ab139480

P4HB (PDIA1) Inhibitor Screening Assay Kit

Instructions for Use

For the detection of protein disulfide isomerase activity in microplates

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

Version 2 Last Updated 10 May 2013

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1. Background

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 μM, 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. 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,

P4HB (PDIA1) inhibitors represent an important class of compounds that may enhance the efficacy of chemotherapy in a wide range of cancers. In addition to serving as a redox catalyst and isomerase, P4HB (PDIA1)-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. P4HB (PDIA1) inhibitors, through blocking reductive cleavage of disulfide bonds associated with these pathogens, can prevent infectivity.

2. Principle of the Assay

Abcam's P4HB (PDIA1) Inhibitor Screening Assay Kit (ab139480) provides a simple, homogenous assay for screening modulators of P4HB (PDIA1) enzymatic activity in microplates. This is accomplished by monitoring the P4HB (PDIA1)-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 P4HB (PDIA1) Detection Reagent.

Relative to the analogous turbidimetric assays of P4HB (PDIA1) 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%.

Abcam's P4HB (PDIA1) Inhibitor Screening Assay Kit (ab139480) is capable of providing a quantitative readout of P4HB (PDIA1) enzymatic activity in a robust and high-throughput fashion and can be applied to identification of P4HB (PDIA1) inhibitors from chemical libraries.

3. Protocol Summary

Prepare Assay reagents.

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Combine Assay reagents and DTT in wells of microplate

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Incubate at RT for 30 minutes, protected from light.

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Disense 10uLof stop Reagent to each well

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Incubate in dark at RT for 15 minutes

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Read plate using fluorescent microplate reader using Ex/Em $500/603_{nm}$.

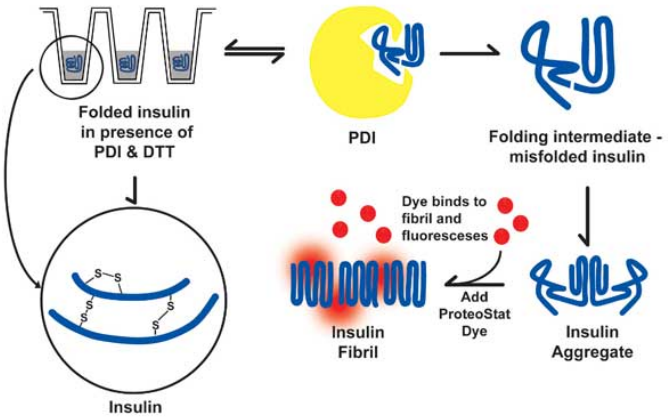

Figure 1: Schematic diagram of P4HB (PDIA1) Inhibitor Screening Assay Kit

4. Materials Supplied

Item	Quantity	Storage
P4HB (PDIA1) (Human, Recombinant)	2 x 165 µL	-80°C
P4HB (PDIA1) Detection Reagent	1 x 20 µL	-80°C
Insulin (from bovine pancreas) (1.8 µmol)	2 x 1 Vial	-80°C
Inhibitor Control (Bacitracin) (4.0 µmol)	1 Vial	-80°C
PBE Buffer	1 x 25 mL	-80°C
Stop Reagent	1 x 1 mL	-80°C
DTT	2 x 1.3 mL	-80°C
Deionized Water	1 x 5 mL	-80°C

5. Storage and Stability

- All kit components should be stored at -80°C to ensure stability and activity. Avoid multiple freeze/thawing. NOTE: P4HB (PDIA1) Detection Reagent is light sensitive. Avoid direct exposure to intense light.

- The reagents provided in the kit are sufficient for 2 x 96-well microplates.

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

6. Materials Required, Not Supplied

- Fluorescence microplate reader with a filter set of Excitation ~500_{nm}/Emission ~ 603_{nm}

- 96-well or 384-well microplates: black wall microplates, preferably with clear bottom.

- Calibrated, adjustable precision pipettes, preferably with disposable plastic tips.

7. Assay Protocol

A. Reagent Preparation

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.

Note: The procedures described in this manual are NOT suitable for detection of P4HB (PDIA1) activity in complex cell or tissue lysates.

