ab190804 – Thioredoxin Reductase 1 (TXNRD1) Activity Assay Kit

Instructions for Use

For the sensitive and accurate measurement of Thioredoxin Reductase 1 (TXNRD1) Activity in Human cell and tissue extracts.

This product is for research use only and is not intended for diagnostic use.

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1. BACKGROUND

Abcam's Thioredoxin Reductase 1 (TXNRD1) Activity Assay kit is designed for the sensitive and accurate measurement of Thioredoxin Reductase 1 activity in Human cell and tissue extracts.

The assay uses a 96-well plate with an antibody specific to isoform 1 of thioredoxin reductase to isolate the isozyme pre-coated onto the wells. Samples are added to the wells and incubated at room temperature, any Thioredoxin Reductase 1 present in the sample will be immobilized in the well. After washing, the Reaction Buffer is added to each well and enzyme activity is measured. By analyzing the enzyme's activity in an isolated context, outside of the cell and free from other isoforms, an accurate measurement of the enzyme's functional state can be understood.

Thioredoxin Reductase 1 (TXNRD1) is a selenium-containing enzyme part of the thioredoxin system responsible for regulating oxidative stress and redox signaling via reduction of disulfide bonds. The dimer, Thioredoxin Reductase 1, reduces thioredoxin and other substrates using NADPH and an FAD cofactor. Thioredoxin Reductase 1 involvement in protecting against oxidative stress and injury, regulation of cellular development and growth, and various other cellular processes make it an interesting target for studies of various cancers, AIDS, and other diseases. Humans express three different isozymes of thioredoxin reductase, isoform 1 is the cytosolic form, isoform 2 is the mitochondrial form, and isoform 3 is a testes specific form.

The enzyme activity is determined by following the NADPH-assisted reduction of 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) to 5-thio-2-nitrobenzoic acid (TNB) which leads to increased absorbance at 412 nm. Two moles of TNB are formed from the oxidation of one mole of NADPH according to the reaction shown below.

$$DTNB + NADPH + H+ \leftrightarrow 2\ TNB + NADP+$$

The molar extinction coefficient of TNB is 14.15 X 10^3 $mM^{-1}cm^{-1}$. Thioredoxin Reductase 1 activity is controlled by enzyme amount. An antibody specific to isoform 1 of thioredoxin reductase is used to isolate the enzyme from a bulk protein source for isoform-specific enzymatic activity measurements. Because the activity measured is specific to the isolated enzyme, a thioredoxin reductase inhibitor is not required for background signal subtraction.

2. ASSAY SUMMARY

Prepare samples as instructed.
Determine the protein concentration of extracts.

Equilibrate all reagents to room temperature.

Dilute samples to desired protein concentration in 1X Incubation Buffer.
Add 100 µL sample to each well used. Incubate 2 hours at room temperature.

Aspirate and wash each well three times using 300 µL 1X Wash Buffer per wash.

Add 200 µL Reaction Buffer to each well.
Pop bubbles and immediately record the color development at 412nm for 10 to 45 minutes.
Alternatively, measure the endpoint at a user-determined time.

3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store kit at 2-8ºC immediately upon receipt.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in sections 9&10.

5. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)
10X Buffer	25 mL	2-8ºC
Extraction Buffer (Buffer A)	15 mL	2-8ºC
10X Blocking Buffer	8 mL	2-8ºC
5X Activity Solution	5 mL	2-8ºC
200X NADPH (Lyophilized)	1 vial	2-8ºC
200X DTNB	110 µL	2-8ºC
Pre-coated Microplate (12 x 8 well strips)	96 Wells	2-8ºC

6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully utilize this assay:

- Microplate reader capable of measuring absorbance at 412nm.
- Method for determining protein concentration (BCA assay recommended).
- Deionized water.
- Multi and single channel pipettes.
- Tubes for sample dilution.
- Phenylmethylsulfonyl Fluoride (PMSF) (or other protease inhibitors) and/or phosphatase inhibitors.

