Thioredoxin Fluorometric Activity Assay Kit

Item No. 500228

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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -80°C kit. After opening the kit, store individual components as stated below.

Item Number	Item	Quantity	Storage
32584	Trx Assay Buffer (10X)	1 vial/5 ml	-20°C
32587	NADPH Assay Reagent	1 vial	-20°C
32585	Trx Reductase	1 vial/80 µl	-80°C
32586	Trx Positive Control	1 vial/10 µl	-80°C
32588	Eosin Standard	3 vials	-20°C
15495	Eosin-Labeled Insulin	1 vial	-20°C
400091	Half-Volume 96-Well Solid Plate (black)	1 plate	RT
400012	96-Well Cover Sheet	1 ea	RT

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.

This kit may not perform as described if any reagent or procedure is replaced or modified.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Email: techserv@caymanchem.com

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 as directed in the **Materials Supplied** section, on page 3, 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 fluorescence with excitation and emission wavelengths of 520 and 560 nm, respectively
2. Adjustable pipettes; multichannel or repeating pipettor recommended
3. A source of pure water; glass-distilled water or HPLC-grade water is acceptable. *NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000).*
4. Protease inhibitor; recommended for sample preparation and dilution *NOTE: Nuclear Extraction Protease Inhibitor Cocktail (100X) is available for purchase from Cayman (Item No. 10009303).*

INTRODUCTION

Background

Thioredoxin (Trx) is a thiol-disulfide oxidoreductase and part of the antioxidant thioredoxin system that is involved in the maintenance of cellular thiol redox homeostasis.[1] Two isoforms, Trx1 and Trx2, are ubiquitously expressed and localized primarily to the cytoplasm and mitochondria, respectively, while a third isoform, Trx3, is expressed only in the testis.[2,3] Each isoform contains active site cysteine residues, and Trx1 contains additional cysteine residues that can form dimers.[4,5] During the catalytic cycle, the active site cysteines are oxidized to a disulfide upon reduction of oxidized protein disulfide substrates and are subsequently restored to their reduced state by thioredoxin reductase (TrxR) and NADPH.[3] Trx provides protection against oxidative stress by scavenging reactive oxygen species (ROS), regulating activation of redox-sensitive transcription factors, and increasing the expression of superoxide dismutase (SOD).[2] It also inhibits apoptosis through redox-sensitive binding and regulation of apoptosis signal-regulating kinase 1 (ASK1). Trx levels are increased in a variety of cancers, and when combined with low levels of the negative Trx regulator Trx-interacting protein, are associated with increased recurrence and lower survival rates in patients with gastric cancer.[6] Inhibition of Trx increases apoptosis in cancer cells *in vitro* and inhibits intratumor angiogenesis *in vivo* in animal models of cancer.[7]

About This Assay

Cayman's Thioredoxin Fluorescent Activity Assay Kit provides a convenient method of detecting Trx activity in cell lysate. Measurement of the Trx activity is based on the reduction of eosin-labeled insulin disulfides by Trx.[8] The reduction of insulin disulfides results in a release of eosin and an increase in fluorescence that can be detected at 560 nm. Trx becomes oxidized during this process, and oxidized Trx is recycled back to the reduced state by Trx reductase. Under circumstances in which the Trx activity rate is limiting, the rate of fluorescence increase is directly proportional to the Trx activity in the sample.

PRE-ASSAY PREPARATION

Reagent Preparation

1. **Trx Assay Buffer (10X) - (Item No. 32584)**

   This vial contains 5 ml of concentrated Trx Assay Buffer. Mix 3 ml of Trx Assay Buffer (10X) with 27 ml of pure water to make 30 ml of Assay Buffer (1X), which will be stable for at least one week when stored at 4°C.

2. **NADPH Assay Reagent - (Item No. 32587)**

   This vial contains lyophilized NADPH. Immediately prior to performing the assay, reconstitute the entire contents of the vial with 2 ml of Assay Buffer (1X). The reconstituted NADPH solution will be stable for one week when stored at -20°C.