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. Unused solutions of Insulin may be stored at -20°C for several weeks.

2. **P4HB (PDIA1) working solution:**
   Two vials of active P4HB (PDIA1) (Human, recombinant) are provided in the kit. Each vial containing 165 µL P4HB (PDIA1) solution should be diluted with 825 µL of PBE Buffer.

Note: If an alternate source of P4HB (PDIA1) to screen for modulators of enzymatic activity is desired, be sure that the P4HB (PDIA1) 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. **P4HB (PDIA1) inhibitor Control (Bacitracin) Working Solution:**

   Bacitracin is provided as lyophilized powder (4 µmol). It should be reconstituted in 400 µL Deionized Water to generate a 10 mM stock solution. To observe at least 50% inhibition of P4HB (PDIA1) activity, a 1mM final concentration is recommended for use. Unused stock solution of Bacitracin may be stored at -20°C for several weeks.

4. **Stop Reagent working solution:**

   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.

   Note: Avoid repeated freeze-thaw cycles for Stop Reagent.

5. P4HB (PDIA1) Detection Reagent working solution:

For each 96-well plate, add 10 µL of P4HB (PDIA1) Detection Reagent to 1 mL of PBE Buffer. Mix well.

Note: P4HB (PDIA1) 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.

B. Standard assay set-up

1. Prepare the 96-well microplate by adding 50 µL of the diluted insulin solution (from step A-1) to each well.
2. Dispense 10 µL of the working solution of the P4HB (PDIA1) (from step A-2), or buffer, to each well.
3. Dispense 10 µL of test agent, or buffer, to each well. As a positive control for P4HB (PDIA1) inhibition, dispense 10 µL of P4HB (PDIA1) inhibitor control, Bacitracin into wells reserved for this purpose.
4. Dispense 10 µL of DTT to each well of the plate.
5. Incubate the plates for 30 minutes at room temperature, protected from light.
6. Dispense 10 µL of Stop Reagent working solution (from step A-4) and 10 µL of the prepared P4HB (PDIA1) Detection Reagent working solution (from step A-5) into each well. Avoid direct exposure of P4HB (PDIA1) Detection 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}.

   Note: In the absence of enzyme, the fluorescence value should be subtracted from the values for wells containing P4HB (PDIA1).

8. Data Analysis

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 9 nm is recommended.

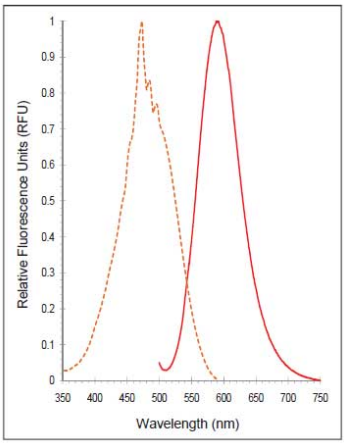

Figure 2: Absorption and fluorescence emission spectra for P4HB (PDIA1) Detection Reagent. All spectra were determined in PBE Buffer.

Expected Results:

The catalytic reduction of insulin by P4HB (PDIA1) 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 P4HB (PDIA1) detection reagent. The P4HB (PDIA1) detection reagent is essentially nonfluorescent until it binds to aggregated protein, wherein it emits brightly at 603_{nm}. Relative to analogous turbidimetric assays of P4HB (PDIA1) 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 P4HB (PDIA1) inhibitor bacitracin was monitored. The IC_{50} of bacitracin for P4HB (PDIA1) activity has previously been shown to be about 250μM using the turbidimetric method. 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 P4HB (PDIA1) activity. These experiments were performed at constant enzyme and substrate concentrations while systematically varying bacitracin concentration. The IC_{50} of the P4HB (PDIA1) inhibitor was determined to be 309 ± 27 μM, which is in good agreement with values reported in literature. 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).

