GLUTATHIONE REDUCTASE ASSAY KIT

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ZMC Catalog #: 0805004

FOR RESEARCH USE ONLY. Not for *in vitro* Diagnostic Use

DESCRIPTION AND INTENDED USE

Glutathione reductase (GR, EC# 1.6.4.2) is a flavoprotein that is required for the conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH). At the same time, it oxidizes nicotinamide adenine dinucleotide phosphate (NADPH). This universally present enzyme is essential for the maintenance of reduced glutathione (GSH) levels *in vivo*[1]. Reduced glutathione plays an important role in oxidoreduction processes and detoxification of H_2O_2 and organic peroxides, which are substances produced in large quantities during inflammatory processes in living cells[2]. Glutathione reductase therefore plays a major role in glutathione peroxidase (GPx) and glutathione s-transferase (GST) reactions as an adjunct in the control of peroxides and free radicals[3]. When levels of catalase, another universally present antioxidant enzyme, are decreased, the glutathione dependant enzymes become activated[4]. A deficiency of glutathione reductase is characterized by hemolysis owing to the increased sensitivity of erythrocyte (RBC) membranes to H_2O_2 that lead to osmotic fragility[5]. This reaction is thus required for the stability and integrity of red cells.

The "Glutathione Reductase Assay Kit" provides an indirect and highly reproducible method of quantifying the activity of total cellular glutathione reductase. The activity of the enzyme is an important measure of the antioxidant status of the cell.

Oxidative stress has been implicated in aging and in the pathogenesis of a number of disorders. The extent of injury is generally related to an increase, or decrease of one or more free radical scavenging enzymes[2]. High levels of glutathione reductase have been found in erythrocytes from patients with rheumatoid arthritis [6].

*** The Glutathione Reductase Assay Kit is for Research Purposes Only.**

PRINCIPLE OF THE PROCEDURE

Glutathione reductase catalyzes the NADPH-dependent reduction of glutathione disulfide (GSSG) to glutathione (GSH).

The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340nm (A_{340}), thus providing a spectrophotometric means of detection which is directly proportional to the GR activity in the sample. The reaction is thus measured by the decrease in absorbance at 340 nm using the extinction coefficient 6220 M^{-1} cm^{-1} for NADPH. One unit of NADPH causes the oxidation of 1.0 µmol NADPH at 25°C at pH 7.0. The Zeptometrix Glutathione Reductase Kit can be used to measure GR activity in plasma, erythrocyte lysates, tissue homogenates, and yeast cell lysates. Preferred sample is diluted RBC hemolysate.

The Kit provides reagents sufficient for 100 (1ml) manual tests. If using an automated system such as the Cobas Mira or Fara, considerably more tests can be run.

PRECAUTIONS

- Please read all instructions carefully prior to performing the assay.
- To avoid cross contamination, use separate pipet tips for each sample.
- Universal safety precautions while working with bio-hazardous materials should be adopted.
- Wear gloves, lab coats and safety glasses at all times.
- All contaminated materials and biohazardous materials should be properly disposed and work surfaces appropriately decontaminated.
- The guidelines provided in this insert are intended to assist the researcher with performing the assay.

REAGENTS

Materials Supplied:

- **GR Assay Buffer (120 ml/kit):** contains potassium phosphate, EDTA.
- **GR Reagent 1 (4 vials/kit):** contains oxidized glutathione
- **GR Reagent 2 (4 vials/kit):** contains β-NADPH
- **GR Reagent 3 (25 ml/kit):** contains Tris/HCl
- **QC Material (1 vial/kit):** contains human source material. **Use Universal Precautions.**

Handling and Storage:

Store the assay buffer at 2-4 ºC. NADPH and oxidized glutathione should be stored at -70°C. The components of the Kit are stable for 1 year when stored properly.

Materials/Equipment/Procedures Required But Not Supplied

- UV/Vis spectrophotometer with a kinetic program. Should be preferably equipped with a temperature controlled cuvette chamber.
- Quartz cuvettes with a 1 cm path length.
- Adjustable pipettors with disposable pipet tips.
- Serological pipets.
- Beaker or flask to make the working solution.
- Deionized distilled water (sterile).
- Hemoglobin measurement for red blood cell hemolysate.
- Protein measurements for clarified homogenates from tissues.

PREPARATION OF REAGENTS AND EQUIPMENT

1. Add 2 ml deionized distilled water to one vial of **GR Reagent 1** (GSSG). Mix well for complete recovery.

2. Add 4 ml **GR Reagent 3** to one vial of **GR Reagent 2** (NADPH). Mix well for complete recovery.

3. **Working Solution:** Aseptically transfer 25 ml GR Assay Buffer to a beaker. Add 2.0 ml of **Reagent 1** and 2.0 ml **Reagent 2** to the GR assay buffer beaker and mix well. Rinse the vial of **Reagent 1** with the working solution to be assured of complete recovery of GSSG. This volume is sufficient for 25 manual tests.