7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not use kit or components if it has exceeded the expiration date on the kit labels.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

8. TECHNICAL HINTS

- A visualized color change right after adding the reaction buffer indicates fast reaction. Samples, therefore, should be further diluted in the appropriate sample dilution buffers.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample and reagent additions.
- As a guide, typical ranges of sample concentration for commonly used sample types are shown below in Sample Preparation (section 10).
- All samples should be mixed thoroughly and gently.
- Avoid multiple freeze/thaw of samples.
- When generating sample dilution series, it is advisable to change pipette tips after each step.
- This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.

9. REAGENT PREPARATION

Equilibrate all reagents to room temperature (18-25°C) prior to use.

9.1 1X Wash Buffer

Prepare Wash Buffer by adding 20 mL 10X Buffer to 180 mL nanopure water. Mix gently and thoroughly.

9.2 1X Incubation Buffer

Prepare 1X Incubation Buffer by adding 5 mL 10X Blocking Buffer to 45 mL 1X Wash Buffer. Mix gently and thoroughly.

9.3 200X NADPH

Resuspend the lyophilized 200X NADPH by adding 110 µL nanopure water. Mix gently and thoroughly. After resuspension any unused 200X NADPH should be aliquoted and stored at -20°C. Avoid multiple freeze/thaws.

9.4 Reaction Buffer

Prepare the Reaction Buffer immediately prior to use. Prepare 1.8 mL Reaction Buffer for each 8 well strip used. Use the table below for instructions on how to prepare the necessary volume of Reaction Buffer:

Note: Add the 200X NADPH to the Reaction Buffer last.

# of strips	Nanopure Water (µL)	5X Activity Solution (µL)	200X DTNB (µL)	200X NADPH (µL)	Total (mL)
1	1,422	360	9	9	1.8
2	2,844	720	18	18	3.6
3	4,266	1,080	27	27	5.4
4	5,688	1,440	36	36	7.2
5	7,110	1,800	45	45	9
6	8,532	2,160	54	54	10.8
7	9,954	2,520	63	63	12.6
8	11,376	2,880	72	72	14.4
9	12,798	3,240	81	81	16.2
10	14,220	3,600	90	90	18
11	15,642	3,960	99	99	19.8
12	17,064	4,320	108	108	21.6

10. SAMPLE PREPARATION

TYPICAL SAMPLE DYNAMIC RANGE -

Typical working ranges	
Sample Type	**Range (µg/mL)**
Hela Cell Lysate	5 – 500
HepG2 Cell Lysate	100 – 1
Human Heart Homogenate (HHH)	100 – 1
Human Liver Homogenate (HLH)	100 – 1

10.1 Preparation of extracts from cell pellets

10.1.1 Collect non adherent cells by centrifugation or scrape to collect adherent cells from the culture flask. Typical centrifugation conditions for cells are 500 x g for 5 minutes at 4ºC.

10.1.2 Rinse cells twice with PBS.

10.1.3 Solubilize cell pellet at $2x10^7$cell/mL in Extraction Buffer.

10.1.4 Incubate on ice for 20 minutes. Centrifuge at 18,000 x g for 20 minutes at 4°C. Transfer the supernatants into clean tubes and discard the pellets. Assay samples immediately or aliquot and store at -80°C.

10.1.5 Quantify protein concentration and dilute samples to within working range of the assay in 1X Incubation Buffer.

10.2 Preparation of extracts from tissue homogenates

10.2.1 Tissue lysates are typically prepared by homogenization of tissue that is first minced and thoroughly rinsed in PBS to remove blood (dounce homogenizer recommended).

10.2.2 Homogenize 100 to 200 mg of wet tissue in 500 µL – 1 mL of the Extraction Buffer. For lower amounts of tissue adjust volumes accordingly.

10.2.3 Incubate on ice for 20 minutes. Centrifuge at 18,000 x g for 20 minutes at 4°C. Transfer the supernatants into clean tubes and discard the pellets. Assay samples immediately or aliquot and store at -80°C.

10.2.4 Quantify protein concentration and dilute samples to within working range of the assay in 1X Incubation Buffer.

11. PLATE PREPARATION

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents.
- Unused well strips should be returned to the plate packet and stored at 4°C.
- For each assay performed, a minimum of 2 wells must be used as the zero control.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).

