Glutathione Reductase Assay Kit

Item No. 703202

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TABLE OF CONTENTS

GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity
703210	GR Assay Buffer (10X)	1 vial
703212	GR Sample Buffer (10X)	1 vial
703214	GR Glutathione Reductase (control)	1 vial
703216	GR GSSG	1 vial
703218	GR NADPH	3 vials
400014	96-Well Solid Plate (Colorimetric Assay)	1 plate
400012	96-Well Cover Sheet	1 cover

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Fax: 734-971-3641

Email: techserv@caymanchem.com

Hours: M-F 8:00 AM to 5:30 PM EST

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored at -20°C and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader capable of measuring absorbance at 340 nm
2. Adjustable pipettes and a repeating pipettor
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable

INTRODUCTION

Background

Glutathione (GSH) is a tripeptide widely distributed in both plants and animals.[1,2] GSH serves as a nucleophilic co-substrate to GSH transferases in the detoxification of xenobiotics and is an essential electron donor to GSH peroxidases in the reduction of hydroperoxides.[2,3] GSH is also involved in amino acid transport across membranes.[4,5] Glutathione reductase (GR, EC 1.6.4.2) is a flavoprotein that catalyzes the NADPH-dependent reduction of oxidized glutathione (GSSG) to GSH.[6] This enzyme is essential for the GSH redox cycle which maintains adequate levels of reduced cellular GSH. A high GSH/GSSG ratio is essential for protection against oxidative stress.

About This Assay

Cayman's Glutathione Reductase Assay Kit measures GR activity by measuring the rate of NADPH oxidation:

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

The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm and is directly proportional to the GR activity in the sample. The Cayman GR Assay Kit can be used to measure GR activity in plasma, erythrocyte lysates, tissue homogenates, and cell lysates.

PRE-ASSAY PREPARATION

Reagent Preparation

1. **GR Assay Buffer (10X) - (Item No. 703210)**

   Dilute 2 ml of Assay Buffer concentrate with 18 ml of HPLC-grade water. This final Assay Buffer (50 mM potassium phosphate, pH 7.5, containing 1 mM EDTA) should be used in the assay. When stored at 4°C, this diluted Assay Buffer is stable for at least two months. Equilibrate the diluted Assay Buffer to 25°C before using in the assay.

2. **GR Sample Buffer (10X) - (Item No. 703212)**

   Dilute 2 ml of Sample Buffer concentrate with 18 ml of HPLC-grade water. This final Sample Buffer (50 mM potassium phosphate, pH 7.5, containing 1 mM EDTA and 1 mg/ml BSA) should be used to dilute the GR control and the GR samples prior to assaying. When stored at 4°C, this diluted Sample Buffer is stable for at least one month.

3. **GR Glutathione Reductase (Control) - (Item No. 703214)**

   This vial contains a solution of GR (from Baker's Yeast). To avoid repeated freezing and thawing, the GR should be aliquoted into several small vials and stored at -20°C. Prior to use, transfer 10 µl of the supplied enzyme to another vial and dilute with 990 µl of diluted Sample Buffer and keep on ice. The diluted enzyme is stable for four hours on ice. A 20 µl aliquot of this diluted enzyme per well causes a decrease of approximately 0.04 absorbance units/minute under the standard assay conditions described in **Performing the Assay** (see page 11).

4. **GR GSSG - (Item No. 703216)**

   This vial contains a 9.5 mM solution of GSSG and should be stored at -20°C when not being used. The reagent is ready to use as supplied.

5. **GR NADPH - (Item No. 703218)**

   The vials contain a lyophilized powder of NADPH. Each reconstituted vial will be enough reagent for 40 wells. Reconstitute the number of vials that you will need by adding 2 ml of HPLC-grade water to each vial and vortex well. The reconstituted reagent should be kept at 25°C while assaying and then stored at 4°C. If stored at 4°C, the reconstituted reagent is stable for two days. *NOTE: Do not freeze the reconstituted reagent.*

Sample Preparation

Tissue Homogenate

1. Prior to dissection, perfuse or rinse tissue with a PBS (phosphate buffered saline) solution, pH 7.4, to remove any red blood cells and clots.
2. Homogenize the tissue in 5-10 ml of cold buffer (*i.e.*, 50 mM potassium phosphate, pH 7.5, 1 mM EDTA) per gram tissue.
3. Centrifuge at 10,000 x g for 15 minutes at 4°C.
4. Remove the supernatant for assay and store on ice. If not assaying on the same day, freeze the sample at -80°C. The sample will be stable for at least one month.

