Thioredoxin Reductase Assay Kit

Catalog Number KA0883

100 assays

Version: 04

Intended for research use only

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Introduction

Background

Thioredoxin reductase (TrxR) (EC 1.8.1.9) is a ubiquitous enzyme which is involved in many cellular processes such as cell growth, p53 activity, and protection against oxidation stress, etc. The mammalian TrxR reduces thioredoxins as well as non-disulfide substrates such as selenite, lipoic acids, lipid hydroperoxides, and hydrogen peroxide. The Thioredoxin Reductase Assay Kit provides a convenient colorimetric assay for detecting TrxR activity in various samples. In the assay TrxR catalyzes the reduction of 5, 5'-dithiobis (2-nitrobenzoic) acid (DTNB) with NADPH to 5-thio-2-nitrobenzoic acid (TNB^{2-}), which generate a strong yellow color (λmax = 412 nm). Since in crude biological samples other enzymes, such as glutathione reductase and glutathione peroxidase, can also reduce DTNB, therefore, TrxR specific inhibitor is utilized to determine TrxR specific activity. Two assays are 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 TrxR specific inhibitor. The difference between the two results is the DTNB reduction by TrxR.

General Information

Materials Supplied

List of component

Component	Amount
TrxR Assay Buffer	25 mL
TNB Standard (lyophilized)	1 vial
DTNB (lyophilized)	1 vial
NADPH (lyophilized)	1 vial
TrxR Positive Control	1 vial
TrxR Inhibitor (lyophilized)	1 vial

Storage Instruction

Store the kit at -20°C, protect from light. Warm Assay Buffer to room temperature before use. Briefly centrifuge vials prior to opening. Read the entire protocol before performing the assay.

Assay Protocol

Reagent Preparation

- TNB Standard: Dissolve lyophilized TNB standard into 0.5 mL Assay Buffer to generate 5 mM TNB Standard. The TNB standard solution is stable for 1 week at 4°C or 2 month at -20°C.
- DTNB Solution: Dissolve DTNB into 0.9 mL Assay Buffer, sufficient for 100 assays. The DTNB solution is stable for 1 week at 4°C or 2 month at -20°C.
- NADPH: Dissolve one vial with 0.22 mL dH_2O; sufficient for 100 assays. The solution is stable for 1 week at 4°C or 2 month at -20°C.
- TrxR Positive Control: Reconstitute with 90 µL Assay Buffer to generate ~0.2 mU/µL TrxR; it is stable for 1 day at 4°C or 2 month at -20°C.
- TrxR Inhibitor: Dissolve TrxR Inhibitor into 1.2 mL Assay Buffer, sufficient for 100 assays. The TrxR Inhibitor solution is stable for 2 month at -20°C.
- Ensure that the Assay Buffer is at room temperature before use. Keep samples, NADPH, TrxR inhibitor, TrxR Positive Control on ice during the assay.

Assay Procedure

1. TNB Standard Curve: Add 0, 2, 4, 6, 8, 10 µL of the TNB Standard into 96-well plate in duplicate to generate 0, 10, 20, 30, 40, 50 nmol/well standard. Bring the final volume to 100 µL with Assay Buffer.
2. Sample and Positive Control Preparations: Take 20 mg Tissues or 2 x 10^6 Cells and homogenize in 100-200 µL cold Assay Buffer on ice (It is recommended to add Protease Inhibitor Cocktail to the buffer); Centrifuge at 10,000 x g for 15 min at 4°C; Collect the supernatant for assay and store on ice.
3. Serum can be tested directly. Determine the protein concentration of the supernatant using the Bradford Reagent. Keep samples at -80°C for storage.
4. Assay Procedure: Add 2-50 µL sample or 10 µL TrxR positive control into each well, adjust volume to 50 µL with assay buffer. 2 sets of samples should be tested as with or without TrxR Inhibitor. Add 10 µL of TrxR Inhibitor to one set of the sample for testing background enzyme activity, and add 10 µL of Assay Buffer to the other set of sample for testing total DTNB reduction, mix well.
5. Reaction Mix: Mix enough reagents for the number of assays to be performed. For each well, prepare a total 40 µL Reaction Mix:

   30 µL Assay Buffer
   8 µL DTNB Solution
   2 µL NADPH
6. Add 40 µL of the Reaction Mix to each test sample, mix well. Measure O.D.412 nm at T_1 to get A_{1t} and A_{1l}, measure O.D.412 nm again at T_2 after incubating the reaction at 25°C for 20 min (The incubate time can vary depend on the sample concentration) to get A_{2t} and A_{2l}, protect from light. The O.D. of TNB^{2-} generated by TrxR is $\Delta A_{412\ nm} = (A_{2t} - A_{1t}) - (A_{2l}-A_{1l})$.

Note: It is essential to read A_{1t}, A_{1l}, A_{2t} and A_{2l} in the reaction linear range. It will be more accurate if you read the reaction kinetics. Then choose A_{1t}, A_{1l}, A_{2t} and A_{2l} in the reaction linear range.

