Product Manual

PROTEOSTAT® Thioredoxin-1 Assay Kit

Catalog #: ENZ-51033-KP002

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Product Manual

TABLE OF CONTENTS

Please read entire booklet before proceeding with the assay.

Carefully note the handling and storage conditions of each kit component.

Please contact Enzo Life Sciences Technical Support if necessary.

INTRODUCTION

The thioredoxin family (Trx) is a group of small globular proteins, distributed in the cytosol, nucleus and mitochondria of eukaryotic cells. The enzyme family is capable of catalyzing disulfide exchange, leading to the rearrangement of disulfide bonds within proteins. As oxidoreductases, Trx family members serve as antioxidants to maintain the redox homeostasis of the cell. The enzymes possess an active site composed of two neighboring cysteine residues in a conserved active site motif, CGPC. To date, three different Trx isoenzymes have been identified: Trx-1 (cytosolic Trx), Trx-2 (mitochondria Trx) and SpTrx (Trx highly expressed in spermatozoa).[1,2] Numerous *in vitro* substrates for Trx have been identified, including coagulation factors, ribonuclease, choriogonadotropins, glucocorticoid receptor, and insulin. Reduction of insulin has typically been employed in *in vitro* assays of thioredoxin activity. Physiologically, Trx family members are maintained in the reduced state by the flavoenzyme, thioredoxin reductase, in an NADPH-dependent fashion. Trx serves as an electron donor to peroxidases and ribonucleotide reductase.

Trx-1, the most commonly investigated member of the mammalian Trx family, is thought to be a defensive protein, induced by various cellular stresses and possessing anti-oxidative, anti-apoptotic and anti-inflammatory activities. Trx-1 activity has been monitored under a variety of clinical conditions. For example, Trx-1 is known to inhibit pathological hypertrophy in the heart. Understanding the role that Trx-1 plays under physiological and pathophysiological situations is crucial for the development of rational therapeutic approaches for manipulating the activity of Trx-1.[3]

Enzo Life Sciences' PROTEOSTAT® Thioredoxin-1 Assay Kit provides a simple, homogenous assay for screening modulators of Trx-1 enzymatic activity in microplates. This is accomplished by monitoring the Trx-1 catalyzed reduction of insulin in the presence of Dithiothreitol (DTT), resulting in the formation of insulin aggregates which then bind avidly to the red-emitting fluorogenic PROTEOSTAT® PDI detection dye (see Figure 1). The fluorescence-based assay provides a vastly improved assay signal window, improved lower detection limit, and superior Z'-score (>0.8) relative to turbidity measurements. Intra-plate and inter-plate CVs using this assay are typically 3-6%. Human recombinant thioredoxin-1 enzyme and the thioredoxin inhibitor, aurothiomalate, are included in the kit, as well as all reagents necessary for monitoring changes in Trx-1 activity.

The PROTEOSTAT® Thioredoxin-1 Assay Kit is capable of providing a quantitative readout of Trx-1 enzymatic activity in a robust and high-throughput fashion and can be applied to identification of Trx-1 inhibitors from chemical libraries.

