

## **PROTEOSTAT<sup>®</sup> PDI Assay Kit**

Catalog No. ENZ-51024

For screening modulators of protein disulfide isomerase (PDI) enzymatic activity in microplates

ENZ-51024-KP002

2 x 96-well tests

ENZ-51024-KP050

50 tests



# Product Manual

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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.

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## INTRODUCTION

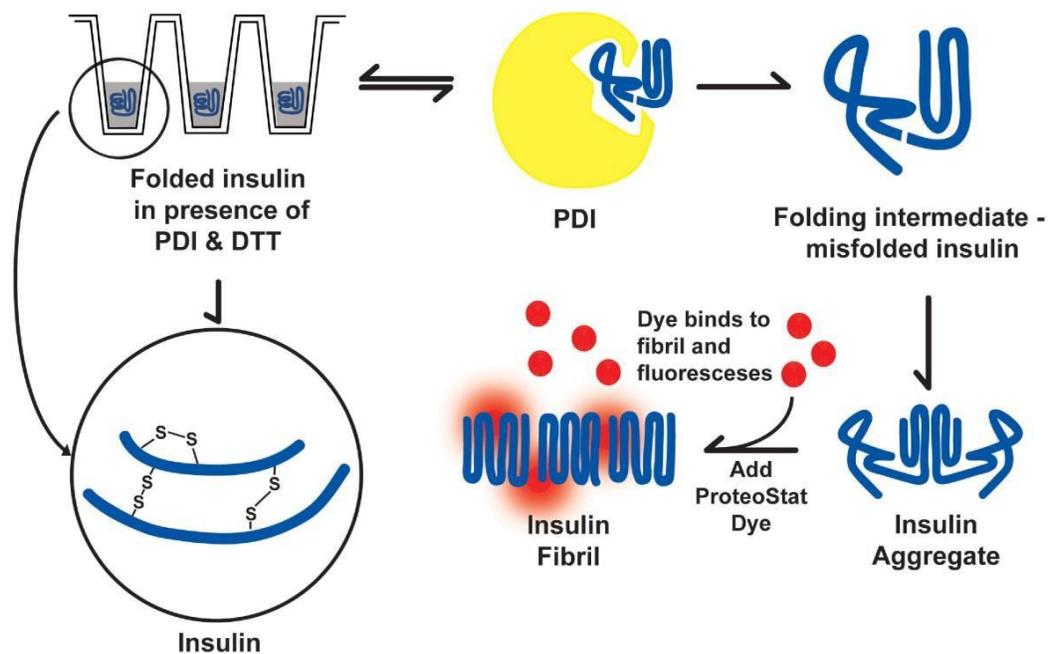
Protein disulfide isomerase (PDI) is a widely expressed enzyme, broadly distributed in eukaryotic tissues. PDI is relatively abundant, being found in the lumen of the endoplasmic reticulum (ER) at concentrations exceeding 400  $\mu\text{M}$ , where it catalyzes the formation and rearrangement of disulfide bonds of secreted proteins. PDI is also known to be secreted from a variety of cell types.

One key strategy for developing novel anti-cancer drugs is to take advantage of the vulnerabilities innate in the intracellular signaling pathways of tumor cells. The activation of cellular stress responses, mediated by the endoplasmic reticulum (ER), promotes survival of cancer cells. The unfolded protein response (UPR) is an important ER stress-mediated phenomenon which rescues the cell by increasing its capacity for protein folding, reducing newly translated protein entry into the ER, and increasing the degradation of unfolded and aggregated proteins.<sup>1</sup> However, ER stress will induce programmed cell death if protein homeostasis mechanisms are insufficient to protect or repair the cell. Since many of the proteins that protect cells against ER stress are PDIs, PDI inhibitors represent an important class of compounds that may enhance the efficacy of chemotherapy in a wide range of cancers.<sup>2-4</sup>

In addition to serving as a redox catalyst and isomerase, PDI-mediated reductive cleavage of disulfide bonds at the cell surface is critical to the entry and subsequent infectivity of a number of disease-causing agents, including human immunodeficiency virus (HIV), cholera toxin, diphtheria toxin, *Chlamydia trachomatis* and *Leishmania chagasi* promastigotes.<sup>5-9</sup> PDI inhibitors, through blocking reductive cleavage of disulfide bonds associated with these pathogens, can prevent infectivity.

Enzo Life Sciences' PROTEOSTAT® PDI Assay kit provides a simple, homogenous assay for screening modulators of PDI enzymatic activity in microplates. This is accomplished by monitoring the PDI-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**). Relative to the analogous turbidimetric assays of PDI activity, the fluorescence-based assay provides a vastly improved assay signal window, improved lower detection limit, and superior Z'-score (>0.8). Intra-plate and inter-plate CVs using this assay are typically 3-5%. Human recombinant PDI enzyme (EC 5.3.4.1) and the PDI inhibitor, bacitracin, are included in the kit, as well as all reagents necessary for monitoring changes in PDI activity.

