Technical Bulletin

Thioredoxin Reductase Assay Kit

Catalog Number CS0170

Product Description

Thioredoxin reductase is a ubiquitous enzyme that is thought to be involved in many cellular processes such as cell growth, p53 activity, and protection against oxidation stress.[1] The mammalian thioredoxin reductase reduces thioredoxins as well as non-disulfide substrates such as selenite, lipoic acids, lipid hydroperoxides, and hydrogen peroxide.[2]

The Thioredoxin Reductase Assay Kit uses a colorimetric assay for the determination of thioredoxin reductase activity. It is based on the reduction of 5,5′-dithiobis(2-nitro-benzoic) acid (DTNB) with NADPH to 5-thio-2-nitrobenzoic acid (TNB), which produces a strong yellow color that is measured at 412 nm.[3]

The kit contains all the reagents needed for an easy and simple colorimetric assay of mammalian thioredoxin reductase. The kit also includes an inhibitor solution for specific inhibition of mammalian thioredoxin reductase. Since several enzymes present in biological samples can reduce DTNB, the specific inhibitor is used to determine the reduction of DTNB due only to thioredoxin reductase activity.

This kit has been tested on samples prepared from mammalian tissues such as liver, kidney, brain, spleen, and heart muscle, as well as lysates from cell lines such as HeLa, A549, Jurkat, U937, A431, COS, CHO, and NIH 3T3 cells.

Components

The kit is sufficient for 100 one ml assays

- Assay Buffer 5× — 30 ml
  for Thioredoxin Reductase
  Catalog Number A4478
  500 mM Potassium phosphate pH 7.0, containing 50 mM EDTA
- Thioredoxin Reductase — 50 µl protein
  Catalog Number T9074
  rat liver thioredoxin reductase in 50 mM Tris HCl, pH 7.4, containing 1 mM EDTA, 300 mM NaCl, and 10% glycerol
- Thioredoxin Reductase Inhibitor Solution, Catalog Number T9199 — 0.05 ml
- 5,5′-Dithiobis(2-nitrobenzoic) acid (DTNB), Catalog Number D8130 — 150 mg
- NADPH
  Catalog Number N6505 — 25 mg
- Dimethyl Sulfoxide (DMSO), Catalog Number D8418 — 7.5 ml

Reagents and Equipment Required but Not Provided

- Ultrapure (17 MΩ·cm) water
- Spectrophotometer and 1 ml cuvette
- Microcentrifuge tubes

Precautions and Disclaimer

For R&D use only. Not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

It is recommended to use ultrapure (17 MΩ·cm or equivalent) water when preparing the reagents.

1× Assay Buffer (sufficient for 10 reactions) - Dilute 2 ml of the Assay Buffer 5× for Thioredoxin Reductase (Catalog Number A4478) to 10 ml with ultrapure water. Keep the diluted 1× Assay Buffer at room temperature.

DTNB Solution - Dissolve 39.6 mg of DTNB [5,5′-Dithiobis(2-nitrobenzoic) acid, Catalog Number D8130] in 1 ml of Dimethyl Sulfoxide (DMSO, Catalog Number D8418). Prepare this solution the day before use and store at 2–8 °C. During the assay the DTNB Solution should be at room temperature. For long term storage, the DTNB Solution may be stored at -20 °C for up to 4 weeks.

NADPH Solution - Add 0.625 ml of water to the bottle containing 25 mg of NADPH (Catalog Number N6505). Ensure that the contents are completely dissolved. The NADPH Solution (40 mg/ml) may be kept at 2-8 °C for up to 5 hours during the assay. It is recommended to aliquot the unused NADPH Solution and store at –20 °C for up to 6 months.

Working Buffer (sufficient for 10 reactions) - Prepare 10 ml of Working Buffer by adding 50 µl of the NADPH Solution to 2 ml of the Assay Buffer 5× for Thioredoxin Reductase and bring the final volume to 10 ml with ultrapure water. The final concentration of the Working Buffer is 100 mM potassium phosphate with 10 mM EDTA and 0.24 mM NADPH. Keep the Working Buffer at room temperature and use within 2 hours.

Diluted Inhibitor Solution (sufficient for 5-10 reactions) - Dilute 10 µl of the Thioredoxin Reductase Inhibitor Solution (Catalog Number T9199) 20-fold with DMSO (Catalog Number D8418) to a final volume of 200 µl. Keep the solution at room temperature during the assay. The Diluted Inhibitor Solution may be stored at -20 °C for up to 4 weeks. Thioredoxin reductase activity is totally inhibited by addition of 20 µl of the Diluted Inhibitor Solution to a 1 ml reaction mixture.

Thioredoxin Reductase Positive Control - Dilute 5 µl of the Thioredoxin Reductase (Catalog Number T9074) 20-fold with 1× Assay Buffer to a final volume of 100 µl. The solution may be kept at 2-8 °C for the duration of the assay (up to 2 hours). Use 10 µl of the prepared thioredoxin reductase solution as a positive control for the reaction. Discard the remaining diluted enzyme solution.