3. **Eosin-Labled Insulin - (Item No. 15495)**

   This vial contains a lyophilized powder of Eosin-Labeled Insulin, a Trx substrate. Immediately prior to performing the assay, reconstitute the entire contents of the vial with 2 ml of Assay Buffer (1X). The reconstituted Eosin-Labeled Insulin will be stable for one week when stored at -20°C.

4. **Trx Reductase - (Item No. 32585)**

   This vial contains 1.6 U of Trx Reductase in 80 µl buffer. To prepare 100 µl of diluted Trx Reductase, which is a sufficient volume for 10 wells, thaw the enzyme on ice and mix 10 µl with 90 µl Assay Buffer (1X). Scale volumes as needed for the number of wells to be assayed. Discard any unused diluted enzyme. Aliquot the undiluted enzyme and store at -80°C where it will be stable for at least six months, and avoid repeated freeze and thaw cycles.

5. **Trx Positive Control - (Item No. 32586)**

   This vial contains 12 µg of Trx in 10 µl buffer. Thaw the enzyme on ice and mix 5 µl with 995 µl Assay Buffer (1X); this diluted Trx Positive Control will provide a volume sufficient for 50 wells. Scale volumes as needed for the number of wells to be assayed. Discard any unused diluted positive control. Aliquot the undiluted positive control and store at -80°C where it will be stable for at least six months. Avoid repeated freeze and thaw cycles.

6. **Eosin Standard - (Item No. 32588)**

   Each vial of Eosin Standard contains lyophilized standard. Immediately prior to performing the assay, reconstitute the contents of one vial with 2 ml of Assay Buffer (1X) to make 10 µM of Eosin Standard solution. This will be used for making the eosin standard curve dilutions. Discard any unused reconstituted standard.

Sample Preparation

Cell Lysate

1. Collect cells (~5 x 10^6) by centrifugation (*i.e.*, 1,000-2,000 x g for 10 minutes at 4°C). For adherent cells, use a cell scraper instead of proteolytic enzymes.
2. Homogenize the cell pellet in 0.5-1 ml cold buffer (*i.e.*, 100 mM Tris-HCl, pH 7.5, 1 mM EDTA), supplemented with protease inhibitor.
3. Centrifuge at 10,000 x g for 15 minutes at 4°C.
4. Remove the supernatant and store on ice. If not assaying on the same day, store the samples at -80°C.
5. Determine protein concentration. It is recommended to use Cayman's Protein Determination (BCA) Kit (Item No. 701780) or a similar protein determination assay to measure the total protein concentration.
6. Dilute cell lysate to 1-0.5 mg/ml with Assay Buffer (1X), supplemented with protease inhibitor, prior to the assay.

ASSAY PROTOCOL

Preparation of Assay-Specific Reagents

Eosin Standard

To prepare the standard for use in the assay: Obtain seven clean test tubes and label them #1-7. Aliquot the Eosin Standard and diluent to each tube as described in Table 1, below.

Tube	Eosin Standard (µl)	Assay Buffer (1X) (µl)	Final Eosin Concentration (µM)
1	0	400	0
2	50	450	1
3	100	400	2
4	200	300	4
5	300	200	6
6	400	100	8
7	400	0	10

Table 1. Preparation of eosin standards

Pipetting Hints

- It is recommended that a multichannel pipette be used to deliver reagents to the wells. This saves time and helps maintain more precise incubation times.
- 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

- All reagents except Trx Reductase and Trx Positive Control must be equilibrated to room temperature before beginning the assay. Trx Reductase and Trx Positive Control need to be thawed on ice.
- The final volume of the assay is 100 µl in all of the wells.
- Use the diluted assay buffer in the assay.
- It is not necessary to use all the wells on the plate at one time.
- We recommend assaying samples in triplicate, but it is at the user's discretion to do so.
- 11 samples can be assayed in triplicate or 19 in duplicate.
- Protect the plate from light after addition of Eosin-Labeled Insulin.
- The assay requires pre-incubation at 37°C.
- Monitor the fluorescence with excitation and emission wavelengths of 520 and 560 nm, respectively, for 60 minutes at room temperature.

