Thioredoxin Reductase Colorimetric Assay Kit

Item No. 10007892

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity
10009092	TrxR Assay Buffer (10X)	1 vial
10009093	Thioredoxin Reductase Control	1 vial
10009094	TrxR Inhibitor	2 vials
10009096	TrxR DTNB	1 vial
10009095	TrxR NADPH	2 vials
10009097	TrxR DMSO	1 vial
400014	96-Well 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 absorbances between 405-414 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

The thioredoxin system is one of the key cellular redox regulators. Together with the glutathione system, it controls the redox state of cysteine residues in proteins and has numerous other roles in redox regulation of cellular processes. The thioredoxin system is composed of thioredoxin reductase (TrxR) and thioredoxin. TrxRs are a family of selenium-containing pyridine nucleotide-disulfide oxidoreductases.[1] TrxR transfers electrons from NADPH to thioredoxin, which in turn reduces thioredoxin peroxidase, methionine sulfoxide reductase, ribonucleotide reductase, and other important redox proteins.[1] TrxRs are able to reduce a number of substrates other than thioredoxin, including selenite, lipid hydroperoxides, vitamin K, and hydrogen peroxide.[1,2] To date, two forms of mammalian TrxRs have been characterized. One is present in the cytosol (called TrxR1, TR1, or TxnRd1) and the other resides in the mitochondria (called TrxR2, TR3, or TxnRd2).[3] TrxRs have been implicated in playing a role in protecting against oxidative injury, cell growth and transformation, and the recycling of ascorbate.[2,4] Determining the involvement of TrxR in both normal and pathological cell function, as well as its drug interaction, may provide new insights into diseases and provide new treatments for cancer, AIDS, and autoimmune diseases.[1,2]

About This Assay

Cayman's Thioredoxin Reductase Colorimetric Assay Kit provides a convenient method for detecting mammalian TrxR activity in tissue homogenates and cell lysates. It is based on the reduction of DTNB (5,5'-dithio-*bis*(2-dinitrobenzoic acid); Ellman's reagent) with NADPH to 5-thio-2-nitrobenzoic acid (TNB) which produces a yellow product that is measured at 405-414 nm.[5,6] The kit includes all reagents needed to assay mammalian TrxR activity. Measurement of TrxR activity by DTNB reduction in the absence and in the presence of aurothiomalate, a specific TrxR inhibitor included in the kit, allows for correction of non-thioredoxin reductase-independent DTNB reduction (*i.e.*, presence of glutathione).[7] The difference between the two results is the DTNB reduction due to TrxR activity.

$$TrxR + DTNB + NADPH + H^+ \longrightarrow 2TNB + NADP^+$$

PRE-ASSAY PREPARATION

Reagent Preparation

1. **TrxR Assay Buffer (10X) - (Item No. 10009092)**

   Dilute 3 ml of Assay Buffer concentrate with 27 ml of HPLC-grade water. This final Assay Buffer (50 mM potassium phosphate, pH 7.0, containing 50 mM potassium chloride, 1 mM EDTA, and 0.2 mg/ml BSA) should be used in the assay and for reconstituting the TrxR Inhibitor and NADPH. When stored at -20°C, this diluted Assay Buffer is stable for at least six months.

2. **Thioredoxin Reductase (control) - (Item No. 10009093)**

   The vial contains 100 µl of a solution of rat liver thioredoxin reductase (TrxR). The thawed enzyme should be stored on ice. The enzyme is ready to use as supplied.

3. **TrxR Inhibitor - (Item No. 10009094)**

   The vial contains a lyophilized powder of sodium aurothiomalate (ATM). Reconstitute the vial with 1 ml of diluted Assay Buffer before use. The reconstituted Inhibitor is stable for four hours. ATM is a specific thioredoxin inhibitor. Since several enzymes present in biological samples can reduce DTNB, the ATM is used to determine the reduction of DTNB specific to thioredoxin reductase. The concentration of Inhibitor used in the assay, 20 µM, will effectively remove all thioredoxin reductase activity.[8]

4. **TrxR DTNB - (Item No. 10009096)**

   Weigh 4 mg of DTNB into another vial, add 2 ml of dimethylsulfoxide (DMSO) and vortex until dissolved. Store the reagent at room temperature in the dark and use within four hours.

5. **TrxR NADPH - (Item No. 10009095)**

   The vial contains a lyophilized powder of NADPH. Reconstitute the vial with 2 ml of diluted Assay Buffer and store on ice. The reconstituted NADPH is stable for six hours.

6. **TrxR DMSO - (Item No. 10009097)**

   The vial contains 10 ml of DMSO. It is ready to use as supplied. Once thawed, DMSO can be stored at room temperature for six months.

Sample Preparation

Tissue Homogenate

The amount of TrxR activity in animal tissues varies from organ to organ. Values range from 0.05-0.6 units per mg of protein for crude extracts.

