
2. Microcentrifuge the disrupted cell suspension at 10,000 x g for 10 minutes at 4°C to remove insoluble material. Recover the supernatant to a fresh tube prechilled on ice. Occasionally, the pellet may float and can easily be removed with a pipet tip.
3. Determine the protein concentration of the cleared cell lysate. Note that the 1X GR Buffer contains BSA at a concentration of 0.1 mg/ml and this should be subtracted from your observed protein concentration.
4. If not assaying for GR immediately, snap-freeze the cleared cell extract in 100 µl aliquots by immersing the aliquots in liquid nitrogen and store at -80°C. Avoid repeated freezing and thawing of the extract.

VII. Glutathione Reductase Assay Protocol

A. GR Standard Curve

1. In 0.5 ml or 1.5 ml microtubes, prepare serial dilutions of the GR standard with 1X GR Buffer. Each level of GR is performed in triplicate and requires 50 µl per well for a total of 150 µl. It is recommended that you make at least 200 µl of each serial dilution of GR at the following concentrations: 0.02 units/50 µl, 0.01 units/50 µl, 0.005 units/50 µl, 0.0025 units/50 µl, 0.001 units/50 µl, and 0.0005 units/50 µl.
2. Controls:
Activity control: Triplicate wells without GR. These wells take into account the spontaneous oxidation of NADPH during the reaction. Distribute 50 µl of **1X** GR Buffer into these wells as indicated in **Figure 2**.
3. Add 50 µl of the serial dilutions of the GR standard to each of triplicate wells as indicated in **Figure 2**.

B. Biological Extracts

1. Make serial dilutions of your cell or tissue extracts with 1X GR Buffer between 0.5 µg/50 µl to 50 µg/50 µl protein.
2. Add 50 µl of the extract dilutions to triplicate wells of the 96-well plate as indicated in **Figure 2**. Include triplicate control wells without cell or tissue extract.
3. Add 100 µl of Master Mix to all the wells.
4. The volume in each well is 150 µl:

Diluted cell/tissue extracts, GR standards or 1X GR Buffer:	50 µl
Master Mix:	<u>100 µl</u>
Total volume:	150 µl

C. Initiation of Reactions

1. Initiate the reactions by adding 50 µl of NADPH solution to all the wells using a multichannel pipettor.
2. Immediately transfer the plate to a plate reader and take 10 absorbance readings at 340 nm at every 1 min interval (0-9 min) at room temperature.

Figure 2: Suggested 96 well plate setup.