The PROTEOSTAT<sup>®</sup> PDI Assay Kit is capable of providing a quantitative readout of PDI enzymatic activity in a robust and high-throughput fashion and can be applied to identification of PDI inhibitors from chemical libraries.



**Figure 1.** Schematic diagram of PROTEOSTAT<sup>®</sup> PDI Assay Kit.

## MATERIALS SUPPLIED

Reagent	ENZ-51024-KP002	ENZ-51024-KP050
PROTEOSTAT <sup>®</sup> PDI Detection Reagent	20 $\mu$ L	5 $\mu$ L
PDI (recombinant, human)	2 x 165 $\mu$ L	82.5 $\mu$ L
Insulin (lyophilized)	2 x 1.8 $\mu$ mol	0.9 $\mu$ mol
Bacitracin (Inhibitor Control, Lyophilized)	4 $\mu$ mol	1 $\mu$ mol
PBE Buffer	25 mL	6.25 mL
Stop Reagent	1 mL	0.25 mL
DTT (10 mM)	2 x 1.3 mL	0.65 mL
Deionized Water	5 mL	1.25 mL



Reagents require separate storage conditions.

## STORAGE

All reagents are shipped on dry ice. Upon receipt, remove the vial of PDI from the box and store at  $-80^{\circ}\text{C}$ . Store the remaining reagents at  $\leq -20^{\circ}\text{C}$  protected from light. When stored properly, these reagents are stable for at least twelve months. **Avoid repeated freezing and thawing.**

## OTHER MATERIALS NEEDED

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

## SAFETY WARNINGS & PRECAUTIONS

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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 PDI 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.*

### A. REAGENT PREPARATION

#### 1. Making diluted insulin working solution

Insulin is supplied as lyophilized powder (1.8  $\mu\text{mol}$  x 2 vials for **ENZ-51024-KP002** and 0.9  $\mu\text{mol}$  x 1 vial for **ENZ-51024-KP050**). Each vial should be reconstituted in 180  $\mu\text{L}$  (**ENZ-51024-KP002**) or 90  $\mu\text{L}$  (**ENZ-51024-KP050**) 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  $\mu\text{M}$  working solution of Insulin.

To prepare a 320  $\mu\text{M}$  solution, add 160  $\mu\text{L}$  of the 10 mM solution into 4840  $\mu\text{L}$  of PBE Buffer for **ENZ-51024-KP002** or add 80  $\mu\text{L}$  of the 10 mM solution into 2420  $\mu\text{L}$  of PBE Buffer for **ENZ-51024-KP050**. Unused solutions of insulin may be stored at  $-20^{\circ}\text{C}$  for several weeks.

#### 2. Making PDI working solution

**ENZ-51024-KP002:** Two vials of active PDI (recombinant, human) are provided. Each vial containing 165  $\mu\text{L}$  PDI solution should be diluted with 825  $\mu\text{L}$  of PBE Buffer.

**ENZ-51024-KP050:** One vial of active PDI (recombinant, human) is provided. Each vial containing 82.5  $\mu\text{L}$  PDI solution should be diluted with 412.5  $\mu\text{L}$  of PBE Buffer.

**NOTE:** *If an alternate source of PDI to screen for modulators of enzymatic activity is desired, be sure that the PDI enzyme to be used is purified. The enzyme should be diluted to a final concentration of 2-4 units per ml in the assay.*

### 3. Making Bacitracin working solution

Assay validation can be performed using the PDI inhibitor, Bacitracin. Bacitracin is provided in the kit as lyophilized powder (4  $\mu\text{mol}$  for **ENZ-51024-KP002** and 1  $\mu\text{mol}$  **ENZ-51024-KP050**). For **ENZ-51024-KP002**, it should be reconstituted in 400  $\mu\text{L}$  deionized water to generate a 10 mM stock solution. For **ENZ-51024-KP050**, it should be reconstituted in 100  $\mu\text{L}$  deionized water to generate a 10 mM stock solution.

To observe at least 50% inhibition of PDI activity, a final concentration of 1mM is recommended.

Unused stock solution of Bacitracin may be stored at  $-20^{\circ}\text{C}$  for several weeks.

### 4. Making Stop Reagent working solution

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

Prepare working solution of Stop Reagent as follows: Add 4 parts Stop Reagent to 6 parts deionized water. Mix well and protect from light. For **ENZ-51024-KP002**, add 1000  $\mu\text{L}$  Stop Reagent to 1500 $\mu\text{L}$  deionized water. For **ENZ-51024-KP050**, add 250  $\mu\text{L}$  Stop Reagent to 375  $\mu\text{L}$  deionized water.

