TREVIGEN® Instructions

For Research Use Only. Not For Use In Diagnostic Procedures

Glutathione Reductase Assay Kit

Reagent kit for the analysis of Glutathione Reductase in cell extracts and tissue homogenates

Sufficient reagents for 100 Assays

Catalog# 7510-100-K

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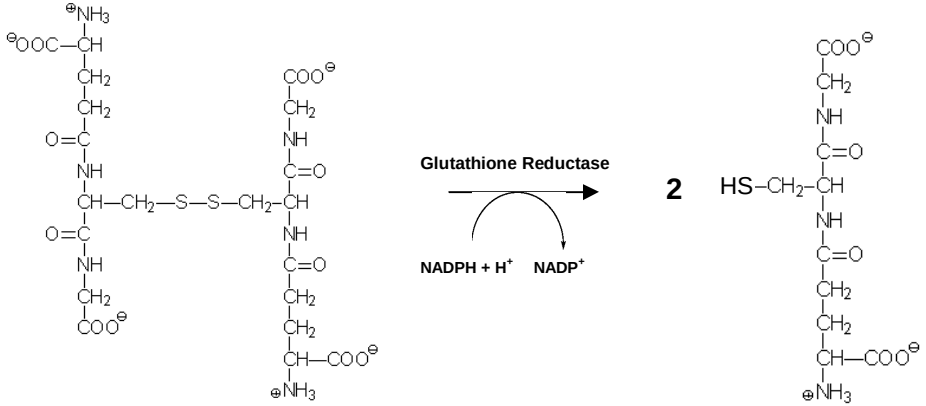
I. Background

Glutathione reductase, 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 glutathione (GSH). Glutathione reductase together with its co-factor, NADPH, catalyzes the reduction of oxidized glutathione (glutathione disulfide, GSSG) to glutathione (**Figure 1**). Glutathione is a water phase sulfhydryl antioxidant and antitoxin. GSH is responsible for converting hydrogen peroxide (H_2O_2) into water within erythrocytes (with glutathione peroxidase acting as the catalyst).

The method employed in Trevigen's Glutathione Reductase Assay Kit is a spectrophotometric assay, wherein the oxidation of NADPH to $NADP^+$ (Figure 1) is monitored as a decrease in absorbance at 340 nm. This rate of decrease in A_{340} is directly proportional to the glutathione reductase activity in the sample because the enzyme is present at rate limiting concentrations. 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. Glutathione reductase activity is slightly inhibited by ammonium sulfate at concentrations greater than 100 mM, and is unaffected by 0.5 M urea, 0.1 M KCl,



Glutathione disulfide (GSSG) **Glutathione (GSH)**

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 provided products may not yet have been fully investigated, therefore, Trevigen recommends the use of gloves, lab coats, and eye protection while using 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

Component	Quantity	Storage	Catalog#
Glutathione Reductase*	500 µl	4 °C	7510-100-01
25X Reaction Buffer	20 ml	4 °C	7510-100-02
NADPH**	10 tubes	-20 °C	7510-100-03
GSSG Solution	6 ml	4 °C	7510-100-04
25X Sample Dilution Buffer	20 ml	4 °C	7510-100-05
25X Tissue Homogenization Buffer	40 ml	4 °C	7510-100-06

***** 0.5 U/ml in 3.6 M $(NH_4)_2SO_4$, 50 mM potassium phosphate, pH 7.5
****** sufficient amount of reagent for 10 assays and one 5 point standard curve (each point in duplicate) per tube using 1 ml assay volumes.

IV. Reagents/Equipment Required but not Supplied

Equipment

1. Spectrophotometer to read absorbance at 340 nm, preferably with a temperature-regulated cuvette holder set at 25°C.
2. Quartz cuvettes with 1 cm path length and at least a 1 ml volume
3. Pipettor
4. Pipette tips
5. Pasteur pipettes and bulb
6. Centrifuge (for cell lysis)
7. Dounce Homogenizer
8. Timer
9. Vortex

Reagents

1. Deionized H_2O (dH_2O)
2. 1 X PBS

V. Reagent Preparation

1. NADPH: Add 525 µL dH_2O to one vial of NADPH and vortex to dissolve. May be kept for up to 24 hours at 0°C - 4°C protected from light.

2. 1 X Tissue Homogenization Buffer: Prepare necessary volume from the 25 X stock solution as needed with cold deionized water.

VI. Sample Preparation

Note: The final concentration of protein (from the clarified lysate/ homogenate) in each sample should be adjusted to 0.1 - 0.75 mg/ mL initially. Samples may be further diluted with 1 X Sample Dilution Buffer if necessary.