Plate Set Up

There is no specific pattern for using the wells on the plate. It is recommended that at least two wells be designated for the Trx Positive Control. It is suggested that each sample be assayed in triplicate and that the contents of each well are recorded on the template sheet provided on page 22. A typical layout of samples to be measured in triplicate is provided below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	Blk	Blk	4	4	8	8	12	12	16	16
B	S2	S2	PC	PC	B4	B4	B8	B8	B12	B12	B16	B16
C	S3	S3	1	1	5	5	9	9	13	13	17	17
D	S4	S4	B1	B1	B5	B5	B9	B9	B13	B13	B17	B17
E	S5	S5	2	2	6	6	10	10	14	14	18	18
F	S6	S6	B2	B2	B6	B6	B10	B10	B14	B14	B18	B18
G	S7	S7	3	3	7	7	11	11	15	15	19	19
H	Blk	Blk	B3	B3	B7	B7	B11	B11	B15	B15	B19	B19

S1-S7 = Standard Wells
PC = Positive Control Wells
Blk = Blank Wells
1-19 = Sample Wells
B1-B19 = Background Wells

Figure 1. Sample plate format

Performing the Assay

1. **Standard Wells**: Add 100 µl of Eosin Standard (tubes 1-7) per well to the designated wells on the plate (see Sample Plate Format, Figure 1, page 11).
2. **Positive Control (PC) Wells**: Add 40 µl of Assay Buffer (1X), 10 µl of diluted Trx Reductase, and 20 µl of diluted Trx Positive Control to the designated wells on the plate.
3. **Sample Wells**: Add 40 µl of Assay Buffer (1X), 10 µl of diluted Trx Reductase, and 20 µl of sample to the designated wells on the plate.
4. **Sample Background Wells**: Add 50 µl of Assay Buffer (1X) and 20 µl of sample to the designated wells on the plate.
5. **Blank (Blk) Wells**: Add 60 µl of Assay Buffer (1X) and 10 µl of diluted Trx Reductase to the designated wells on the plate.

Wells	Assay Buffer (1X)	Trx Reductase	Positive Control	Sample
Positive Control	40 µl	10 µl	20 µl	
Sample	40 µl	10 µl		20 µl
Sample Background	50 µl			20 µl
Blank	60 µl	10 µl		

Table 2. Pipetting summary

6. Add 10 µl of reconstituted NADPH Assay Reagent to all of the wells except the standard wells, pipet to mix, cover the plate with the 96-Well Cover Sheet (Item No. 400012), and incubate at 37°C for 30 minutes.
7. Remove plate cover and add 20 µl of reconstituted Eosin-Labeled Insulin to all of the wells except the standard wells, and mix.
8. Measure the fluorescence at excitation and emission wavelengths of 520 and 560 nm, respectively, once every minute for 60 minutes at room temperature.

ANALYSIS

Calculations

1. Determine the average fluorescence of each standard at time 0.
2. Plot the average relative fluorescence unit (RFU) of each standard as a function of the final concentration of eosin from Table 1, page 9. See Figure 2 for a typical standard curve. The slope of this curve is called the f-slope.

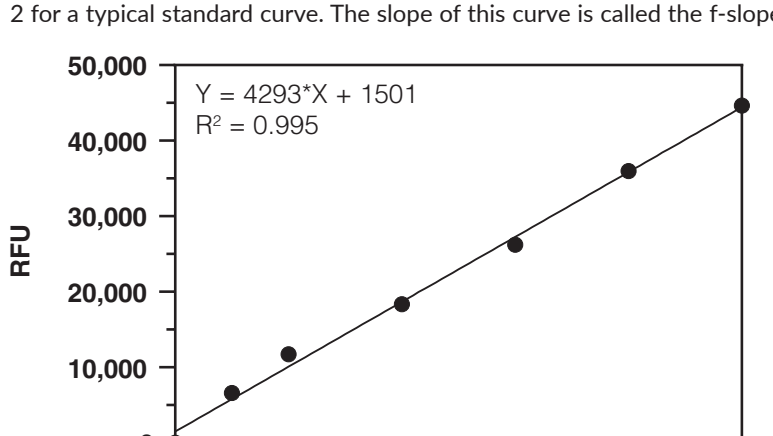

Figure 2. Eosin standard curve
NOTE: The actual slope will vary depending on gain settings and equipment variances.