4. **QC Material:** Add 0.5 ml of deionized water to the vial. Allow to sit for 5 min, then vortex gently until completely resuspended. Keep on ice.

5. If samples are frozen, remove them from the freezer and allow to thaw to room temperature (RT). Vortex samples once they are at RT. Fresh samples may be kept at 4ºC 4-6 hrs.

6. Turn on spectrophotometer and allow to warm up for at least 15 minutes. Set kinetic parameters as follows: wavelength 340 nm, lag time 40 sec, rate time 60 sec, total measurement time 100 sec, and read intervals every 15-30 sec. The assay is to be run at 25ºC.

Notes:

- Samples should be run in duplicate.
- Reactions are very sensitive to temperature changes.
- Blanks (DI water replaces sample) should be run.
- For samples suspended in medium, a reagent blank should be run.
- Sodium azide inhibits the reaction.
- Thawed and resuspended QC Material cannot be refrozen. Recommend each lab have their own plasma they can run each time.

SAMPLE PREPARATION

PLASMA

1. Collect blood using EDTA, heparin or citrate as the anticoagulant.
2. Centrifuge at 3000 rpm for 10 min at 4ºC.
3. Remove plasma from the cells by drawing it off from the top.
4. Freeze at -70º C for up to 6 months if not used immediately.
5. Thaw out samples before analysis. Vortex well to mix.

RED BLOOD CELLS:

1. Collect blood using EDTA, heparin or citrate as the anticoagulant.
2. Collect the RBCs by centrifugation at 3000 rpm for 10 min at 4ºC.
3. Discard the plasma and buffy coat (the white interface between the pelleted RBCs and the plasma) by removing from the top. Optional: aliquot and save the plasma.
4. Wash RBC pellet once with cold saline at 4ºC filled to the top of the tube. Invert several times, then centrifuge at 3000 rpm for 10 min at 4ºC. Discard clear saline and any remaining buffy coat from the top. Repeat once.

5. Lyse the RBCs by adding cold deionized water (1:1) to the packed cells. Allow to mix 10 min.
6. Perform a hemoglobin measurement of this 1:2 diluted RBC hemolysate. Commercial standards and Drabkins reagent (Sigma or Randox) provide a simple spectrophotometric method of measuring hemoglobin in g/dl. Convert units to g/L.
7. Aliquot and freeze at -70ºC for up to 6 months if not used immediately.
8. Thaw out frozen samples before analysis. Vortex well.
9. Dilute a small aliquot of the red blood cell hemolysate to 7 g/L with deionized water.
    - Mix well.
    - Leave diluted samples at RT for at least 10 min before analysis.
    - Diluted hemolysate samples are stable for 4-6 hrs at 4ºC.

TISSUES

1. Homogenize or sonicate tissue samples that have been flash-frozen in liquid nitrogen in 4-6 volumes (per wet weight of tissues) of cold Glutathione Reductase Assay Buffer and 1 mM β-mercaptoethanol.
2. Centrifuge mixture for 10 -15 min at 8000 rpm at 4ºC.
3. Draw off supernatant from the top of the tube for the assay.
4. Freeze samples at -70ºC for up to 6 months if not used directly.
5. Determine protein concentration of the supernatant.

YEAST CELLS (Maximum sample: 10^9 cells)

1. Pellet yeast cells (10^9 cells) by centrifugation at 2500 rpm for 10 min in 13 X 100 mm glass test tubes.
2. Resuspend cell pellet in 1.25 ml cold extraction buffer (20mM Tris pH 8.0).
3. Add 0.48 g glass beads (0.22 mm).
4. Vortex samples for 5 min.
5. Centrifuge samples at 2500 rpm for 10 min.
6. Aliquot supernatants to Eppendorf tubes.
7. If not used directly, quick freeze samples in either liquid nitrogen or ethanol-dry ice bath and store at -70° C for up to 6 months until analysis.
8. Thaw out samples before use. Vortex well.

ASSAY PROCEDURE

1. Turn on spectrophotometer and set to parameters described above.

2. Zero at 340 nm with deionized water.

3. Pipette the following into the sample cuvette:
    a. 935 µl Working Solution
    b. 35 µl sample

4. Place parafilm on top and gently invert the cuvette several times to mix. Avoid bubbling.

5. Place cuvette in the correct position in the spectrophotometer.

6. Record the change in A_{340} for 100 sec at 15-30 sec intervals. Most spectrophotometers allowing kinetic parameters will show the reaction as it proceeds. For manual plotting of absorbance points, user should have at least 3 points. For automated runs, such as with the Cobas Mira or Fara, the user may view all raw data and their plots.