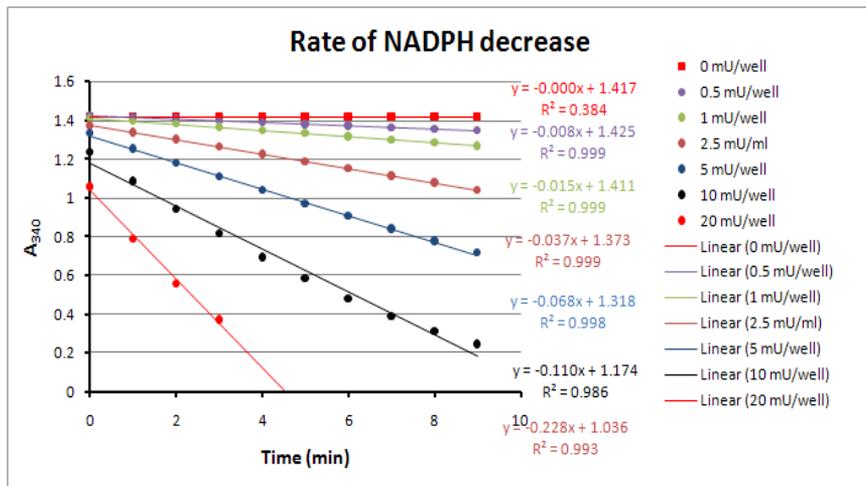
	12	No sample (Buffer)	0.5 µg protein Extract 3	1 µg protein Extract 3	2 µg protein Extract 3	5 µg protein Extract 3	10 µg protein Extract 3	20 µg protein Extract 3	50 µg protein Extract 3
	11	No sample (Buffer)	0.5 µg protein Extract 3	1 µg protein Extract 3	2 µg protein Extract 3	5 µg protein Extract 3	10 µg protein Extract 3	20 µg protein Extract 3	50 µg protein Extract 3
	10	No sample (Buffer)	0.5 µg protein Extract 3	1 µg protein Extract 3	2 µg protein Extract 3	5 µg protein Extract 3	10 µg protein Extract 3	20 µg protein Extract 3	50 µg protein Extract 3
	9	No sample (Buffer)	0.5 µg protein Extract 2	1 µg protein Extract 2	2 µg protein Extract 2	5 µg protein Extract 2	10 µg protein Extract 2	20 µg protein Extract 2	50 µg protein Extract 2
	8	No sample (Buffer)	0.5 µg protein Extract 2	1 µg protein Extract 2	2 µg protein Extract 2	5 µg protein Extract 2	10 µg protein Extract 2	20 µg protein Extract 2	50 µg protein Extract 2
	7	No sample (Buffer)	0.5 µg protein Extract 2	1 µg protein Extract 2	2 µg protein Extract 2	5 µg protein Extract 2	10 µg protein Extract 2	20 µg protein Extract 2	50 µg protein Extract 2
	6	No sample (Buffer)	0.5 µg protein Extract 1	1 µg protein Extract 1	2 µg protein Extract 1	5 µg protein Extract 1	10 µg protein Extract 1	20 µg protein Extract 1	50 µg protein Extract 1
	5	No sample (Buffer)	0.5 µg protein Extract 1	1 µg protein Extract 1	2 µg protein Extract 1	5 µg protein Extract 1	10 µg protein Extract 1	20 µg protein Extract 1	50 µg protein Extract 1
	4	No sample (Buffer)	0.5 µg protein Extract 1	1 µg protein Extract 1	2 µg protein Extract 1	5 µg protein Extract 1	10 µg protein Extract 1	20 µg protein Extract 1	50 µg protein Extract 1
	3	No GR (Buffer)	0.0005 Units GR	0.001 Units GR	0.0025 Units GR	0.005 Units GR	0.01 Units GR	0.02 Units GR	
	2	No GR (Buffer)	0.0005 Units GR	0.001 Units GR	0.0025 Units GR	0.005 Units GR	0.01 Units GR	0.02 Units GR	
	1	No GR (Buffer)	0.0005 Units GR	0.001 Units GR	0.0025 Units GR	0.005 Unit GR	0.01 Units GR	0.02 Units GR	
A		No sample (Buffer)	0.5 µg protein Extract 1	1 µg protein Extract 1	2 µg protein Extract 1	5 µg protein Extract 1	10 µg protein Extract 1	20 µg protein Extract 1	50 µg protein Extract 1
B		No sample (Buffer)	0.5 µg protein Extract 2	1 µg protein Extract 2	2 µg protein Extract 2	5 µg protein Extract 2	10 µg protein Extract 2	20 µg protein Extract 2	50 µg protein Extract 2
C		No sample (Buffer)	0.5 µg protein Extract 3	1 µg protein Extract 3	2 µg protein Extract 3	5 µg protein Extract 3	10 µg protein Extract 3	20 µg protein Extract 3	50 µg protein Extract 3
D		No sample (Buffer)	0.5 µg protein Extract 3	1 µg protein Extract 3	2 µg protein Extract 3	5 µg protein Extract 3	10 µg protein Extract 3	20 µg protein Extract 3	50 µg protein Extract 3
E		No sample (Buffer)	0.5 µg protein Extract 3	1 µg protein Extract 3	2 µg protein Extract 3	5 µg protein Extract 3	10 µg protein Extract 3	20 µg protein Extract 3	50 µg protein Extract 3
F		No sample (Buffer)	0.5 µg protein Extract 3	1 µg protein Extract 3	2 µg protein Extract 3	5 µg protein Extract 3	10 µg protein Extract 3	20 µg protein Extract 3	50 µg protein Extract 3
G		No sample (Buffer)	0.5 µg protein Extract 3	1 µg protein Extract 3	2 µg protein Extract 3	5 µg protein Extract 3	10 µg protein Extract 3	20 µg protein Extract 3	50 µg protein Extract 3
H		No sample (Buffer)	0.5 µg protein Extract 3	1 µg protein Extract 3	2 µg protein Extract 3	5 µg protein Extract 3	10 µg protein Extract 3	20 µg protein Extract 3	50 µg protein Extract 3

VIII. Data Interpretation.

A. Time Course of the Change in Absorbance at 340 nm of the GR Standard

- Plot the mean of the triplicate absorbance values at 340 nm versus time of the GR standard (**Figure 3**). For your convenience, a calculation worksheet (MS Excel required) is provided at http://www.trevigen.com/docs/1427816818.7513-500-k_calculation_worksheet.xls

Figure 3: Change in absorbance at 340 nm with time for selected concentrations of the GR standard.