12. ASSAY PROCEDURE

- **Equilibrate all materials and prepared reagents to room temperature prior to use.**
- **It is recommended to assay all controls and samples in duplicate.**

12.1 Prepare all reagents as directed in section 9 & 10.

12.2 Add 100 µL sample into each well.

12.3 Incubate for 2 hours at room temperature.

12.4 Wash each well with 3 x 300 µL 1X Wash Buffer. Wash by aspirating or decanting from wells then dispensing 300 µL 1X Wash Buffer into each well. Complete removal of liquid at each step is essential for good performance. After the last wash invert the plate and blot it against clean paper towels to remove excess liquid.

12.5 Add 200 µL of Reaction Buffer to each well and proceed directly to the next step.

12.6 Immediately record the yellow color development with elapsed time in the microplate reader prepared with the following settings:

Mode:	Kinetic
Wavelength:	412 nm
Time:	up to 45 min.
Interval:	20 sec.
Shaking:	Shake before and between readings

Alternative– In place of a kinetic reading, at a ***user defined time point***, record the endpoint O.D. data at 412 nm.

13. CALCULATIONS

Thioredoxin Reductase 1 activity in each well is proportional to the increase in absorbance at 412 nm within each well. Thioredoxin Reductase 1 specific activity is calculated by subtracting the OD value of the sample well from the OD value of the background well. The activity is expressed as the change in absorbance per minute per amount of sample loaded into the well. Examine the linear rate of increase in absorbance at 412 nm over time. Most microplate software is capable of performing this function.

14. TYPICAL DATA

An example is shown below where the rate/slope is calculated between different time points.

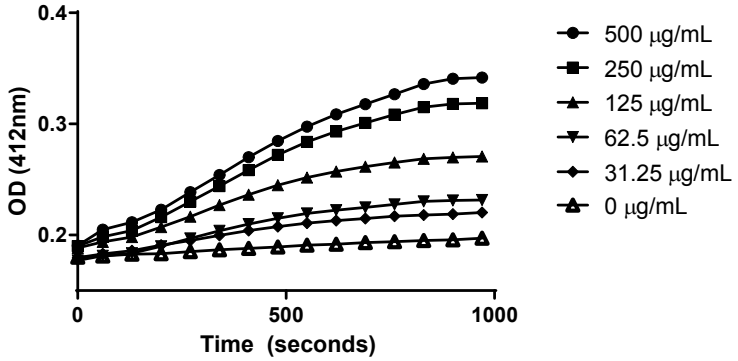


Figure 1. Raw data from HeLa cell extract. After the rate/slope of each lane is extracted from the linear range of the time point data, it is expressed as rate (mOD/min) per microgram of cell lysate added per well. The extinction for the DTNB dye is 9.9/ mM / well.

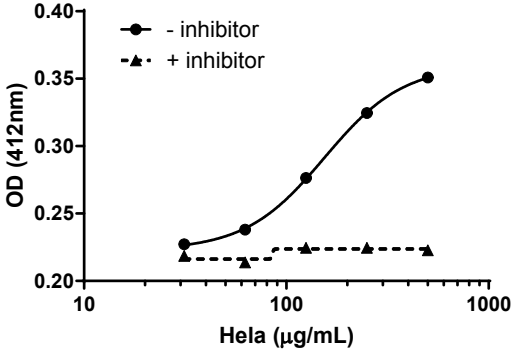


Figure 2. Thioredoxin Reductase 1 activity in HeLa cell lysates with or without the addition of a thioredoxin reductase activity inhibitor, aurothiomalate (ATM), at 20 µM. The data shown above was collected at the endpoint after 30 minutes.