CALCULATIONS

1. The net rate of decrease in A_{340} for the sample can be calculated by subtracting the rate observed for a blank (where water is used instead of sample).

2. The net A_{340}/min for the test sample can be converted to NADPH consumed using the following relationship:

    1 unit of Glutathione Reductase will cause the formation of 1 µmol $NADP^+$ from NADPH per min at pH 7.0 at 25º C.

    Extinction coefficient for NADPH is 0.00622 μM^{-1} cm^{-1} at 340 nm.

3. Activity of GR can be expressed as International Unit/Liter (U/L) of the sample or in terms of the protein or hemoglobin content.

4. A theoretical unique factor is determined to convert change in absorbance (ΔA) to corresponding units of enzyme activity. This factor can be calculated using the following equation:

    U/L = ΔA/minute X F; where F= factor

    F = (TV/SV) x 10^3/ 6.22 where

    TV = Total Volume in ml
    SV = Sample Volume in ml
    10^3 = converts ml to L
    6.22 = millimolar absorbance coefficient

    For this assay, with a 1 cm light path, the factor calculates to be 4455. This factor can be programmed into the spectrophotometer and the machine directly converts the change in absorbance at 340 nm (ΔA/min) to activity in U/L, or alternately, results can be calculated manually.

EXAMPLE WITH MANUAL CALCULATION

A RBC hemolysate sample diluted to 7g/L hemoglobin was assayed for glutathione reductase activity at 25ºC with a cuvette path length of 1 cm using the assay procedure above. Change in absorbance was recorded every 20 sec. The first 40 seconds were not taken into consideration.

Time (sec)	Absorbance 340 nm (Sample)	Absorbance 340 nm (Blank)
40	1.0230	0.557
60	1.0200	0.557
80	1.0170	0.557
100	1.0132	0.5574

Figure 1 shows the slope (rate) of the linear portion of the curve when the absorbance (A_{340}) values are plotted as a function of time (in seconds) in the sample above.

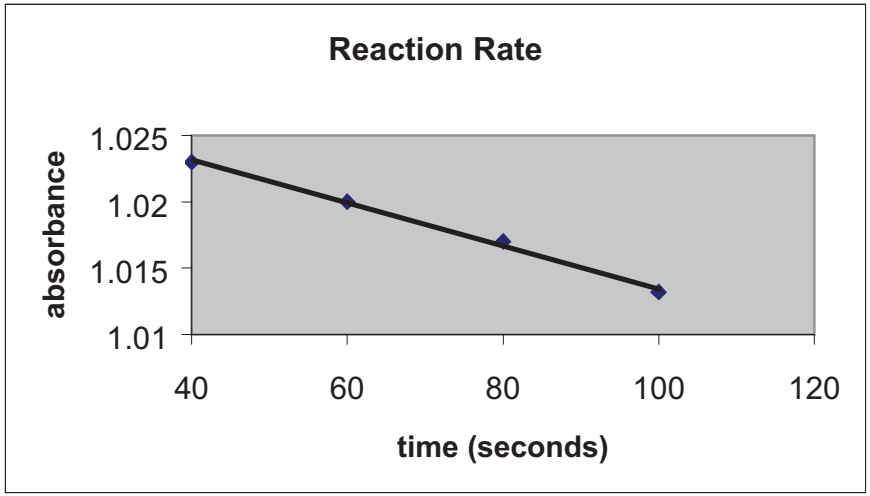

Figure 1

Select the highest and lowest points of the linear curve and determine the change in absorbance at 340 nm during the time interval. In this example it is: A_{340} (Time 2)-A_{340} (Time 1)/T2-T1

1.023-1.0132/60 sec = 0.0098/min

Sample Rate ΔA_{340}/min = 0.0098
Blank Rate ΔA_{340}/min = 0.0004
Net Rate ΔA_{340}/min = 0.0094

Calculation of activity

GR Activity U/L = 1µmol/min/L = (ΔA_{340}/min)/0.00622 x d x (TV/SV in µl)

For a 1 cm cuvette path length (d) = (0.0094/0.0062) X (935/35) = 1.52 X 26.7 = 40.58 IU/L = 5.8 U/g hemoglobin.

Unit definition: 1 unit of glutathione reductase will form 1.0 µmol $NADP^+$ from NADPH per min at pH 7.0 at 25ºC

Note: Enzyme activity can be decreased by negative feedback from excess substrate or from damage by oxidative modification[7]. For very high or very low results, check the plots of absorbance points over time. Lines should be linear. A curve followed by a plateau indicates substrate depletion and will require diluting samples.