Storage/Stability

The kit ships on dry ice and storage at -20 °C is recommended. The kit, as supplied, is stable for 24 months when stored properly. Upon arrival, the DTNB (Catalog Number D8130) and DMSO (Catalog Number D8418) should be stored at room temperature.

Procedure

The *in vivo* thioredoxin reductase (EC 1.6.4.5) reduction reaction is shown below with thioredoxin (TRX) as the substrate:

$$\text{TRX-S-S} + \text{NADPH} + \text{H}^+ \xrightarrow{\text{Thioredoxin reductase}} \text{TRX-(SH)}_2 + \text{NADP}^+$$

$$\text{TRX-(SH)}_2 + \text{protein} \xrightarrow{\text{Spontaneous}} \text{TRX-S-S} + \text{protein-(SH)}_2$$

Mammalian thioredoxin reductase activity is determined with the kit using DTNB as the substrate:[2,3]

$$\text{DTNB} + \text{NADPH} + \text{H}^+ \xrightarrow{\text{Thioredoxin reductase}} 2\ \text{TNB} + \text{NADP}^+$$

Two moles of 5-thio-2-nitrobenzoic acid (TNB) are formed for every 1 mole of NADPH oxidized. The assay is performed at room temperature (25 °C) and the TNB has an absorption maximum at 412 nm (molar extinction coefficient [εM] of 14,150 M-1cm-1).4 In crude biological samples, other enzymatic activities, such as glutathione reductase and glutathione peroxidase, also reduce DTNB and will increase the observed rate of DTNB reduction. The contribution of these activities to the total DTNB reduction may be estimated by using a specific thioredoxin reductase inhibitor. In order to determine the DTNB reduction due only to the thioredoxin reductase activity present in the sample, two assays need to be performed: the first measurement is of the total DTNB reduction by the sample and the second one is the DTNB reduction by the sample in the presence of the thioredoxin reductase inhibitor solution. The difference between the two results is the DTNB reduction due to thioredoxin reductase activity.

The reaction scheme for a 1 ml reaction measured with a cuvette is summarized in Table 1. The assay is performed at room temperature (25 °C). The reaction rate is linear between 0.005-0.10 unit of thioredoxin reductase activity for the 1 ml reaction. See the Appendix for guidelines regarding sample preparation and amounts of protein to be used in the reaction.

Table 1.
Reaction Scheme for a 1 ml Assay

Sample Category	Enzyme	1× Assay Buffer	Diluted Inhibitor Solution	Working Buffer	DTNB
Blank	0	70 µl	0	900 µl	30 µl
Positive Control	10 µl	60 µl	0	900 µl	30 µl
Unknown Sample	x µl	70–x µl	0	900 µl	30 µl
Unknown Sample with Inhibitor	x µl	50–x µl	20 µl	900 µl	30 µl

x = Volume of unknown sample (**do not** exceed 50 µl)

The positive control is not required for every set of assays.

1. Set the spectrophotometer at 412 nm using an enzymatic kinetic program as follows:

   Delay = 120 seconds
   Interval = 10 seconds
   Number of readings = 6

2. Add 900 µl of Working Buffer to each 1-ml cuvette.
3. Add the other components according to the reaction scheme in Table 1:

   For the **total activity** of the unknown Sample add x µl of the sample and 70-x µl of 1× Assay Buffer to the designated cuvette. Mix by inversion.

   For the activity of the **Positive Control** add 10 µl of the Thioredoxin Reductase Positive Control and 60 µl of 1× Assay Buffer to the designated cuvette. Mix by inversion. The activity in 10 µl of the Thioredoxin Reductase Positive Control will be totally inhibited by 20 µl of the Diluted Inhibitor Solution.

   For the **Inhibition reaction** add x µl of the Sample, 50-x µl of 1× Assay Buffer, and 20 µl of Diluted Inhibitor Solution to the designated cuvette. Mix by inversion.

4. Start the reactions by addition of 30 µl of the DTNB Solution to each cuvette. Mix by inversion.
5. Determine the rate of formation of the yellow color by measuring the increase in absorption (ΔA_{412}/min) for each reaction.

7. Calculate the amount of thioredoxin reductase (TR) activity present:

TR Activity =

$$\frac{Unit}{ml} = \frac{\Delta A_{412}/_{min}\ (TR) \times D\ \times V}{S}$$

$$\Delta A_{412}/_{min}\,(TR)\ = \Delta A_{412}/_{min}\ (Sample) - \Delta A_{412}/_{min}\ (Sample + Inhibitor)$$

where

D = Sample dilution factor

V = Total volume of reaction in ml

S = Volume of Sample enzyme in ml

Unit definition: One unit of mammalian thioredoxin reductase will cause an increase in A_{412} of 1.0 per minute per ml (when measured in a non-coupled assay containing DTNB alone) at pH 7.0 at 25 °C.

Note: The 1 ml assay may be modified for use with 96-well plates by using the reaction scheme in Table 2.