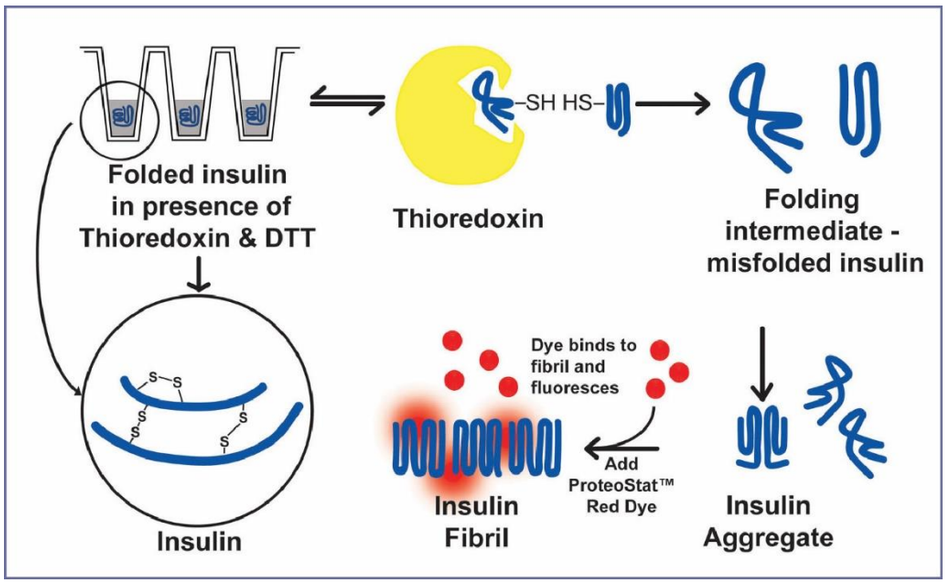

Figure 1. Schematic diagram of PROTEOSTAT® Thioredoxin-1 Assay Kit.

MATERIALS SUPPLIED

Reagent	Quantity
PROTEOSTAT® PDI Detection Reagent	20 µL
Thioredoxin-1 (recombinant, human)	2 vials x 80 µL
Insulin	2 vials x 1.8 µmol
Aurothiomalate (Inhibitor)	2 vials x 25 nmol
PBE Buffer	25 mL
Stop Reagent	1 mL
DTT	2 vials x 1.3 mL
Deionized Water	5 mL

Reagants require separate storage conditions.

STORAGE

All reagents are shipped on dry ice. Upon receipt, remove the vial of Thioredoxin-1 from the box and store at -80°C. Store the remaining reagents at ≤-20°C protected from light. When stored properly, these reagents are stable for at least twelve months. Avoid repeated freezing and thawing. The reagents provided in the kit are sufficient for 2 x 96-well microplates.

OTHER MATERIALS NEEDED

- Fluorescence microplate reader with a filter set or monochromator setting of Excitation = ~500nm / Emission = ~603nm.
- 96-well or 384-well microplates: black wall microplates, preferably with clear bottom.
- Calibrated, adjustable precision pipetters, preferably with disposable plastic tips.

SAFETY WARNINGS & PRECAUTIONS

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

Handle with care

1. Some components of this kit may contain hazardous substances. Reagents can be harmful if ingested or absorbed through the skin and may cause irritation to the eyes. They should be treated as possible mutagens, should be handled with care and disposed of properly.
2. Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas. All blood components and biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.
3. To avoid photobleaching, perform all manipulations in low light environments or protected from light by other means.

METHODS AND PROCEDURES

***NOTES**: (1) The procedures described in this manual are NOT suitable for detection of Trx activity in complex cell or tissue lysates.*

(2) Allow all reagents to thaw at room temperature before starting with the procedures. Upon thawing, gently hand-mix or vortex the reagents prior to use to ensure a homogenous solution. Briefly centrifuge the vials at the time of first use, as well as for all subsequent uses, to gather the contents at the bottom of the tube.

REAGENT PREPARATION

1. Insulin Working Solution

Insulin is supplied as lyophilized powder (1.8 µmol x 2 vials). Each vial should be reconstituted in 180 µL deionized water to generate a 10 mM stock solution. The 10 mM stock solution should be further diluted in the supplied PBE Buffer to generate a 320 µM working solution of Insulin.

To prepare a 320 µM solution, add 160 µL of the 10 mM solution into 4840 µL of PBE Buffer. Unused solutions of insulin may be stored at -20°C for several weeks.

2. Thioredoxin-1 Working Solution

Two vials of active Thioredoxin-1 (recombinant, human) are provided in the kit. Each vial containing 80 µL of Thioredoxin-1 solution should be diluted with 920 µL of PBE Buffer.