B. Determination of GR Activity in Experimental Samples from the GR Standard Curve.

For your convenience, a calculation worksheet (MS Excel required) is provided at http://www.trevigen.com/docs/1427816818.7513-500-k_calculation_worksheet.xls

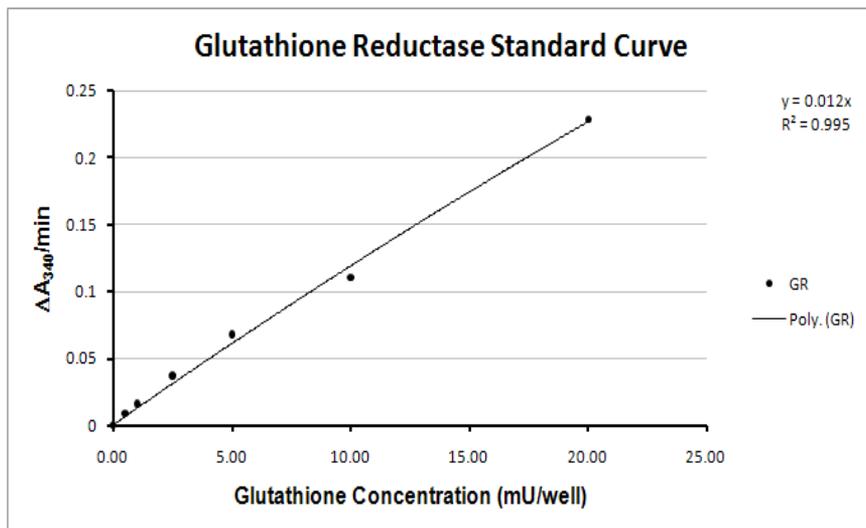
- Determine the rate of decrease in absorbance at 340 nm per minute ($\Delta A_{340} \text{ nm/min}$) for each of the standards and the blank (Buffer control):

$$\frac{(A_{340} \text{ nm @ 1 min.}) - (A_{340} \text{ nm @ 10 min.})}{9 \text{ min}} = \Delta A_{340} \text{ nm / min}$$

Note: Our plate reader takes absorbance readings beginning at the 1 minute time point and ends at 10 minutes.

- Calculate the net rate for each standard by subtracting the rate obtained for the blank (the blank measures the spontaneous oxidation of NADPH. This value is usually small and may be negligible compared to sample values).
- Plot the number of units of GR/well versus these new $\Delta A_{340} \text{ nm/ min}$ values (**Figure 4**):

Figure 4: Graph of the rate of decrease in absorbance at 340 nm versus the units of GR in each well.



4. Plot the absorbance values for your samples versus time as shown in **Figure 3**. **Take the slope of the linear portion of the curves.**
 5. Calculate the net rate for each level of your experimental samples by subtracting the rate obtained for the blank (the blank measures the spontaneous oxidation of NADPH. This value is usually small and may be negligible compared to sample values).
 6. Determine the number of units/well of GR in your samples from the linear portion of the GR standard curve of **Figure 4**.
- C. Determination of GR Activity in Experimental Samples from the Molar Extinction Coefficient of NADPH**

The GR activity in the sample(s) may be calculated by using the extinction coefficient of NADPH:

1. Determine the rate of decrease in absorbance per minute for both samples and blanks as described above.
2. Calculate the net rate of your samples by subtracting the blank rate from the sample rate.
3. Using Beer-Lambert Law one can determine the concentration of NADPH in solution. Convert the net rate ($\Delta A_{340} \text{ nm}/\text{min}$) to concentration of NADPH consumed, which is equal to the activity of GR in mU/ ml. The molar extinction coefficient (E^M) for NADPH is $6220 \text{ M}^{-1}\text{cm}^{-1}$ and $E^M = 6.22 \times 10^{-3} \text{ nmol/mL}$ if the pathlength is 1 cm. One unit of glutathione reductase is defined as the amount of enzyme required to catalyze the reduction of one μmole of GSSG per minute at pH 7.5 and 25°C . One molecule of NADPH is oxidized per molecule of GSSG reduced. Therefore, the oxidation of NADPH (measured by loss of $A_{340} \text{ nm}$) directly correlates with GSSG reduction.

$$\begin{aligned}
 1 \text{ U of GR} &= 1 \mu\text{mol GSSG reduced/min} \\
 &= 1 \mu\text{mol NADPH oxidized/min} \\
 1 \text{ mU of GR} &= 1 \times 10^{-3} \mu\text{mol NADPH oxidized/min} \\
 1 \text{ mU of GR} &= 1 \text{ nmol NADPH oxidized/min}
 \end{aligned}$$