Table 2.
Reaction Scheme for a 96-Well Plate Assay

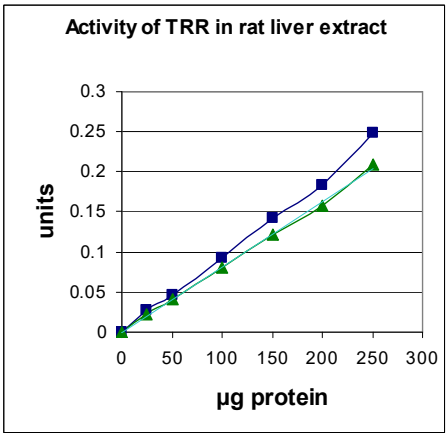
Sample	Enzyme	1× Assay Buffer	Diluted Inhibitor Solution	Working Buffer	DTNB
Blank	0	14 µl	0	180 µl	6 µl
Positive Control	2 µl	12 µl	0	180 µl	6 µl
Unknown Sample	x µl	14–x µl	0	180 µl	6 µl
Unknown Sample with Inhibitor	x µl	10–x µl	4 µl	180 µl	6 µl

The calculation of the enzymatic activity in this case needs to be adjusted for the difference in path length between a 1 ml cuvette (1 cm) and the plate used. A standard 96-well polystyrene plate containing 200 µl of liquid will have a path length of ~0.55 cm. The calculated activity (unit/ml) obtained with a 96-well plate needs to be divided by 0.55 to be compared to activity determined with a 1 ml cuvette.

Results

An example of the activity found in a rat liver extract is shown in Figure 1. Protein concentration was determined with the Bradford Reagent.

Figure 1.
Thioredoxin reductase (TRR) activity in rat liver



Thioredoxin reductase (TRR) activity measured in a rat liver extract (1,000 × *g* supernatant) assayed in the presence (triangles) or absence (squares) of Diluted Inhibitor Solution.

References

1. Mustacich, D., and Powis, G., Thioredoxin Reductase. *Biochem. J.*, **346**, 1-8 (2000).
2. Arner, E.S.J. et al., Preparation and assay of mammalian thioredoxin and thioredoxin reductase. *Meth. Enzymol*., **300**, 226-239 (1999).
3. Holmgren, A., and Bjornstedt, M., Thioredoxin and thioredoxin reductase. *Meth. Enzymol*., **252**, 199-208 (1995).
4. Riddles, P.W. et al., Reassessment of Ellman's Reagent. *Meth. Enzymol*., **91**, 49-60 (1983).

Appendix

The amount of thioredoxin reductase activity in animal tissues varies from organ to organ. Values range from 1-14 units per gram of tissue (0.05-0.6 unit per mg of protein) for crude extracts (1,000 × *g* supernatants). The residual activity measured in the presence of the inhibitor, due to other NADPH reductases, varies in the range of 5-50%.

Cell culture extracts have a range of 0.4-4 units per 10^8 cells (0.04-0.25 unit per mg of protein). The residual activity measured in the presence of the inhibitor, due to other NADPH reductases, varies in the range of 15–40%.

Sample preparation

Reagents and Equipment Required but Not Provided

- Protease Inhibitor Cocktail for use with mammalian cell and tissue culture extracts (Catalog Number P8340)
- Dulbecco's Phosphate Buffered Saline (PBS, Catalog Number D8537)
- CelLytic™ M, Mammalian Cell Lysis/Extraction Reagent (Catalog Number C2978)
- SORVALL® RC-5C centrifuge with SS-34 head or equivalent
- Microcentrifuge
- Pestle and glass tube, Potter-Elvehjem (PTFE in glass homogenizer), 8 ml (Catalog Number P7859)
- Overhead electric motor
- Bradford Reagent (Catalog Number B6916)

Extraction Procedures

1. Samples extracted from animal tissues or cultured cells should have a protease inhibitor cocktail (Catalog Number P8340) added to the extract buffer at 1:100 dilution to prevent unwanted proteolysis of the sample.
2. Cell lines should be washed with PBS (Catalog Number D8537) and then centrifuged in a conical tube. Approximately 0.5-1 × 10^8 cells are needed to measure activity. It is recommended to extract the packed cell volume with 1 volume of CelLytic M (Catalog Number C2978). The lysate is centrifuged at 10,000 × *g* for 10 minutes and the supernatant is used as the enzyme sample.
3. Animal tissues that contain large amounts of blood need to be washed with PBS (Catalog Number D8537) prior to extraction. The tissue may be extracted with 4 volumes of 0.25× Assay Buffer containing the protease inhibitor cocktail (Catalog Number P8340) using a Potter-Elvehjem homogenizer. The sample may be centrifuged either for 5 minutes at 1,000 × *g* to remove crude cell debris or for 15 minutes at 10,000 × *g* to remove mitochondria and other subcellular organelles. In both cases the supernatant is used as the enzyme sample.
4. Determine the protein concentration of the supernatant using the Bradford Reagent.

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