### 5. Making 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  $-20^{\circ}\text{C}$ , protected from light. Avoid repeated freeze/thaw cycles.

PDI reagent needs to be diluted 1:100 with PBE Buffer prior to use. **ENZ-51024-KP002:** for each 96-well plate add 10  $\mu\text{L}$  of PROTEOSTAT® PDI Reagent to 1 mL of PBE Buffer. Mix well. **ENZ-51024-KP050:** add 5  $\mu\text{L}$  of PROTEOSTAT® PDI Reagent to 0.5 mL of PBE Buffer. Mix well and protect from light.

## B. PDI 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<sup>®</sup> 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 96-well microplate by adding 50  $\mu$ L of the diluted insulin solution to each well.
2. Dispense 10  $\mu$ L of the working solution of the PDI, or buffer, to each well.

**NOTE:** *Additional PDI can be ordered from Enzo Life Sciences (Prod. No. ADI-SPP-891). If using PDI from another source, make sure to pre-dilute the enzyme such that the final concentration in the assay is 2-4 units per mL.*

3. Dispense 10  $\mu$ L of test agent, or buffer, to each well. As a positive control for PDI inhibition, dispense 10  $\mu$ L of Bacitracin into wells reserved for this purpose.
4. Dispense 10  $\mu$ L of DTT to each well of the plate.
5. Incubate the plates for 30 minutes at room temperature, protected from light.
6. Dispense 10  $\mu$ L of Stop Reagent working solution and 10  $\mu$ L of the prepared PROTEOSTAT<sup>®</sup> PDI Detection Reagent working solution into each well.

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

7. Incubate the microplate in the dark at room temperature for 15 minutes.
8. Read the generated signal with a fluorescent microplate reader using an excitation setting of about 500 nm and an emission filter of about 603 nm.



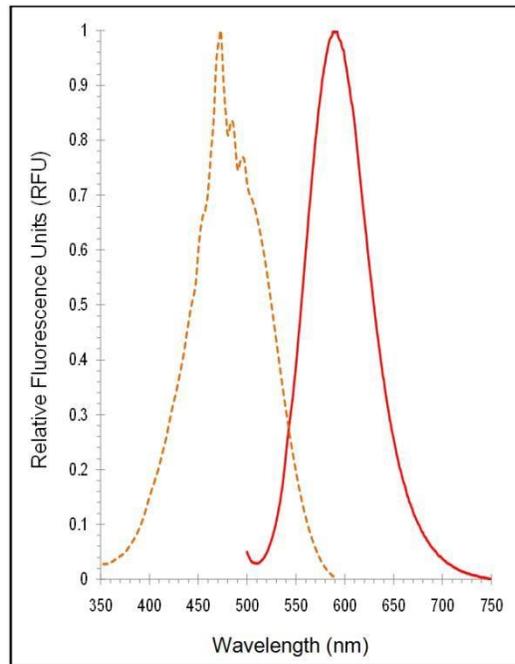
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**NOTE:** *In the absence of enzyme, the fluorescence value should be subtracted from the values for wells containing PDI.*

## APPENDICES

### A. MICROPLATE FILTER SET SELECTION

The selection of optimal settings for a fluorescence microplate

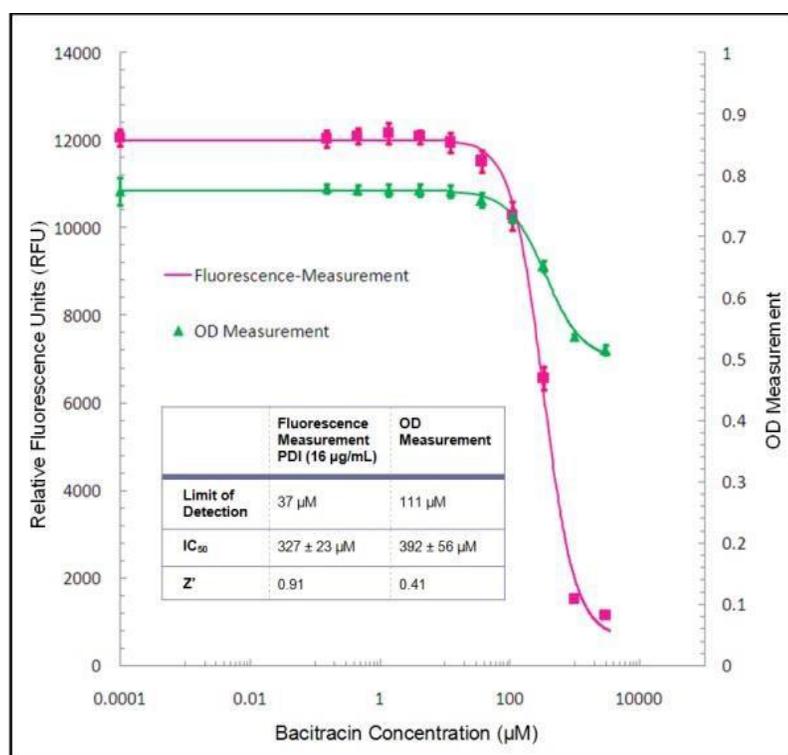


reader application requires matching the monochromator or optical filter specifications to the spectral characteristics of the dyes employed in the analysis. Please consult your instrument or filter set manufacturer for assistance in selecting optimal filter sets. Pre-designed filter sets for Texas Red 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.