A. Cell Lysate Preparation

1. Detach adherent cells using a rubber policeman. Centrifuge at 250 x g for 10 minutes at 4°C. Wash the cells once with cold 1 X PBS.

2. Suspend the cell pellet in cold 1 X Tissue Homogenization Buffer. Homogenize the suspension using a Dounce homogenizer or a sonicator.

3. Centrifuge at 8,500 x g for 10 minutes at 4°C. Adjust protein concentration as needed (see note pg. 2). Transfer the supernatant to a clean tube and place on ice (if performing the assay the same day), or store at -80°C for future use.

B. Erythrocyte Lysate Preparation

1. Centrifuge blood at 2,500 x g for 5 minutes at 4°C. (discard supernatant)

2. Wash the red blood cell pellet twice with 1 X PBS.

3. Lyse the erythrocyte pellet in 4 pellet volumes of cold deionized H_2O. Mix well by pipetting.

4. Centrifuge the suspension at 8,500 x g for 10 minutes at 4°C. Adjust protein concentration as needed (see note pg. 2). Transfer the supernatant to a clean tube and place on ice (if performing the assay the same day) or store at -80°C for future use.

C. Tissue Homogenate Preparation*

1. Wash tissue 3 times with 1X PBS and then homogenize tissue in 5-10 mL of 1X Tissue Homogenization Buffer per gram of tissue.

2. Centrifuge the suspension at 8,500 x g for 10 minutes at 4°C. Adjust protein concentration as needed (see note pg. 2). Transfer the supernatant to a clean tube and place on ice (if performing the assay the same day), or store at -80°C for future use.

VII. Assay Protocol

The assay is performed at room temperature and in duplicate. Keep cell lysates, tissue homogenates, and the glutathione reductase solution on ice; all other reagents should be brought to room temperature before use.
Note: immediately prior to use, briefly vortex each reagent. Perform each reaction seperately.

A. Blank
Include a blank to account for the endogenous oxidation of NADPH over the period of the reaction.

1. Add the following components in order to a quartz cuvette:

	Blank
dH_2O	930 µL
25X Assay Buffer	40 µL
GSSG Solution	20 µL

2. Mix thoroughly by repeated pipetting with a clean Pasteur pipette.

3. Place each cuvette individually into a spectrophotometer set at 340 nm and 25°C. Adjust the absorbance reading to zero.

4. Add 10 µL of the NADPH solution prepared in Section V to the cuvette, mix thoroughly by repeated pipetting with a clean Pasteur pipette, and start a timer or a stopwatch. Record the absorbance every 60 seconds for 5 minutes.

B. Sample
1. Add the following components in order to a quartz cuvette:

	Sample
dH2O	930 µL - X µL
25X Assay Buffer	40 µL
Sample	X µL
GSSG Solution	20 µL

2. Mix thoroughly by repeated pipetting with a clean Pasteur pipette.

3. Place each cuvette individually into a spectrophotometer set at 340 nm and 25°C. Adjust the absorbance reading to zero.

4. Add 10 µL of the NADPH solution prepared in Section V to the cuvette, mix thoroughly by repeated pipetting with a clean Pasteur pipette, and start a timer or a stopwatch. Record the absorbance every 60 seconds for 5 minutes.

C. Standard Curve
The kit contains sufficient Glutathione Reductase to generate ten 5-point standard curves with each point done in duplicate. The Glutathione Reductase has an activity of 0.5 U/mL (1 unit will cause the oxidation of 1 µmole of NADPH in one minute at 25°C at pH 7.5). The concentrations of Glutathione Reductase (GR) to be used are: 1, 3, 5, 7, and 10 mU/mL.

1. In the table below are the reagent volumes required for each of the five standards. Follow the procedure for preparing these solutions starting with step C-2 below:

	1	2	3	4	5
dH_2O	928 µL	924 µL	920 µL	916 µL	910 µL
25X Assay Buffer	40 µL	40 µL	40 µL	40 µL	40 µL
GSSG Solution	20 µL	20 µL	20 µL	20 µL	20 µL
GR solution	2 µL	6 µL	10 µL	14 µL	20 µL
NADPH Solution	10 µL	10 µL	10 µL	10 µL	10 µL
GR concentration (mU/mL)	1	3	5	7	10

2. To a quartz cuvette add the required amounts of dH_2O, 25X Assay buffer, and GSSG solution in that order.

3. Mix thoroughly by repeated pipetting with a clean Pasteur pipette.

4. Add the required amount of glutathione reductase solution according to the Table in step C-1 and mix thoroughly by repeated pipetting with a clean Pasteur pipette.