***NOTE**: If an alternate source of thioredoxin-1 to screen for modulators of enzymatic activity is desired, be sure that the enzyme is purified. Thioredoxin-1 should be diluted to a final concentration of 9 units/mL in the assay.*

3. Aurothiomalate Working Solution

Assay validation can be performed using the Trx-1 inhibitor, aurothiomalate. Aurothiomalate is provided in the kit as 2 vials of lyophilized powder (2 x 25 nmol). Each vial contains enough inhibitor control for 1 microplate. As needed, the contents of one vial should be reconstituted in 100 µL deionized water to generate a 250 µM stock solution.

To observe at least 50% inhibition of Trx-1 activity, a 25 µM final concentration is recommended for use.

Discard unused stock solution of aurothiomalate.

4. Stop Reagent Working Solution

***NOTE**: Avoid repeated freeze/thaw cycles for Stop Reagent.*

For each 96-well plate, prepare 1.0 mL of working solution of Stop Reagent as follows: Add 400 µL of Stop Reagent to 0.6 mL deonized water. Mix well.

5. PROTEOSTAT® PDI Detection Reagent Working Solution

***NOTE**: The PROTEOSTAT® PDI Detection Reagent is light sensitive. Avoid direct exposure of the reagent to intense light. Aliquot and store unused reagent at -20°C, protected from light. Avoid repeated freeze/thaw cycles.*

For each 96-well plate to be assayed, prepare the working solution of PROTEOSTAT® PDI Detection Reagent as follows: Add 10 µL of the provided PROTEOSTAT® PDI Detection Reagent to 1 mL of PBE Buffer. Mix well.

THIOREDOXIN-1 ACTIVITY ASSAY

***NOTES**: (1) The procedure described below is a homogenous, mix-and-read assay for 96-well plate applications. No removal of assay buffer from the wells should be performed after addition of PROTEOSTAT® PDI Detection Reagent.*

(2) For 384-well plate applications, the volume for each step should be reduced by 50% from 96-well plate assay.

1. Prepare the Thioredoxin-1 Assay Master Mix by combining the reagents listed in Table 1. The volumes of reagents indicated in Table 1 are sufficient for a 96-well plate.

Table 1. Thioredoxin-1 Assay Master Mix

Reagent	Vol. per 96-well plate
Dilute insulin solution (from Reagent Preparation step 1)	5 mL
Thioredoxin-1 working solution (from Reagent Preparation step 2)	1 mL
PROTEOSTAT® PDI Detection Reagent working solution (from Reagent Preparation step 5)	1 mL
Total	**7 mL**

***NOTE**: If using thioredoxin-1 from another source, make sure to pre-dilute the enzyme such that the final concentration in the assay is about 9 units/mL.*

***NOTE**: PROTEOSTAT® PDI Detection Reagent is light sensitive. Avoid direct exposure of the reagent to intense light.*

2. Dispense 10 µL of test agent, or buffer, to each well. As a positive control for Trx-1 inhibition, dispense 10 µL of Aurothiomalate working solution (from Reagent Preparation step 3) into wells reserved for this purpose.
3. Dispense 70 µL of Thioredoxin-1 Assay Master Mix (from step 1, above) and 10 µL of DTT to each well of the plate.
4. Incubate the microplate(s) for 30 minutes at room temperature, protected from light.
5. Dispense 10 µL of Stop Reagent working solution (from Reagent Preparation step 4).

6. Read the generated signal with a fluorescent microplate reader using an excitation setting of about 500nm and an emission filter of about 603nm.

   ***NOTE**: In the absence of enzyme, the fluorescence value should be subtracted from the values for wells containing Trx-1.*

APPENDICES

MICROPLATE FILTER SET SELECTION

The selection of optimal settings for a fluorescence microplate reader application requires matching the monochromator or optical filter specifications to the spectral characteristics of the dyes employed in the analysis. Please consult your instrument or filter set manufacturer for assistance in selecting optimal filter sets. Pre-designed filter sets for Texas Red should work well for this application. For monochromator-based detection, a slit width of approximately 9nm is recommended.