RESULTS

Table 1 shows the precision statistics of this assay for the QC material in terms of coefficient of variation (%CV) for 2 lots of kits. The tests were carried out both manually and via automation over 3 days of 10 tests each in 2 batches. GR activity is expressed in terms of IU/L. The GR activity range for the QC material varies between 23.39 and 30.02 IU/L for the Cobas Mira automated system and 21.73 and 38.56 done manually. Manual data is as follows:

	Intraassay; N=5	Interassay N=10	Day to Day (3 day) variation N=29	Lot to Lot variability N=20
Mean GR Activity (IU/L)	30.68	30.45	30.15	31.66
+/- SD	2.12	2.48	4.21	4.25
%CV	6.92	8.15	13.95	13.42

Linearity:

Shown below is the activity in U/L for a RBC hemolysate spiked with increasing volume of purified Glutathione Reductase (Sigma) enzyme (250 U/L stock solution). The assay is linear within a change of absorbance range (Δ A/min) of approximately 0.0061 to beyond 0.02 which in our experiment corresponds to 27 U/L and 200 U/L enzyme activity respectively. Accordingly, change in absorbance values below or above the lower and upper limits (0.003 and 0.025 A_{340}/min) indicate the need for the use of a more concentrated or diluted sample.

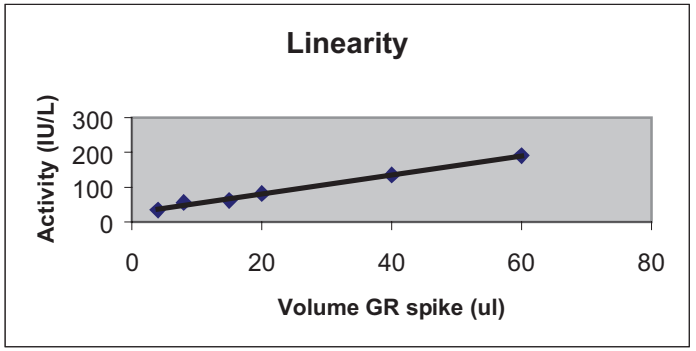

Figure 2

Figure 3 shows the range of dilutions that the Glutathione Reductase Kit can accurately and consistently measure GR activity in human RBC hemolysate. Dilutions of 1:10,1:20,1:30,1:40, 1:50 and 1:100 of a 166.56 g/L hemoglobin in a RBC hemolysate was tested. Activity is expressed in U/g hemoglobin. The Kit is effectively accurate over a range of 2.0 fold dilution from 1:20 to 1:40 of the hemolysate that corresponds to 8.3 g/L and 4.1 g/L hemoglobin respectively.

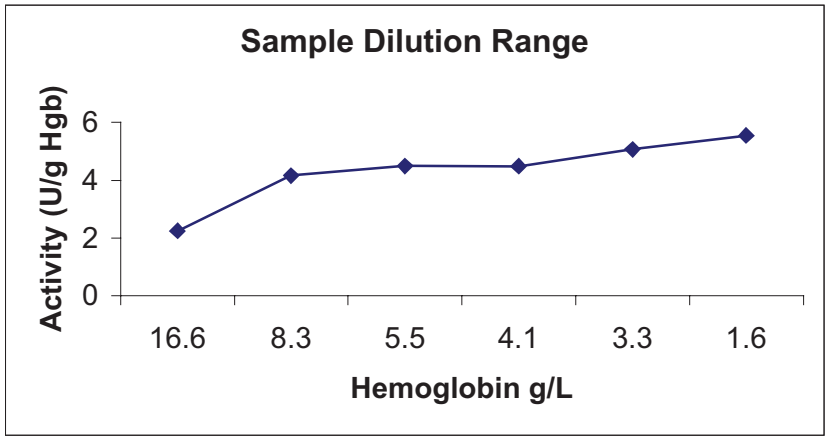

Figure 3

Experiment:

The Glutathione Reductase Assay Kit was used to measure GR activity in 15 random samples of blood that were obtained commercially. The plasma and red blood cells were separated and appropriate dilutions of RBC hemolysates were made. The GR activity was measured both manually and by automation. Correlation between the 2 methods was 0.93.

*Parameters for the automated system are available upon request.

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PROCEDURAL FLOW CHART

TURN ON SPECTROPHOTOMETER AND SET AT KINETIC PARAMETERS

↓

SET ASSAY TEMPERATURE AT 25ºC

↓

PREPARE REAGENTS

↓

PREPARE SAMPLES

↓

AUTO ZERO at 340 nm WITH DEIONIZED WATER

↓

PIPETTE FOLLOWING REAGENTS INTO A CUVETTE

935 µl WORKING SOLUTION
35 µl SAMPLE
MIX BY INVERSION

↓

PLACE CUVETTE IN CORRECT POSITION IN SPECTROPHOTOMETER

↓

RECORD THE CHANGE IN A_{340} FOR 1 MIN

NOTES:

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1004665