5. Place the cuvette into a spectrophotometer set at 340 nm and adjust the absorbance reading to zero.

6. Add 10 µL of the NADPH solution prepared in Section V to the cuvette, mix thoroughly by repeated pipetting with a clean Pasteur pipette, and start a timer or a stopwatch.

7. Record the absorbance every 60 seconds for 5 minutes.

D. Standard Curve Calculations

1. Determine the rate of decrease in absorbance @ 340nm per minute (ΔA_{340} /min) for each of the standards and the blank:

$$\frac{(A_{340} \text{ @ 1 min.}) - (A_{340} \text{ @ 5 min.})}{4 \text{ min}} = \Delta A_{340}/\text{min}$$

2. Determine the average of the duplicate ΔA_{340} /min values for each standard and blank.

3. Calculate the net rate for each standard by subtracting the rate obtained for the blank.

4. Plot GR concentration versus these new ΔA_{340}/ min. values.

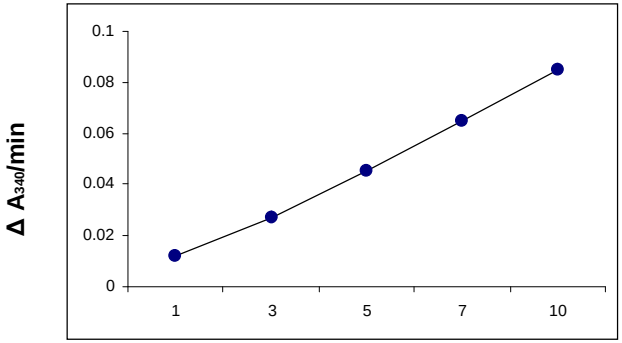


Figure 2. Plot of glutathione reductase concentration vs. the rate of decrease in absorbance at 340 nm due to the oxidation of NADPH by GR.

VIII. Glutathione Reductase Activity Determination

The GR activity in the sample(s) may be calculated by one of two methods: (1) substituting the net ΔA_{340}/min value obtained for the sample into the equation generated by the standard curve; or (2) using the extinction coefficient of NADPH. The procedure for using the extinction coefficient of NADPH is as follows:

1. Determine the rate of decrease in absorbance per minute (ΔA_{340}/min) for both samples and blanks.

2. Calculate the net rate by subtracting the blank rate from the sample rate.

3. Convert the net rate (ΔA_{340}/min) to concentration of NADPH consumed, which is equal to the activity of Glutathione Reductase in mU/mL. The molar extinction coefficient (E^M) for NADPH is 6220 $M^{-1}cm^{-1}$ and $E^M = 6.22 \times 10^{-3}$ 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 micromole of GSSG per minute at pH 7.5 and 25°C. One molecule of NADPH is consumed per molecule of GSSG reduced. Therefore, the reduction of NADPH (measured by loss of A_{340}) directly correlates with GSSG reduction.

   1 U of Glutathione Reductase = 1 µmol GSSG/min =1 µmol NADPH/min
   or 1 mU of Glutathione Reductase = 1 nmole NADPH consumed/min

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

$$E^M = 6220\ M^{-1}cm^{-1} = 6.22 \times 10^{-3} \mu M^{-1}cm^{-1} = 6.22 \times 10^{-3}\ L/\mu mol/cm$$
$$= 6.22 \times 10^{-3}\ mL/nmol \text{ if the pathlength is 1 cm}$$

$$\frac{\Delta A_{340}/\text{ min}}{(6.22 \times 10^{-3}\ \mu M^{-1}cm^{-1})} = \frac{\Delta A_{340}/\text{ min}}{6.22 \times 10^{-3}\ mL/nmol} = \text{x nmol NADPH/ mL}$$

   = x mU/mL Glutathione Reductase

4. 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 200 µL and was diluted 1/50 prior to the assay,

Mean Sample Rate, ΔA_{340}/min	= 0.0325 ΔA_{340}/min
Mean Blank Rate, ΔA_{340}/min	= 0.0005 ΔA_{340}/min
Net Rate, ΔA_{340}/min	= 0.0320 ΔA_{340}/min (0.0325 - 0.0005)
Glutathione Reductase Activity	= 0.0320/6.22 x 10^{-3} = 5.14 mU/mL
Assay Dilution Correction	= 1000 µL/200 µL x 5.14 = 25.7 mU/ml
Sample Dilution Correction	= 50 x 25.7 = 1285 mU/ml

IX. References

1. Tietze, F., Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Anal. Biochem., **27**, 502-522 (1969).
2. Carlberg, I., Mannervik, B., Glutathione reductase. Meth. Enzymol., **113**, 485-490 (1985).
3. Smith IK, Vierheller TL, Thorne CA, Assay of glutathione reductase in crude tissue homogenates using 5,5'-dithiobis(2-nitrobenzoic acid). Anal. Biochem., **175**, 408-413 (1988).
4. Dolphin, D., Poulson, R., and Avramovic, O. (Eds.), Glutathione: Chemical, Biochemical, and Metabolic Aspects, Volumes A and B, Wiley and Sons (1989).
5. Dringen R, Gutterer JM. Glutathione reductase from bovine brain. Methods Enzymol. **348**, 281-288 (2002).