3. Determine the change in RFU per minute for each PC, sample, sample background, and Blk well by plotting the average fluorescence values as a function of time (see Figure 3, page 16), and either:

   Obtain the slope (rate) of the linear portion of the curve (typically after the first 10-20 minutes)

   OR

   Select two points on the linear portion of the curve and determine the change in fluorescence during that time using the following equation:

$$\frac{\text{RFU}}{\text{min.}} = \frac{\text{RFU (Time 2) - RFU (Time 1)}}{\text{Time 2 (min.) - Time 1 (min.)}}$$

4. Subtract the rate (RFU/min.) of the Blk wells from the sample and PC wells to calculate the c-slope.
5. Subtract the rate (RFU/min.) of the background wells from the c-slope of the corresponding sample wells.
6. Use the following formula to calculate the Trx activity.

$$\text{TRX Activity}\left(\frac{\mu\text{M}}{\text{min.}}\right) = \frac{\text{c-slope - background}\left(\frac{\text{RFU}}{\text{min.}}\right)}{\text{f-slope}} \times \text{sample dilution}$$

7. Optional: divide Trx activity by protein concentration to determine Trx specific activity.

NOTE: If the sample background slope value is higher than 15% of the sample slope value, the linearity of the assay may be affected and sample purification may be required. The purification protocol will need to be determined by the end user.

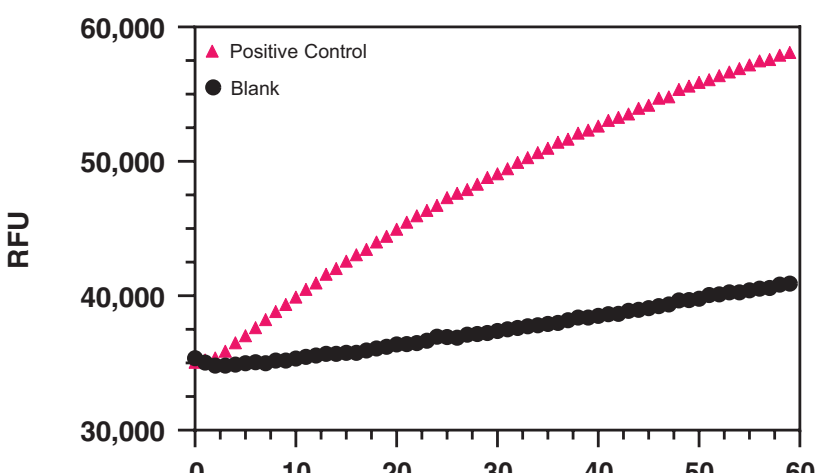

Figure 3. Trx Positive Control Activity

Performance Characteristics

Sensitivity:

The limit of detection for this assay is 0.01 ±0.002 µM/min.

Precision:

When a series of 40 Blk and Trx Positive Control measurements were performed on the same day under the same experimental conditions, the intra-assay coefficients of variation were 6 and 2%, respectively. When a series of sample measurements were performed on three different days under the same experimental conditions, the inter-assay coefficient of variation was 13.3%.

