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Novel Fluorescence-Based Microplate Assay for Screening Inhibitors of Thioredoxin and Protein Disulfide Isomerase

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ABSTRACT

Thioredoxin (Trx) is a small globular protein, present in the cytosol, nucleus and mitochondria, which catalyzes reduction of protein disulfide bonds, while protein disulfide isomerase (PDI) is primarily located in the endoplasmic reticulum where it is also capable of catalyzing disulfide exchange, leading to the rearrangement of disulfide bonds within proteins. Trx possesses a highly active site made up of two neighboring cysteine residues in a conserved active site motif, CGPC, while the redox active sites in PDI are slightly different, being CGHC. The motif difference leads to an increase in PDI's redox potential, which in turn dramatically increases its catalytic activity for disulfide formation. The enzymatic activities of Trx and PDI were measured using a homogenous fluorescence-based assay wherein the reduction of insulin in the presence of dithiothreitol leads to the formation of insulin aggregates which subsequently bind avidly to a novel red-emitting fluorogenic dye. Relative to analogous turbidometric assays, the fluorescence-based assay provides a superior assay signal window, improved lower detection limit, and higher Z'-score (>0.8). Intra-plate and inter-plate CVs using the assay are typically 3-4%. Concentration-response plots were employed to determine the effects of bacitracin on PDI and Trx activity. These experiments were performed at constant enzyme and substrate concentrations while systematically varying bacitracin concentration. Compared with PDI, Trx was determined to be relatively insensitive to bacitracin. The described assay has been specifically developed for use with a fluorescence microplate reader and can potentially be applied to the identification of inhibitors that selectively act upon PDI or Trx.

The Thioredoxin Fold and Enzymatic Activity

Human Trx: DFSATWCGPCKM
Human PDI: FFFAPWCGHCKA

- Eukaryotic PDI is homologous to Trx, a ubiquitous small dithiol-disulfide oxidoreductase and each PDI molecule contains two Trx fold domains per polypeptide.
- Overall, PDI and Trx share approximately 30% amino acid identity and contain similar active sites (CXXC).
- As shown above, the amino acid residues that surround the CXXC motifs of PDI and Trx are those most conserved between the two proteins.
- The active site disulfide bonds of these two proteins differ by as much as 90 mV (or ~4 kcal/mol) in stability and the enzymes also differ substantially in substrate specificities.
- Recent advances in the treatment of certain cancers has been predicated upon movement away from traditional to more targeted chemotherapy. Selective Trx and PDI inhibitors offer the potential to display a broad range of anticancer activities, and are thus potentially attractive anticancer therapeutic agents.

Assay Workflow

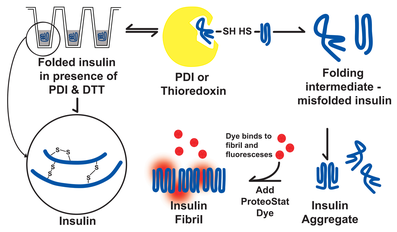
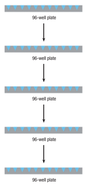


FIGURE 1A: Schematic diagram of the PDI and Thioredoxin Assay Mechanism.

Assay Workflow



Prepare the 96-well microplate by adding 50 µL of the diluted insulin solution to each well.

Dispense 10 µL of the working solution of PDI or Thioredoxin or buffer, to each well.

Dispense 10 µL of test agent, or buffer, to each well.

Dispense 10 µL of DTT to each well and ncubate the plates for 30 minutes at room temperature, protected from light.

Dispense 10 µL of Stop Reagent working solution and then 10 µL of the prepared ProteoStat™ PDI Detection Reagent working solution. Incubate the microplate in the dark at room temperature for 15 minutes. Read the generated signal with a fluorescent microplate reader, using an excitation setting of 500 nm and an emission filter of 600 nm.

FIGURE 1B: Generalized workflow for the PDI and Thioredoxin Assay in 96-well microplates. For 384-well plate applications, the cited volumes for each step should be reduced by 50%.

Protein Disulfide Isomerase Activity Assessment

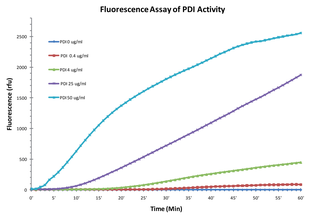


FIGURE 2: PDI titration: Reactions were performed with varying PDI concentrations (0-50 µg/ml), as indicated. Fluorescence was measured in 1-min increments at room temperature using a BioTek Synergy MX fluorescence microplate reader.