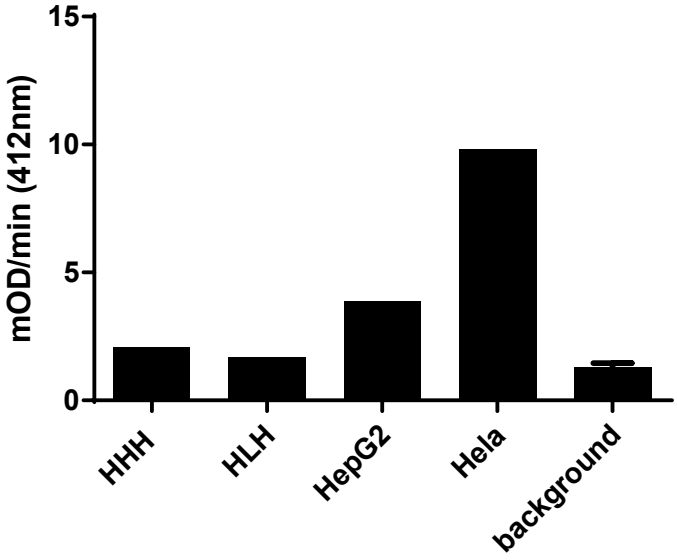


Figure 3. The assay was used to determine the Thioredoxin Reductase 1 activity in a series of normal cell lysates and tissue homogenates loaded at 250 µg/mL. The data shown above was connected kinetically.

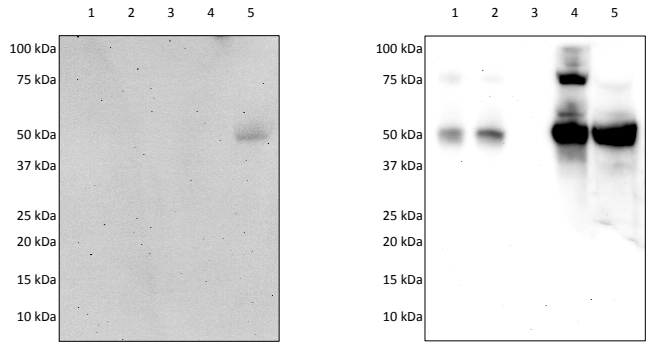
PRECISION –

	Intra-Assay	Inter-Assay
n=	4	4
%CV	2.9	3.2

15. ASSAY SPECIFICITY

To demonstrate assay specificity to isoform 1 of Thioredoxin Reductase, the in-well extraction (IWE) technique was used to visualize the isolated proteins via Western Blot. HeLa lysates were added to the precoated plate included in the kit. The lysates were allowed to incubate for two hours and were then washed before extracting the bound analyte with SDS. The extracted proteins were then analyzed with Western Blot using SDS-PAGE.

4A **4B**



Lane 1: In-well extracted recombinant TXNRD1
Lane 2: In-well extracted HeLa lysate
Lane 3: Blank
Lane 4: Recombinant TXNRD1 (100 ng)
Lane 5: Hela lysate (60 µg)

Figure4. Thioredoxin Reductase 1 assay specificity shown by Western Blot. Both SDS-PAGE membranes were loaded identically. **A)** Membrane blotted with an Anti-TXNRD1 specific antibody. **B)** Membrane blotted with an Anti-TXNRD2 specific antibody (ab180493). Although the overall signal is lower in Figure 4B, the lack of signal in the in-well extracted HeLa sample (Lane 2) illustrates that the assay specifically captures TXNRD1. Additionally, the lack of signal against recombinant TXNRD1 (Lane 4) indicates that the Western Blot antibody in 4B is specific to TXNRD2.

16. TROUBLESHOOTING

Problem	Cause	Solution
Assay not working	Assay buffer at wrong temperature	Assay buffer needs to be at room temperature
	Component missed in the Reaction Buffer	Prepare fresh buffers and follow protocol exactly
	Insufficient amount of enzyme	Try loading samples at a higher concentration
Unexpected results	Plate read at incorrect wavelength	Use appropriate reader and filter settings described in datasheet
	Sample readings are outside linear range	Adjust concentrations of samples to be within the linear range of the assay
	Sample prepared in an unsuitable extraction reagent	Use the extraction buffer included in the kit to prepare lysates
Inconsistent readings	Sample has undergone too many freeze/ thaw cycles	Aliquot samples to reduce the number of freeze-thaw cycles
	Samples are too old or incorrectly stored	Use freshly made samples and store at recommended temperature until use
	Kit components not fully thawed prior to beginning the assay	Wait for components to thaw completely and gently mix prior use
	Inaccurate Pipetting	Check pipettes
	Air bubbles in wells	Air bubbles will interfere with readings; try to avoid producing air bubbles and always remove bubbles prior to reading plates
	Use of other reagents than those provided with the kit	Use fresh components from the same kit

17. **NOTES**

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