Interferences

The following reagents were tested for interference in the assay:

Reagent		Will Interfere (Yes or No)
Buffers	HEPES	Yes
	Phosphate	No
	1X Phosphate-Buffered Saline	No
	Tris	No
Detergents	Polysorbate 20 (1%)	Yes
	Polysorbate 20 (0.1%)	Yes
	Triton X-100 (1%)	Yes
	Triton X-100 (0.11%)	Yes
	SDS (1%)	Yes
	SDS (0.1%)	Yes
	CHAPS (1%)	Yes
	CHAPS (0.1%)	Yes
Chelators	EDTA (1 mM)	No
	EGTA (1 mM)	No
Protease Inhibitors/Enzymes	Antipain (10 µg/ml)	No
	Chymostatin (10 µg/ml)	No
	Leupeptin (10 µg/ml)	No
	Trypsin (10 µg/ml)	Yes
Solvents	DMSO (5%)	No
	Ethanol (5%)	No
	Methanol (5%)	No
Others	BSA (0.1%)	Yes
	DTT (1 mM)	Yes
	Glycerol (10%)	No
	Sodium Chloride (150 mM)	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 fluorescence detected above background in the sample wells	A. Trx activity is too low to detect B. The sample does not contain Trx, or the sample contains an interfering reagent	A. Repeat the assay using a more concentrated sample B. Check for possible interference (see page 19)
The fluorometer exhibited 'MAX' values for the wells	The *gain* setting is too high	A. Reduce the *gain* and repeat the assay. B. Establish the gain with the Eosin Standard prior to assaying samples
The fluorescence of the sample wells was higher than the last standard	A. Trx concentration in the sample was too high B. Sample was too concentrated	Dilute the samples with Assay Buffer (1X), supplemented with protease inhibitor, and repeat the assay

References

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4. Watanabe, R., Nakamura, H., Masutani, H., *et al.* Anti-oxidative, anti-cancer and anti-inflammatory actions by thioredoxin 1 and thioredoxin-binding protein-2. *Pharmacol. Ther.* **127(3)**, 261-270 (2010).
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7. Mohammadi, F., Soltani, A., Ghahremanloo, A., *et al.* The thioredoxin system and cancer therapy: A review. *Cancer Chemother. Pharmacol.* **84(5)**, 925-935 (2019).
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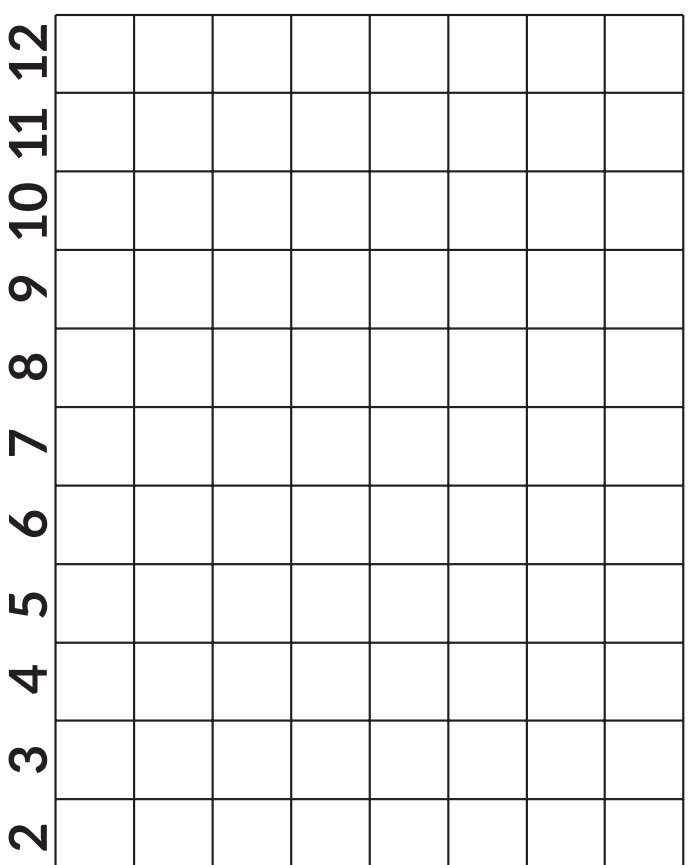

NOTES

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.