H_2O_2 Rapidly Terminates the PDI Reaction Without Impacting Fluorescence Signal

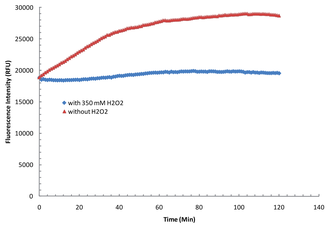


FIGURE 3: Evaluation of hydrogen peroxide (H_2O_2) as a stop reagent. 350 mM H_2O_2 was added to the enzyme/insulin mixture and incubated after the initiation of the reaction with dithiothreitol. H_2O_2 immediately stopped the reaction, providing a stable fluorescent signal for up to 2 hours afterwards.

Standard Curve for PDI Activity

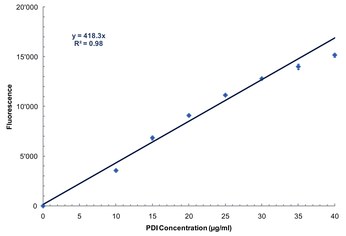
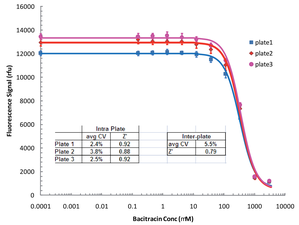


FIGURE 4: The enzymatic end point reaction (45 mins) displays linearity with PDI concentration. End point reactions were performed using varying PDI concentrations(0-40 µg/mL), as indicated.

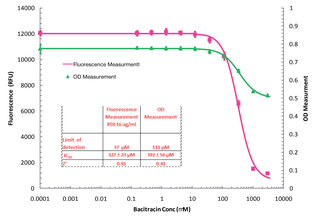
Reproducibility of the Assay



	Intra Plate		Inter-plate	
	avg CV	Z'	avg CV	5.5%
Plate 1	2.4%	0.92	Z'	0.79
Plate 2	3.8%	0.88		
Plate 3	2.5%	0.92		

FIGURE 5: Intra-plate and inter-plate reproducibility of the PDI assay using bacitracin as an inhibitor. Dose response curves were generated using 0 to 3000 µM bacitracin, added 15 min prior to the initiation of the enzymatic reaction. Intra-plate and inter-plate CVs using the assay are typically 3-5%.

Comparison of Fluorescence and Turbidity Assays



	Fluorescence Measurement PDI 16 ug/ml	OD Measurement
Limit of detection	12 µM	111 µM
IC_{50}	327 ± 23 µM	392 ± 54 µM
Z'	0.91	0.61

FIGURE 6: Assay validation using bacitracin as an inhibitor. Dose response assay was performed with 0 to 3000 µM bacitracin added 15 min prior to the initiation of reaction. The fluorescence-based assay provides a vastly improved assay signal window and improved lower detection limit, In addition, Z-factor (0.90 for assay with and without PDI) obtained using the assay demonstrates excellent signal-to-noise and signal-to-background ratio.

Validation in 384-well Microplates

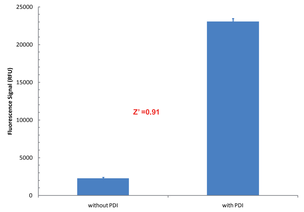


FIGURE 7: Assay validation in 384-well microplates. The Z-factor Value (0.91 for the assay with and without PDI) using the assay demonstrates excellent signal-to-noise and signal-to-background ratio.

Thioredoxin Activity Assay

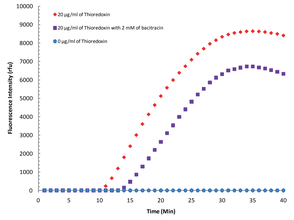


FIGURE 8: Trx activity assay, using bacitracin as an inhibitor. Only 20% enzyme inhibition was observed using 2 mM of bacitracin. This concentration of bacitracin inhibits PDI by more than 95%. Thus, compared with PDI, Trx was determined to be relatively insensitive to bacitracin.

CONCLUSION

- A high-throughput fluorescence-based assay suitable for screening inhibitors of PDI or Trx activity was developed.
- The assay was successfully converted from a kinetic to an end-point measurement using hydrogen peroxide as a stop reagent.
- The assay provides a linear response relative to enzymatic activity over a 40-fold range.
- The assay is homogenous, robust, and cost-effective, typically providing intra-plate and inter-plate CVs of 3-5%.
- The fluorescence-based activity assay provides superior signal generation, lower detection limit, and higher Z'-factor score relative to analogous turbidimetric assays of enzymatic activity.
- The assay should be suitable for 96-well or 384-well high-throughput screening for inhibitors from compound libraries.
- Compared with PDI, Trx was determined to be relatively insensitive to bacitracin.

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