Cell Lysate

1. Collect cells by centrifugation (*i.e.*, 1,000-2,000 x g for 10 minutes at 4°C). For adherent cells, do not harvest using proteolytic enzymes; rather use a rubber policeman.
2. Homogenize cell pellet in cold buffer (*i.e.*, 50 mM potassium phosphate, pH 7.5, 1 mM EDTA).
3. Centrifuge at 10,000 x g for 15 minutes at 4°C.
4. Remove the supernatant for assay and store on ice. If not assaying on the same day, freeze the sample at -80°C. The sample will be stable for at least one month.

Plasma and Erythrocyte Lysate

1. Collect blood using an anticoagulant such as heparin, citrate, or EDTA.
2. Centrifuge the blood at 700-1,000 x g for 10 minutes at 4°C. Pipette off the top yellow plasma layer without disturbing the white buffy layer. Store plasma on ice until assaying or freeze at -80°C. The plasma sample will be stable for at least one month. Dilute the plasma 1:2 with Sample Buffer before assaying.
3. Remove the white buffy layer (leukocytes) and discard.
4. Lyse the erythrocytes (red blood cells) in four times its volume of ice-cold HPLC-grade water.
5. Centrifuge at 10,000 x g for 15 minutes at 4°C.
6. Collect the supernatant (erythrocyte lysate) for assaying and store on ice. If not assaying the same day, freeze at -80°C. The sample will be stable for at least one month. Dilute the erythrocyte lysate 1:10-1:20 with Sample Buffer before assaying.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. However, it is necessary to have three wells designated as non-enzymatic or background wells. The absorbance rate of these wells must be subtracted from the absorbance rate measured in the GR sample and control wells. We suggest that there be at least three wells designated as positive controls and that you record the contents of each well on the template sheet provided on page 18.

	1	2	3	4	5	6	7	8	9	10	11	12
A	BW	BW	BW	7	7	7	15	15	15	23	23	23
B	+	+	+	8	8	8	16	16	16	24	24	24
C	1	1	1	9	9	9	17	17	17	25	25	25
D	2	2	2	10	10	10	18	18	18	26	26	26
E	3	3	3	11	11	11	19	19	19	27	27	27
F	4	4	4	12	12	12	20	20	20	28	28	28
G	5	5	5	13	13	13	21	21	21	29	29	29
H	6	6	6	14	14	14	22	22	22	30	30	30

BW - Background Wells
+ - Positive Control Wells
1-30 - Sample Wells

Figure 1. Sample plate format

Pipetting Hints

- It is recommend that a repeating pipettor be used to deliver Assay Buffer (dilute), GSSG, and NADPH to the wells. This saves time and helps to maintain more precise incubation times.
- Use different tips to pipette the GSSG, enzyme, and NADPH.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- The final volume of the assay is 190 µl in all the wells.
- It is not necessary to use all the wells on the plate at one time.
- The assay temperature is 25°C.
- Use the Assay Buffer (dilute) in the assay.
- Monitor the decrease in absorbance at 340 nm using a plate reader.

Performing the Assay

1. **Background or Non-enzymatic Wells** - add 120 µl of Assay Buffer and 20 µl of GSSG to three wells.
2. **Positive Control Wells (Baker's yeast GR)** - add 100 µl of Assay Buffer, 20 µl of GSSG, and 20 µl of diluted GR (control) to three wells.
3. **Sample Wells** - add 100 µl of Assay Buffer, 20 µl of GSSG, and 20 µl of sample to three wells. To obtain reproducible results, the amount of GR added to the well should cause an absorbance decrease between 0.008 and 0.1/min. When necessary, samples should be diluted with Sample Buffer or concentrated with an Amicon centrifuge concentrator with a molecular weight cut-off of 10,000 to bring the enzymatic activity to this level. *NOTE: The amount of sample added to the well should always be 20 µl. To determine if an additional sample control should be performed, see the* ***Interferences*** *section (page 14).*
4. Initiate the reactions by adding 50 µl of NADPH to all the wells you are using. Make sure to note the precise time you started and add the NADPH as quickly as possible.
5. Carefully shake the 96-well plate for a few seconds to mix.
6. Read the absorbance once every minute at 340 nm using a plate reader to obtain at least 5 time points. *NOTE: The initial absorbance of the sample wells should not be above 1.2 or below 0.5.*

