Thioredoxin Reductase Assay Kit

(Catalog #K763-100; 100 reactions; Store kit at –20˚C)

I. Introduction:

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. BioVision's 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 (λ_{max} = 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.

II. Kit Contents:

Components	K763-100	Cap Code	Part Number
TrxR Assay Buffer	25 ml	WM	K763-100-1
TNB Standard (lyophilized)	1 vial	Brown	K763-100-2
DTNB (lyophilized)	1 vial	Red	K763-100-3
NADPH (lyophilized)	1 vial	Blue	K763-100-4
TrxR Positive Control	1 vial	Green	K763-100-5
TrxR Inhibitor (lyophilized)	1 vial	Clear	K763-100-6

III. Storage and Handling:

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.

IV. Reagent Reconstitution and General Consideration:

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: Dilute 10 µl TrxR 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.

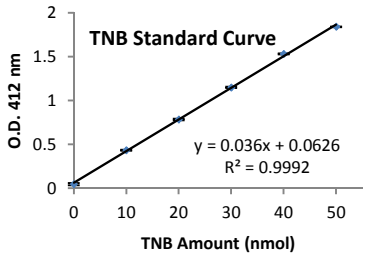
V. Thioredoxin Reductase Activity Assay:

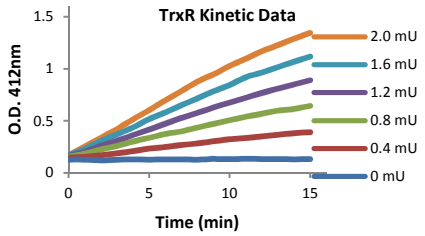
1. **TNB Standard Curve:**
   Add 0, 2, 4, 6, 8, 10 µl of the TNB Standard into 96-well plate in duplicate to generate 0, 10, 20, 30, 40, 50 nmol/well standard. Bring the final volume to 100 µl with Assay Buffer.
2. **Sample and Positive Control Preparations:**
   Take 20 mg Tissues or 2 x 10^6 Cells and homogenize in 100-200 µl cold Assay Buffer on ice (It is recommended to add Protease Inhibitor Cocktail (Cat.# K271-500) 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 (BioVision Cat # K810-100). 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 OD 412 nm at T_1 to get A_{1t} and A_{1l}, measure OD 412 nm again at T2 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 OD 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.
7. **Calculation:** 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).

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

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.





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GENERAL TROUBLESHOOTING GUIDE:

Problems	Cause	Solution
Assay not working	• Use of ice-cold assay buffer	• Assay buffer must be at room temperature
	• Omission of a step in the protocol	• Refer and follow the data sheet precisely
	• Plate read at incorrect wavelength	• Check the wavelength in the data sheet and the filter settings of the instrument
	• Use of a different 96-well plate	• Fluorescence: Black plates (clear bottoms) ; Luminescence: White plates ; Colorimeters: Clear plates
Samples with erratic readings	• Use of an incompatible sample type	• Refer data sheet for details about incompatible samples
	• Samples prepared in a different buffer	• Use the assay buffer provided in the kit or refer data sheet for instructions
	• Cell/ tissue samples were not completely homogenized	• Use Dounce homogenizer (increase the number of strokes); observe for lysis under microscope
	• Samples used after multiple free-thaw cycles	• Aliquot and freeze samples if needed to use multiple times
	• Presence of interfering substance in the sample	• Troubleshoot if needed
	• Use of old or inappropriately stored samples	• Use fresh samples or store at correct temperatures until use
Lower/ Higher readings in Samples and Standards	• Improperly thawed components	• Thaw all components completely and mix gently before use
	• Use of expired kit or improperly stored reagents	• Always check the expiry date and store the components appropriately
	• Allowing the reagents to sit for extended times on ice	• Always thaw and prepare fresh reaction mix before use
	• Incorrect incubation times or temperatures	• Refer datasheet & verify correct incubation times and temperatures
	• Incorrect volumes used	• Use calibrated pipettes and aliquot correctly
Readings do not follow a linear pattern for Standard curve	• Use of partially thawed components	• Thaw and resuspend all components before preparing the reaction mix
	• Pipetting errors in the standard	• Avoid pipetting small volumes
	• Pipetting errors in the reaction mix	• Prepare a master reaction mix whenever possible
	• Air bubbles formed in well	• Pipette gently against the wall of the tubes
	• Standard stock is at an incorrect concentration	• Always refer the dilutions in the data sheet
	• Calculation errors	• Recheck calculations after referring the data sheet
	• Substituting reagents from older kits/ lots	• Use fresh components from the same kit
Unanticipated results	• Measured at incorrect wavelength	• Check the equipment and the filter setting
	• Samples contain interfering substances	• Troubleshoot if it interferes with the kit
	• Use of incompatible sample type	• Refer data sheet to check if sample is compatible with the kit or optimization is needed
	• Sample readings above/below the linear range	• 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.		