1. Prior to dissection, either perfuse tissue or rinse tissue with a phosphate buffered saline (PBS) 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 7.4, containing 1 mM EDTA) per gram of 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

Typically cell culture extracts have a range of 0.4-4 units per 10^8 cells (0.04-0.25 units per mg of protein)

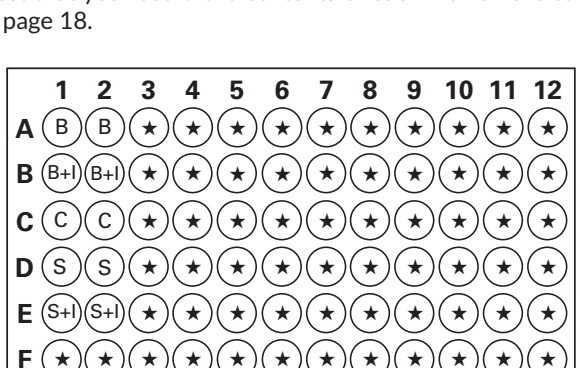
1. Collect cells (1 x 10^8) by centrifugation (*i.e.*, 1,000-2,000 x g for 10 minutes at 4°C). For adherent cells, do not harvest using proteolytic enzymes; rather use a rubber policeman.
2. Homogenize cell pellet in 5-10 ml of cold buffer (*i.e.*, 50 mM potassium phosphate, pH 7.4, containing 1 mM EDTA).
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.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. However, it is necessary to have two wells designated as background wells, two wells designated as positive control wells, and two background wells treated with inhibitor. We recommend that each sample also be treated with inhibitor.

A typical layout of samples to be measured in duplicate is given below (see Figure 1). We suggest that you record the contents of each well on the template sheet provided on page 18.



B - Background
B+I - Background + ATM
C - Positive Control
S - Sample
S+I - Sample + ATM
★ - Additional Samples

Figure 1. Sample plate format

Pipetting Hints

- It is recommended that an adjustable pipette be used to deliver reagents to the wells.
- 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 200 µl in all the wells.
- It is not necessary to use all the wells on the plate at one time.
- The assay temperature is 22°C.
- We recommend assaying samples in the presence and absence of ATM. Since several enzymes present in biological samples can reduce DTNB, ATM is used to determine the reduction of DTNB due only to TrxR activity.
- Twenty-three samples can be assayed in duplicate.
- Monitor the absorbance at 405-414 nm.

Performing the Assay

1. **Background Wells -** add 160 µl of diluted Assay Buffer to two wells.
2. **Background + ATM Wells -** add 140 µl of diluted Assay Buffer and 20 µl of ATM to two wells. *NOTE: ATM does slightly react with DTNB so it is important to subtract this activity from the sample + ATM wells.*
3. **Positive Control Wells (rat liver TrxR) -** add 140 µl of diluted Assay Buffer and 20 µl of rat liver TrxR (control) to two wells.
4. **Sample Wells -** add 140 µl of diluted Assay Buffer and 20 µl of sample to two wells.
5. **Sample + ATM Wells -** add 120 µl of diluted Assay Buffer, 20 µl of sample, and 20 µl of ATM to two wells.
6. Initiate the reactions by adding 20 µl of NADPH and 20 µl of DTNB to all the wells being used. Carefully shake the microtiter plate for 10 seconds to mix.
7. Read the absorbance once every minute at 405-414 nm using a plate reader to obtain at least five time points.

ANALYSIS

Calculations

Determination of the Reaction Rate

1. Determine the change in absorbance (ΔA_{405}) per minute by either:
   a. Plotting the average absorbance values as a function of time to obtain the slope (rate) of the linear portion of the curve (a graph is shown using rat liver TrxR, see Figure 2, page 14) - *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:

$$\Delta A_{405}/\text{min.} = \frac{A_{405}\ (\text{Time 2}) - A_{405}\ (\text{Time 1})}{\text{Time 2 (min.)} - \text{Time 1 (min.)}}$$

2. Determine the rate ΔA_{405}/min. for the background and subtract this rate from all the wells, including the background + ATM, sample, sample + ATM, and positive control wells.
3. Use the following formulas to calculate the TrxR activity. The reaction rate at 405 or 414 nm can be determined using either the DTNB extinction coefficient of 7.92 mM^{-1} (405 nm) or 8.42 mM^{-1} (414 nm). The actual extinction coefficients for DTNB at 405 nm and 414 nm are 12.8 $mM^{-1}cm^{-1}$ and 13.6 $mM^{-1}cm^{-1}$, respectively. The values have been adjusted for the pathlength of the solution in the well (0.619 cm). One unit is defined as the NADPH-dependent production of 2 µmol of 2-nitro-5-thiobenzoate per minute at 22°C.