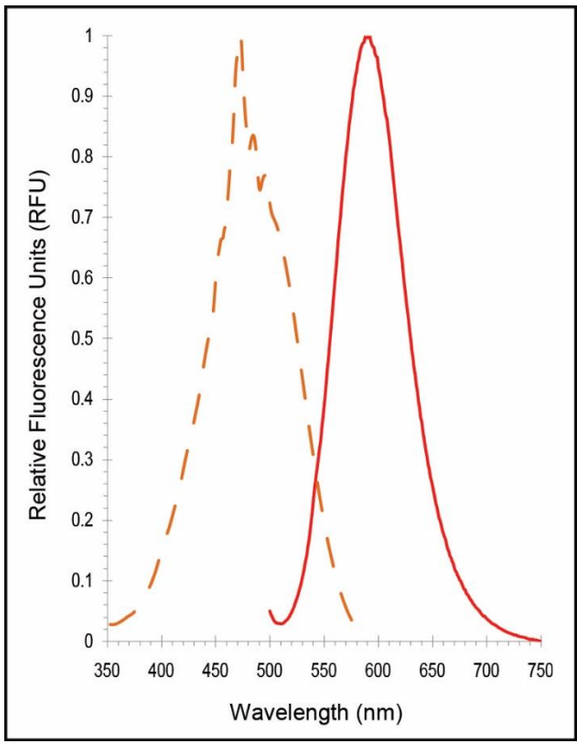

Figure 2. Absorption and fluorescence emission spectra for PROTEOSTAT® PDI Detection Reagent. All spectra were determined in PBE Buffer.

EXPECTED RESULTS

The catalytic reduction of insulin by thioredoxin-1 (Trx-1) in the presence of DTT results in the formation of reduced insulin chains, which spontaneously aggregate. The insulin aggregates, in turn, bind avidly to the PROTEOSTAT® PDI detection dye. The PROTEOSTAT® PDI detection dye is essentially non-fluorescent until it binds to the aggregated protein, wherein it emits brightly at 603nm. Relative to analogous turbidimetric assays of Trx-1 activity, the fluorescence-based assay provides a vastly improved assay signal window, improved lower detection limit, and superior Z'-factor (>0.8). See **Figure 3**.

In order to validate this fluorescence-based assay, the potency of the Trx-1 inhibitor aurothiomalate was monitored. The IC_{50} of aurothiomalate for Trx-1 activity has previously been shown to be about 30 µM.[4] A dose-response assay for aurothiomalate using the high throughput assay was performed. Concentration response plots were employed to determine the effects of aurothiomalate on Trx-1 activity. These experiments were performed at constant enzyme and substrate concentrations while systematically varying the aurothiomalate concentration. The IC_{50} of the aurothiomalate inhibitor was determined to be 25.9 ± 1.7µM, which is in good agreement with values reported in literature for inhibiting cell growth.[4]

Additionally, intra-plate and inter-plate reproducibility were determined in 96-well microplates. The CV values using the assay were typically determined to be 3-6% (see **Figure 3**).

The PROTEOSTAT® Thioredoxin-1 Assay Kit provides an ideal high-throughput approach, enabling sensitive and accurate screening of modulators of thioredoxin-1 activity. The assay is homogenous, robust and cost-effective. It can potentially be applied to high-throughput screening of Trx-1 inhibitors from chemical libraries, identifying agents useful for modulation of the unfolded protein response (UPR), as well as agents that block pathogen entry into cells.