Data Analysis

Calculation of Results

Plot the TNB standard Curve. Apply the ΔA_{412nm} to the TNB standard curve to get B nmol of TNB (TNB amount generated between T1 and T2 in the reaction wells).

$$\text{TrxR Activity} = \frac{B}{(T2-T1) \times V} \text{ X Sample Dilution Factor = nmol/min/ml = mU/mL}$$

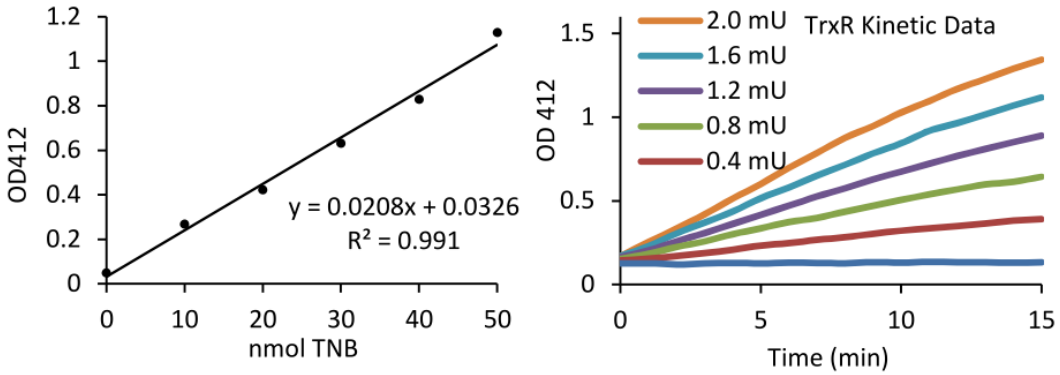
Where: B is the TNB amount from TNB standard Curve (in nmol).

T1 is the time of the first reading (A_{1t} and A_{1l}) (in min).

T2 is the time of the second reading (A_{2t} and A_{2l}) (in min).

V is the pretreated sample volume added into the reaction well (in mL).

TrxR Unit Definition: One unit of TrxR is the amount of enzyme that generates1.0 µmol of TNB per minute at 25°C. The oxidation of 1 mole of NADPH to NADP will generate 2 mole TNB finally, therefore, 1 TNB unit equals 0.5 NADP unit.



Resources

Troubleshooting

GENERAL TROUBLESHOOTING GUIDE:

Problems	Cause	Solution
Assay not working	• Use of ice-cold assay buffer • Omission of a step in the protocol • Plate read at incorrect wavelength • Use of a different 96-well plate	• Assay buffer must be at room temperature • Refer and follow the data sheet precisely • Check the wavelength in the data sheet and the filter settings of the instrument • Fluorescence: Black plates (clear bottoms) ; Luminescence: White plates ; Colorimeters: Clear plates
Samples with erratic readings	• Use of an incompatible sample type • Samples prepared in a different buffer • Cell/ tissue samples were not completely homogenized • Samples used after multiple free-thaw cycles • Presence of interfering substance in the sample • Use of old or inappropriately stored samples	• Refer data sheet for details about incompatible samples • Use the assay buffer provided in the kit or refer data sheet for instructions • Use Dounce homogenizer (increase the number of strokes); observe for lysis under microscope • Aliquot and freeze samples if needed to use multiple times • Troubleshoot if needed • Use fresh samples or store at correct temperatures until use

Lower/ Higher readings in Samples and Standards	• Improperly thawed components • Use of expired kit or improperly stored reagents • Allowing the reagents to sit for extended times on ice • Incorrect incubation times or temperatures • Incorrect volumes used	• Thaw all components completely and mix gently before use • Always check the expiry date and store the components appropriately • Always thaw and prepare fresh reaction mix before use • Refer datasheet & verify correct incubation times and temperatures • Use calibrated pipettes and aliquot correctly
Readings do not follow a linear pattern for Standard curve	• Use of partially thawed components • Pipetting errors in the standard • Pipetting errors in the reaction mix • Air bubbles formed in well • Standard stock is at an incorrect concentration • Calculation errors • Substituting reagents from older kits/ lots	• Thaw and resuspend all components before preparing the reaction mix • Avoid pipetting small volumes • Prepare a master reaction mix whenever possible • Pipette gently against the wall of the tubes • Always refer the dilutions in the data sheet • Recheck calculations after referring the data sheet • Use fresh components from the same kit
Unanticipated results	• Measured at incorrect wavelength • Samples contain interfering substances • Use of incompatible sample type • Sample readings above/below the linear range	• Check the equipment and the filter setting • Troubleshoot if it interferes with the kit • Refer data sheet to check if sample is compatible with the kit or optimization is needed • Concentrate/ Dilute sample so as to be in the linear range
Note: The most probable list of causes is under each problem section. Causes/ Solutions may overlap with other problems.		