ANALYSIS

Calculations

1. Determine the change in absorbance (ΔA_{340}) per minute by:
   a. Plotting the absorbance values as a function of time to obtain the slope (rate) of the linear portion of the curve (a graph is shown on page 13 using Baker's yeast GR)

   OR

   b. Select two points on the linear portion of the curve and determine the change in absorbance during that time using the following equation:

$$\Delta A_{340}/\text{min.} = \frac{^{*}|\, A_{340}\,(\text{Time 2}) - A_{340}\,(\text{Time 1})\,|}{\text{Time 2 (min.)} - \text{Time 1 (min.)}}$$

*Use the absolute value.

2. Determine the rate of ΔA_{340}/min. for the background or non-enzymatic wells and subtract this rate from that of the sample wells.
3. Use the following formula to calculate the GR activity. The reaction rate at 340 nm can be determined using the NADPH extinction coefficient of 0.00373 μM^{-1}*. One unit is defined as the amount of enzyme that will cause the oxidation of 1.0 nmol of NADPH to $NADP^{+}$ per minute at 25°C.

$$\text{GR Activity} = \frac{\Delta A_{340}/\text{min.}}{0.00373\ \mu M^{-1}} \times \frac{0.19\ \text{ml}}{0.02\ \text{ml}} \times \text{Sample dilution} = \text{nmol/min/ml}$$

*The actual extinction coefficient for NADPH at 340 nm is 0.00622 $\mu M^{-1}cm^{-1}$. This value has been adjusted for the pathlength of the solution in the well (0.6 cm).

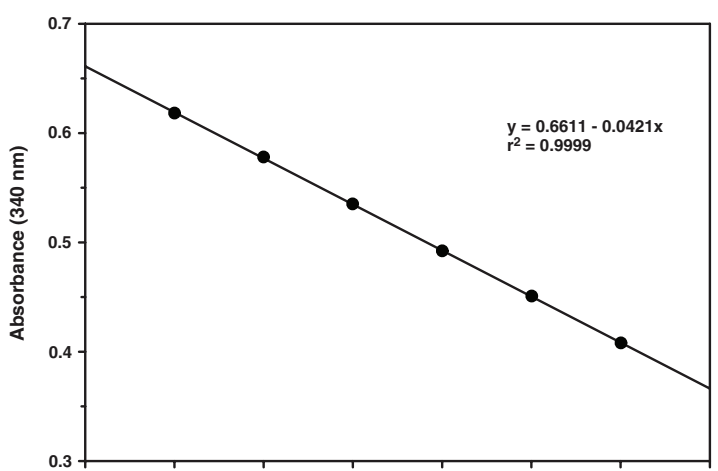


Figure 2. Activity of Baker's yeast GR

Performance Characteristics

Precision:

When a series of 20 GR samples were assayed on the same day, the intra-assay coefficient of variation was 3.7%. When a series of 20 GR samples were assayed on five different days under the same experimental conditions, the inter-assay coefficient of variation was 9.3%.

Assay Range:

Samples containing GR activity between 20-255 nmol/min/ml can be assayed without further dilution or concentration. This GR activity is equivalent to an absorbance decrease of 0.008 to 0.1 per minute.

RESOURCES

Interferences

- Samples that have a high intrinsic absorbance at 340 nm may exceed the absorbance maximum of the plate reader. Therefore, samples with an initial absorbance >1.2 should be diluted with Sample Buffer until the absorbance is lowered. For example, hemoglobin absorbs significantly at 340 nm, and thus erythrocyte lysates must be diluted before assaying.
- Samples containing high levels of GSSG or NADPH consuming enzymes will cause the GR levels to be overestimated. A blank without GSSG should be performed to assess nonspecific oxidation of NADPH. GSSG can be removed from the sample by either dialysis or passing through a gel filtration column.

The following reagents were tested for interference in the assay.