If E^M is the molar extinction coefficient for NADPH at 340 nm,

$$\begin{aligned}
 E^M &= 6220 \text{ M}^{-1}\text{cm}^{-1} \\
 &= 6220 \times 10^{-6} \mu\text{M}^{-1}\text{cm}^{-1} \\
 &= 6.22 \times 10^{-3} \mu\text{M}^{-1}\text{cm}^{-1} \\
 &= 6.22 \times 10^{-3} \text{ L}/\mu\text{mol}/\text{cm} \\
 &= 6.22 \times 10^{-3} \text{ ml}/\text{nmol}/\text{cm}
 \end{aligned}$$

Note: The path length for 200 μl of reaction volume in the 96 well plate is 0.6 cm

$$\begin{aligned}
 \text{Therefore, } E^M &= 6.22 \times 10^{-3} \text{ ml}/\text{nmol}/\text{cm} \times 0.6 \text{ cm} \\
 &= 3.732 \times 10^{-3} \text{ ml}/\text{nmole}
 \end{aligned}$$

$$\begin{aligned}
 \Delta A_{340} \text{ nm}/\text{min} &= \frac{\Delta A_{340} \text{ nm}/\text{min}}{3.732 \times 10^{-3} \text{ ml}/\text{nmole NADPH}} \\
 &= Y \text{ nmole NADPH}/\text{min}/\text{ml} \\
 &= Y \text{ mU}/\text{ml GR}
 \end{aligned}$$

- Correct for the sample dilution in the assay and for the sample dilution performed prior to the assay. For example: If the sample volume was 50 μL and was diluted 1/50 prior to the assay:

$$\begin{aligned}
 \text{Mean } \Delta A_{340} \text{ nm} / \text{min (Sample)} &= 0.0325/\text{min} \\
 \text{Mean } \Delta A_{340} \text{ nm} / \text{min (Blank)} &= 0.0005/ \text{min} \\
 \text{Net Rate, } \Delta A_{340} \text{ nm}/\text{min} &= 0.0320/ \text{min} \\
 \text{Glutathione Reductase Activity} &= 0.0320/\text{min}/3.732 \times 10^{-3} \text{ ml}/\text{nmol NADPH}/\text{min} \\
 &= 8.57 \text{ mU}/\text{mL}
 \end{aligned}$$

$$\begin{aligned}
 \text{Assay Dilution Correction} &= 200 \mu\text{l}/50 \mu\text{l} \times 8.57 \text{ mU}/\text{ml} \\
 &= 34.30 \text{ mU}/\text{ml}
 \end{aligned}$$

$$\begin{aligned}
 \text{Sample Dilution Correction} &= 50 \times 34.30 \text{ mU}/\text{ml} \\
 &= 1715 \text{ mU}/\text{ml}
 \end{aligned}$$

- Divide the GR activity (mU/ml) by the protein concentration to determine the specific activity of GR in your sample (mU GR/mg protein).

IX. References

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X. Troubleshooting

PROBLEM	CAUSE	SOLUTION
No change in absorbance at 340 nm with time in all the wells	Failure to add GSSG to the Master Mix	Add the GSSG reagent to the Master Mix
Absorbance at 340 nm in wells with 1X GR Buffer alone is less than 0.5	Failure to add NADPH to the wells	Follow protocol for making the NADPH reagent and add 50 µl to each well
	NADPH has degraded	Contact Customer Service
Change in absorbance with the GR standard is satisfactory, but no apparent change in absorbance observed in your sample	GR activity in cells and tissues very low	Extend reaction to 20 minutes
		Reduce the amount of dH ₂ O in the Master Mix. Add 50 µl of this modified Master Mix and 100 µl of your extract to each well
Change in absorbance with the GR standard is satisfactory, but absorbance with sample drops to baseline within one or two minutes	GR activity in cells and tissues very high	Increase the dilution of the sample in order to reduce the amount of sample added to the wells

XI. Related Products Available from Trevigen

<u>Catalog #</u>	<u>Description</u>	<u>Size</u>
4870-500-6	10X PBS, pH 7.4	6 X 500 ml
7501-500-K	HT Superoxide Dismutase Assay Kit	480 tests
7511-500-K	HT Glutathione Assay Kit	384 tests
7512-100-K	HT Glutathione Peroxidase Assay Kit	480 tests

The product accompanying this document is intended for research use only and is not intended for diagnostic purposes or for use in humans.

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