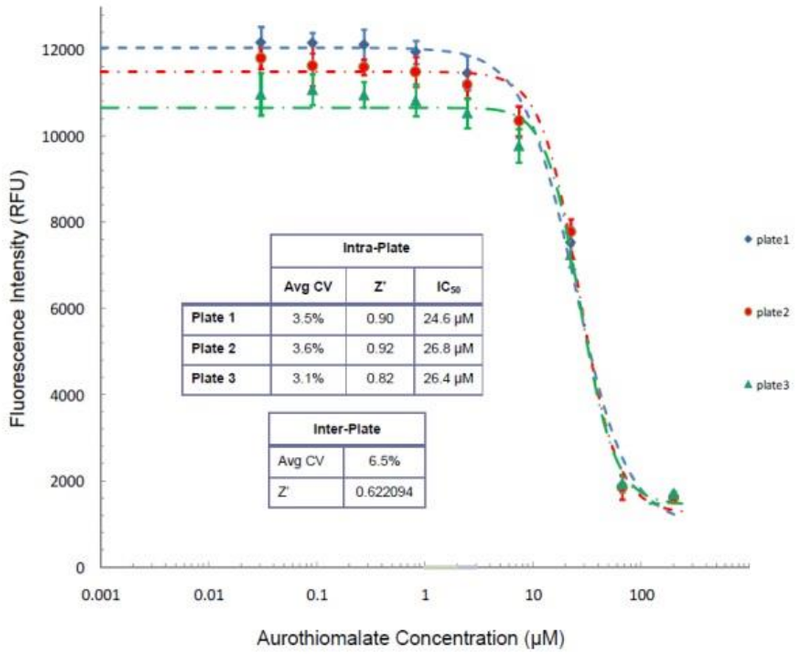

Figure 3. Intra-plate and inter-plate reproducibility using aurothiomalate as an inhibitor. Dose response assay was performed with 0 to 100 µM aurothiomalate added. Reactions were performed as described in Methods and Procedures section. Intra-plate and inter-plate CVs using the assay are typically 3-6%.

REFERENCES

1. Berndt C, Lillig CH, Holmgren A (2008), “Thioredoxins and glutaredoxins as facilitators of protein folding”, Biochimica et Biophysica Acta 1783:641–650.
2. Yamawaki H, Haendeler J, Berk B (2003), “Thioredoxin A Key Regulator of Cardiovascular Homeostasis”, Circ. Res. 93:1029-1033.
3. Burke-Gaffney A, Callister M and Nakamura H (2005), “Thioredoxin: friend or foe inhuman disease?” TRENDS in Pharmacological Sciences 26(8):398-404.
4. Regala, R.P., Thompson, E.A. and Fields, A.P. (2008). “Atypical Protein Kinase Cı Expression and Aurothiomalate Sensitivity in Human Lung Cancer Cells.” Cancer Research 68:5888-5895.

TROUBLESHOOTING GUIDE

Problem	Potential Cause	Suggestion
Poor fluorescence signal observed	Band pass settings are too narrow or not optimal for the fluorescent probe.	Use correct monochromator setting or filter set for the fluorophore. Check Methods and Procedures section of this manual and Appendix A for recommendations.
	PROTEOSTAT® PDI Detection Reagent has been exposed to strong light.	Protect samples from exposure to strong light and analyze them immediately after staining.
	Kit reagent has degraded.	Verify that the reagents are not past their expiration dates before using them.
	Insufficient PROTEOSTAT® PDI dye concentration	Follow the procedures provided in this manual.
	Inappropriate addition of DTT	DTT is required for the reaction. Follow the procedures provided in this manual.
High fluorescent background in the well without Trx-1 enzyme	Inappropriate dye dilution	Follow the procedures provided in this manual. It is important to make certain that there are no particles in the dye. Centrifuge well before use.
Inconsistent results between experiments	Inappropriate Stop Reagent addition	Be sure to pre-incubate with Stop Reagent to terminate both the enzyme reaction and the chemical reaction.
Insulin does not go into solution.	Insulin was not reconstituted in deionized water prior to dilution.	Resuspend Insulin in deionized water before dilution into PBE Buffer.

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