X. Related Products Available From Trevigen

Contact Trevigen for details of our unique product line for studying DNA damage and repair. All of Trevigen's kits include highly qualified enzymes, substrates, buffers, full instructions for use, and a synopsis specific for your kit.

PARP Assay Kits:

Catalog #	Description	Size
4690-096-K	**HT F Homogeneous PARP Inhibition Assay kit**	96 tests
4677-096-K	**HT Universal Colorimetric PARP Assay w/ Histone Coated Strip Wells**	96 samples
4676-096-K	**Universal Chemiluminescent PARP Assay w/Histone Coated Strip Wells**	96 samples
4667-250-01	**Recombinant Human PARP Enzyme**	250 µl
4668-100-1	**Recombinant Human PARP (High Specific Activity)**	1000 Units

DNA Damage Antibodies:

Catalog #	Description	Size
4350-MC-100	UVssDNA	100 µg
4335-MC-100	Anti-PAR polymer mAb (10HA)	100 µl
4336-BPC-100	Anti- PAR polymer polyclonal	100 µl
4338-MC-50	Anti-human/murine-PARP mAb (clone C2-10)	50 µg
4354-MC-050	Anti-8-oxo-dG (clone 2E2)	50 µl
4360-MC-100	Anti-BPDE (clone 8E11)	100 µg
4365-MC-100	Anti-BPDE DNA (clone 5D11)	100 µg
4410-PC-100	Anti-FEN-1 polyclonal	100 µl
4411-PC-100	Anti-γ-H2AX polyclonal	100 µl
4415-PC-100	Anti-APE/Ref-1 (clone 13B8E5C2)	100 µl
4420-PC-100	Anti-Fpg polyclonal	100 µl
4421-MC-100	Anti-XRCC1 (clone 144)	100 µg
4430-MC-100	Anti-XPF (clone CD12)	100 µg
4445-MC-100	Anti-Polymerase β (clone 61)	100 µg
4451-MC-200	Anti-Rad1 (clone 33)	200 µg

Glutathione Kits

Catalog #	Description	Size
7511-100-K	**HT Glutathione Assay Kit**	384 tests
7512-100-K	**HT Glutathione Peroxidase Assay Kit**	480 tests
7513-500-K	**HT Glutathione Reductase Assay Kit**	500 tests

CometAssay™ Kits:

Catalog #	Description	Size
4250-050-K	**CometAssay™ Kit**	**50 samples**
4251-050-K	**CometAssay™ Silver Staining Kit**	**50 samples**
4252-040-K	**CometAssay™ Higher Throughput Kit**	**40 samples**
4253-096-K	**CometAssay™ Kit 96 Wells**	**96 samples**

FLARE™ Assay Kits:

Catalog #	Description	Damage Recognized	Size
4040-100-FK	Fpg Kit	**8-oxoguanine, DNA containing formamidopyrimidine moieties**	75 samples
4040-100-FM			100 samples
4045-01K-FK	Endonuclease III Kit	**Thymine Glycol, 5,6-dihydro-thymine, urea, 5-hydroxy-6-hydrothymine, 5,6-dihydro-uracil, alloxan, 5-hydroxy-6-hydrouracil, uracil glycol, 5-hydroxy-5-methylhydantoin, 5-hydroxycytosine,5-hydroxy-uracil, methyl-tartonylurea, thymine ring saturated or fragmentation product**	75 samples
4045-01K-FM			100 samples
4130-100-FK	hOGG1 Kit	**8-oxoguanine, DNA containing formamidopyrimidine moieties**	75 samples
4130-100-FM			100 samples
4055-100-FK	T4-PDG Kit	**Cis-syn isomers of cyclo-butane pyrimidine dimers**	75 samples
4055-100-FM			100 samples
4065-100-FK	cv-PDG Kit	**Cis-syn and trans-syn isomers of cyclobutane pyrimidine dimers**	75 samples
4065-100-FM			100 samples
4100-100-FK	UVDE Kit	**Cyclobutane pyrimidine dimers, (6-4) photoproducts**	75 samples
4100-100-FM			100 samples

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