Corrected ΔA/min. (sample) =

ΔA/min (sample) - [ΔA/min (sample + ATM) - ΔA/min (Bkg + ATM)]

TrxR Activity (µmol/min/ml) =

$$\frac{\text{Corrected } \Delta A/\text{min. (sample)}}{7.92\ \text{mM}^{-1}} \times \frac{0.2\ \text{ml}}{0.02\ \text{ml}} \times \text{Sample Dilution}$$

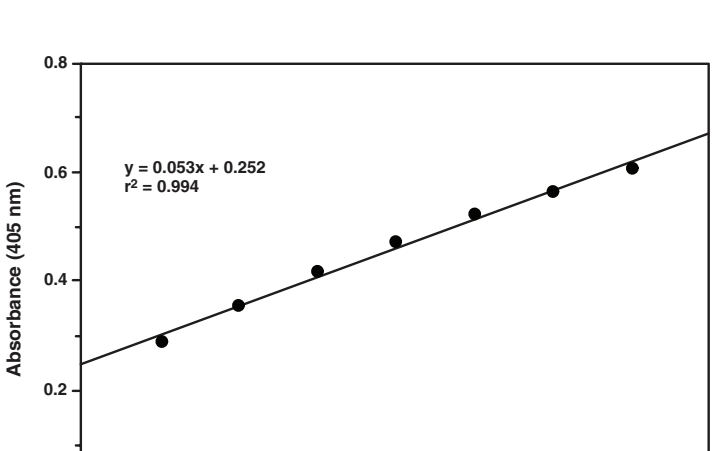


Figure 2. Activity of the rat liver TrxR

Performance Characteristics

Sensitivity:

Under the standardized conditions of the assay described in this booklet, the detection range of the assay is from 0.013-0.063 µmol/min/ml of TrxR activity, which is equivalent to an absorbance increase of 0.01-0.05 per minute.

Precision:

When a series of twenty-one thioredoxin reductase measurements were performed on the same day, the intra-assay coefficient of variation was 2.3%. When a series of twenty-one thioredoxin reductase measurements were performed on five different days under the same experimental conditions, the inter-assay coefficient of variation was 6.1%.

RESOURCES

Interferences

The following reagents were tested for interference in the assay.

	Reagent	Will Interfere (Yes or No)
Buffers:	Tris (pH 8)	No
	HEPES (pH 7)	No
	Phosphate (pH 7)	No
	Borate (pH 9)	Yes
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
	EDTA (≤10 mM)	No
	EGTA (≤1 mM)	No
Solvents:	Ethanol (10 µl)	No
	Methanol (10 µl)	Yes
	Dimethylsulfoxide (10 µl)	No
Others:	Glutathione	Yes
	BSA (≤1%)	No
	Glycerol (≤10%)	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
No activity was detected in the sample	TrxR activity was too low	Concentrate the sample using an Amicon concentrator with a molecular weight cut-off of 30,000 and re-assay
The sample rate did not change in the presense of ATM	TrxR activity was too low	Concentrate the sample using an Amicon concentrator with a molecular weight cut-off of 30,000 and re-assay
The sample starting absorbance is >1.0	There maybe interference due to thiol-containing compounds	Dilute the sample and re-assay

References

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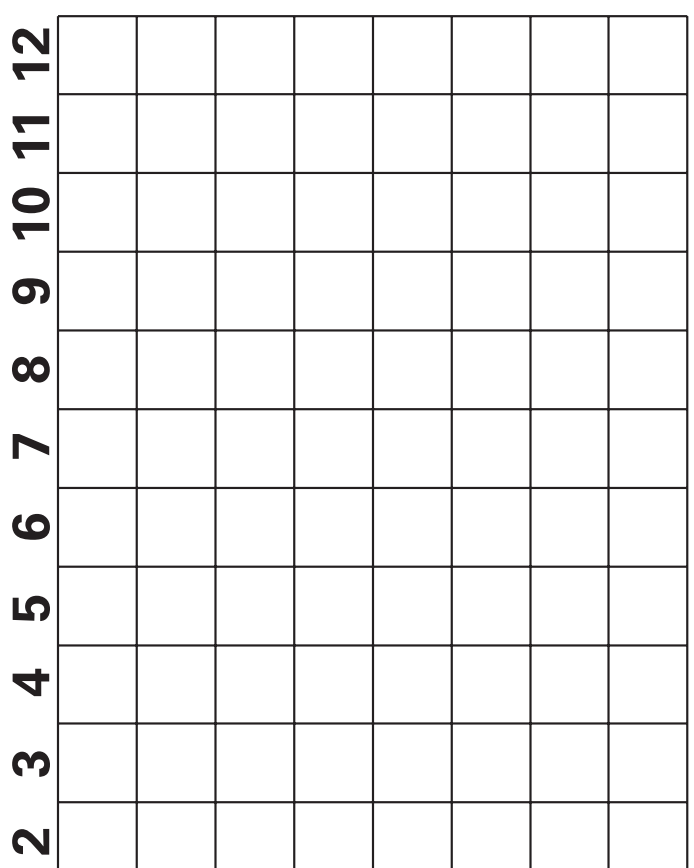

NOTES

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.