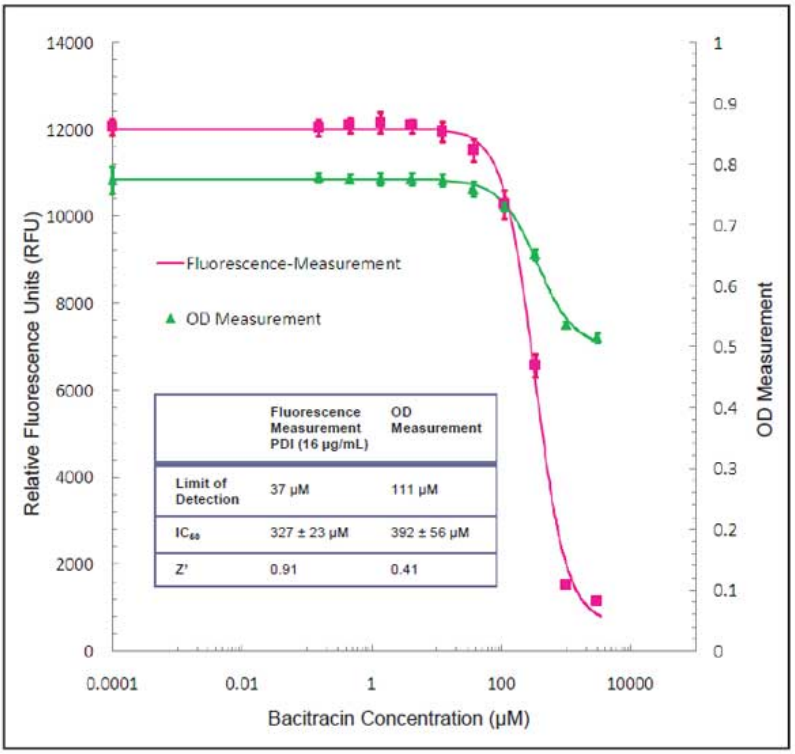

Figure 3: Assay validation using Inhibitor Control (bacitracin).

Dose response assay was performed with 0 to 3000 µM bacitracin added 15 min prior to the initiation of enzymatic reaction. Reactions were performed as described in Assay Protocol 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 P4HB (PDIA1)) demonstrates excellent signal-to-noise and signal-to-background ratio.

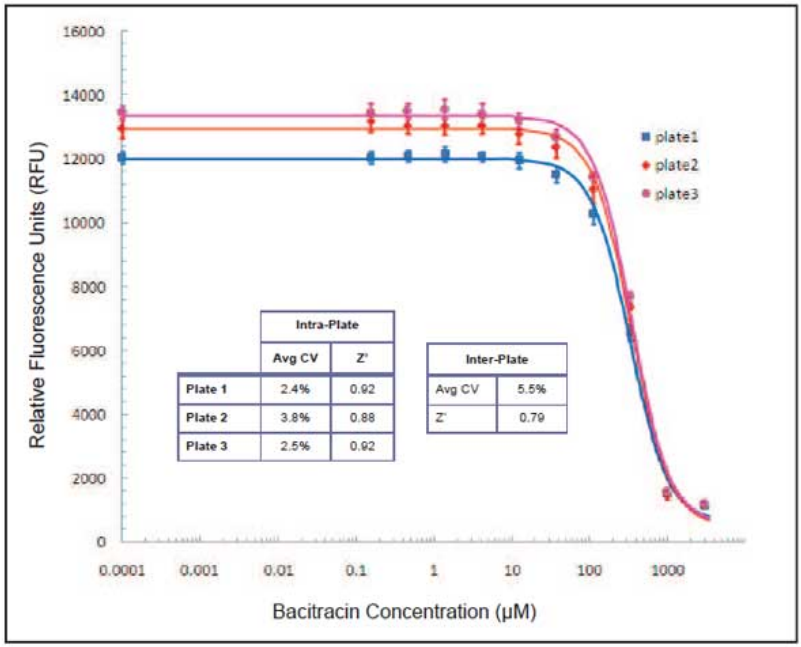

Figure 4: Intra-plate and inter-plate reproducibility using Inhibitor Control (bacitracin)

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. Intra-plate and inter-plate CVs using the assay are typically 3-6%.

9. Troubleshooting

Problem	Reason	Solution
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 Assay Protocol section of this manual and Appendix for recommendations.
	P4HB (PDIA1) 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 P4HB (PDIA1) 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 P4HB (PDIA1) 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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