### B. EXPECTED RESULTS

The catalytic reduction of insulin by PDI 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 nonfluorescent until it binds to aggregated protein, wherein it emits brightly at 603nm. Relative to analogous turbidimetric assays of PDI 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**).

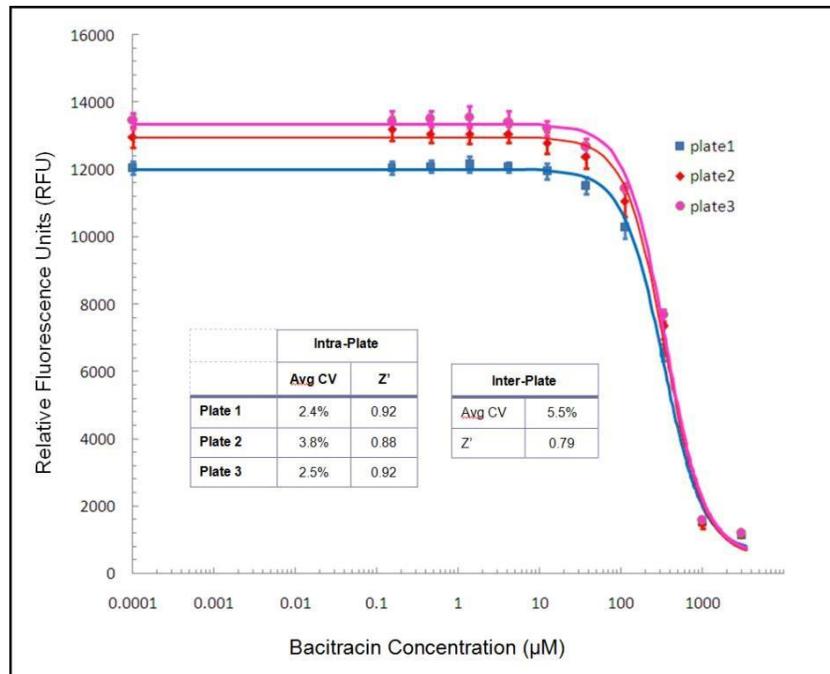


**Figure 3.** Assay validation using bacitracin as an inhibitor. Dose response assay was performed with 0 to 3000 µM bacitracin added 15 minutes prior to the initiation of enzymatic reaction. Reactions were performed as described in Methods and Procedures section. The fluorescence-based assay provides a vastly improved assay signal window and improved lower detection limit. In addition, the Z'-factor score obtained using the assay (0.91 for assay with and without PDI) demonstrates excellent signal-to-noise and signal-to-background ratio.

In order to validate this fluorescence-based assay, the potency of the PDI inhibitor bacitracin was monitored. The IC<sub>50</sub> of Bacitracin for PDI activity has previously been shown to be about 250 µM using the turbidimetric method.<sup>10</sup> A dose-response assay for bacitracin using the high throughput assay was performed. Concentration response plots were employed to determine the effects of bacitracin on PDI activity. These experiments were performed at constant enzyme and substrate concentrations while systematically varying bacitracin concentration. The IC<sub>50</sub> of the PDI inhibitor was determined to be 309 ± 27 µM, which is in good agreement with values reported in literature.<sup>10</sup>

Additionally, intra-plate and inter-plate reproducibility were determined in 96-well microplates. The CV values using the assay were typically determined to be 3-4% (see **Figure 4**).

The PROTEOSTAT<sup>®</sup> PDI Assay Kit provides an ideal high-throughput approach, enabling sensitive and accurate screening of modulators of PDI activity. The assay is homogenous, robust and cost-effective. It can potentially be applied to high-throughput screening of PDI 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 4.** Intra-plate and inter-plate reproducibility using bacitracin as an inhibitor. Dose response assay was performed with 0 to 3000  $\mu\text{M}$  bacitracin added 15 minutes prior to the initiation of enzymatic reaction. Reactions were performed as described in Methods and Procedures section. Intra-plate and inter-plate CVs using the assay are typically 3-6%.

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## 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 <sup>®</sup> 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 <sup>®</sup> 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 PDI 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.



# Product Manual

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