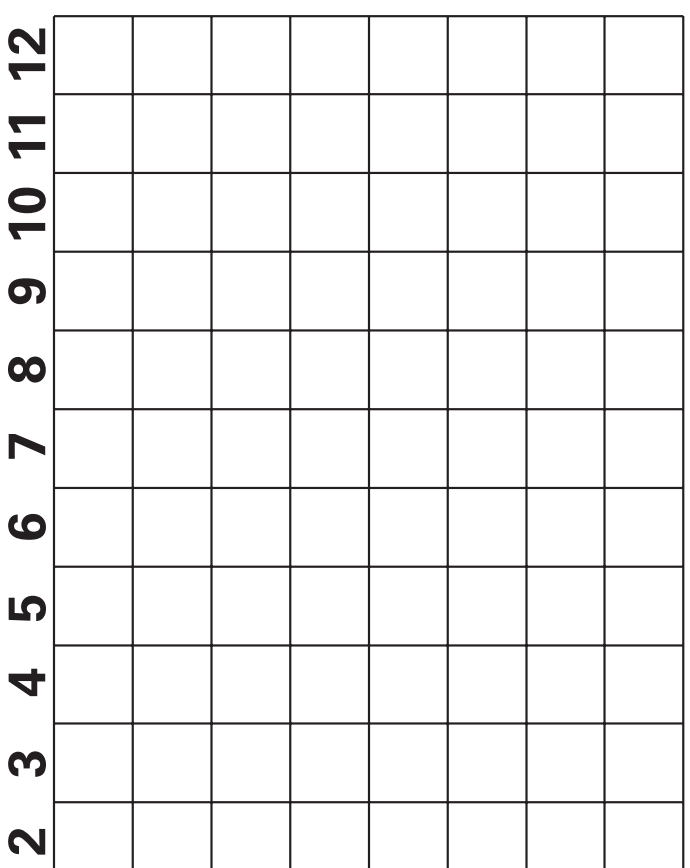
Reagent		Will Interfere (Yes or No)
Buffers	Tris (pH 8.0)	No
	HEPES (pH 7.0)	No
	Phosphate (pH 7.5)	No
Detergents	Triton X-100 (≤1%)	No
	Polysorbate 20 (≤1%)	No
	CHAPS (≤1%)	No
Protease Inhibitors/ Chelators	Antipain (≤0.1 mg/ml)	No
	PMSF (≤200 µM)	No
	Leupeptin (10 µg/ml)	No
	Trypsin (10 µg/ml)	No
	Chymostatin (10 µg/ml)	No
	EGTA (≤1 mM)	No
	EDTA (≤1 mM)	No
Solvents	Ethanol (10 µl)	No
	Methanol (10 µl)	No
	Dimethylsulfoxide (10 µl)	No
Others	Glycerol (≤10%)	No
	BSA (≤1 mg/ml)	No

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
The initial absorbance in the wells is less than 0.1	NADPH was not added to the wells	Make sure to add all components to the wells
No decrease in absorbance was observed in the sample wells	A. Enzyme activity was too low B. GSSG was not added to the wells	A. Concentrate your sample using an Amicon centrifuge concentrator with a 10,000 MW cut-off and re-assay B. Make sure to add all components to the wells
Reaction rate was too fast; the initial absorbance of the sample well is below 0.5	Too much enzyme added to well(s)	Dilute your samples with Sample Buffer and re-assay
The initial absorbance in the sample wells is above 1.2	There may be something in the sample that is interfering (see **Interference** section on page 14)	Dilute your sample with Sample Buffer and re-assay

References

1. Foyer, C.H., Lelandais, M., and Kunert, K.J. Photooxidative stress in plants. *Physiol. Plant.* **92**, 696-717 (1994).
2. Glutathione: Metabolism and function, Arias, I.M. and Jakoby, W.B., editors. Raven Press, New York (1976).
3. Baillie, T.A. and Slatter, J.G. Glutathione: A vehicle for the transport of chemically reactive metabolites *in vivo*. *Acc. Chem. Res.* **24**, 264-270 (1991).
4. Inoue, M., Saito, Y., Hirata, E., *et al.* Regulation of redox states of plasma proteins by metabolism and transport of glutathione and related compounds. *Journal of Protein Chemistry* **6**, 207-225 (1987).
5. Inoue, M. Interorgan metabolism and membrane transport of glutathione and related compounds, Chapter 6, *in* Renal Biochemistry. Kinne, R.K.H., editor. Elsevier Science Publishers B.V., London, 225-269 (1985).
6. Carlberg, I. and Mannervik, B. Glutathione reductase. *Methods Enzymol.* **113**, 484-490 (1985).



NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.