INSTRUCTIONS

Pierce Protease Assay Kit

23263 0478.5

Number	Description
23263	**Pierce Protease Assay Kit**, sufficient reagents to assay ~250 samples (including standards)

Kit Contents:

Succinylated Casein, 5 × 10mg, supplied as lyophilized salt-free powder

TNBSA (2,4,6-trinitrobenzene sulfonic acid), 5% (w/v) in methanol, 2mL

TPCK Trypsin, 50mg; specific activity: > 10,350 BAEE units/mg of protein

BupH™ Borate Buffer Pack, yields 50mM borate, pH 8.5 after reconstitution with 500mL water

Caution: TNBSA is a skin sensitizer and stain. Avoid skin contact with the TNBSA solution.

Storage: Upon receipt store contents at 4°C. Kit is shipped at ambient temperature.

Introduction

The Thermo Scientific™ Pierce™ Protease Assay Kit enables easy, fast and reproducible quantitation of protease activity. The homogeneous assay is performed in a microplate and requires no strong acids or separation steps. TPCK trypsin is provided as a general protease standard and may be used for secondary calibration of other specific protease standards.

The assay method uses succinylated casein and trinitrobenzenesulfonic acid (TNBSA).[1-3] Succinylated casein is native casein that has been treated with succinic anhydride to block primary amines on the surface of the protein. In the presence of protease, the succinylated casein is cleaved at peptide bonds, thereby exposing primary amines (predominantly α-amines). TNBSA reacts with these exposed primary amines to produce an orange-yellow product whose intensity may be measured at 450nm. The increase in color relative to sample without succinylated casein is a measure of protease activity in the sample.[1]

Because TNBSA produces color when it reacts with any primary amine, it is critical that blanks for each sample be included in the assay to correct for the effect of amines (general protein content) in the protease sample. Amine-containing buffers such as Tris will cause high background, although correction for small amounts of Tris (< 50mM) in the sample can be made using the blank. In practice, when the concentration of amines in the buffer or sample is high, the sample must be diluted so that its blank (no succinylated casein) absorbance at 450nm in the assay is less than 0.500. The assay is compatible with up to 1mM DTT in the protease sample; however, the background (blank) absorbance reading will be increased.

Additional Materials Required

- Pipettors capable of accurately dispensing 50µL, 100µL, and 5mL. An 8-channel pipettor is very convenient and well-suited for large numbers of samples.
- Microplate(s). Use standard clear 96-well plates or 8-well strip plates (see Related Thermo Scientific Products).
- Optional: Microtube Racked System (see Related Thermo Scientific Products). This system of 1.1mL tubes in a 96-well format allows for convenient manipulation of samples to be dispensed with a multi-channel pipettor.
- Plate reader capable of measuring absorbance at 450nm.

  Note: Other wavelengths between 400-500nm can be used but will result in decreased sensitivity.

Material Preparation

Assay Buffer	Prepare by dissolving contents of the BupH Borate Buffer Pack in 500mL distilled water. After reconstitution, the composition of this buffer will be 50mM borate, pH 8.5. **Note:** The assay can be performed using other buffers, provided they are free of primary amines (e.g., Tris). Optimal pH is 8.0 or higher.
Succinylated Casein Solution	Dissolve 1 vial (10mg) of lyophilized Succinylated Casein in 5mL of Assay Buffer. Let the vial stand for 5 minutes after addition of buffer, then gently swirl the vial to fully dissolve the protein. Five milliliters of succinylated casein solution will be sufficient to assay 48 samples in a 96-well microplate. **Note:** It is normal for the Succinylated Casein Solution to be slightly opalescent.
Trypsin Stock Solution	Dissolve the lyophilized TPCK Trypsin in 1mL of ultrapure water or Assay Buffer to make a 50mg/mL stock solution. Prepare small (10-50µL) aliquots of this stock and store at -80°C. Thaw a new aliquot each time an assay is performed; do not refreeze a thawed aliquot.
Trypsin Standard	Thaw one aliquot of Trypsin Stock Solution and dilute to 0.5mg/mL in Assay Buffer. Serially dilute this solution to yield 6-8 standards that can be used to construct a standard curve for the assay. A series of 2-fold dilutions may not span the full range of detectable protease activity; consider making a series of 5- or 10-fold dilutions (see Additional Information Section). **Note:** Trypsin serves as a general standard for comparison of overall protease activity among different samples. However, optimum conditions for activity of other proteases are likely to be different than for trypsin. To accurately measure activity of a specific protease, use known amounts of that protease to prepare the standard curve. If standard curves of both trypsin and the protease of interest are prepared, a calibration may be established between the two proteases.
TNBSA Working Solution	Prepare by adding 100µL of the supplied TNBSA stock to 14.9mL of Assay Buffer. **Caution:** TNBSA is a skin sensitizer and stain. Wear gloves. A paper towel dipped in concentrated detergent (see Related Thermo Scientific Products) can be used to wipe up spills.

Procedure

Note: The assay method requires use of a control blank for each unknown and standard sample. Each blank contains buffer and protease sample (unknown or standard) but no Succinylated Casein Solution. When assayed in parallel to the samples, these blanks control for color development caused by amines in the protease sample, including those cause by self-cleavage.

1. Add 100µL Succinylated Casein Solution to one set of microplate wells. Add 100µL Assay Buffer to a duplicate set of wells to serve as blanks.
2. Add 50µL of each unknown or standard sample to both Succinylated Casein wells and corresponding blank wells.
3. Incubate plate for 20 minutes at room temperature (RT).

   Note: Warmer incubation temperatures (up to 37°C) can also be used; in general, this will increase protease activity.
4. Add 50µL TNBSA Working Solution to each well.
5. Incubate plate for 20 minutes at RT.
6. Measure absorbances of wells in a plate reader set to 450nm. Other wavelengths between 400-500nm can be used but will result in decreased sensitivity.
7. For each well calculate the change in absorbance at 450nm (ΔA_{450}) by subtracting the A_{450} of the blank from that of the corresponding Casein well. This ΔA_{450} is the absorbance generated by the proteolytic activity of the protease.

   Note: Samples or buffers containing high concentrations of proteins and other amine-containing compounds may result in blanks that have A_{450} values greater than 0.500. In such cases, assessment of proteolytic activity by the Pierce Method will be inaccurate. Dilute samples in Assay Buffer so that they have blank A_{450} values < 0.500.
8. Plot a standard curve (ΔA_{450} against protease standard concentration) and use to assess relative protease activity of the unknown sample.

Troubleshooting

Problem	Possible Cause	Solution
All absorbance values (for non-trypsin protease standard curve and unknown sample) are low (e.g., < 0.3)	pH and other buffer conditions were not optimal for the protease being tested	Repeat assay using buffer conditions that are optimal for the protease being tested and used to make standard curve
	Protease being tested digests the casein substrate more slowly or less fully than the trypsin standard for which the default assay method was developed.	Perform the digestion step for > 20 minutes (e.g., 40 minutes); if necessary, also perform the TNBSA development step for longer
		Use the specific substrate for the protease being tested (see Rao, *et al.)*
Sample ΔA_{450} values are lower than smallest trypsin or other protease standard ΔA_{450} values	Sample protease activity is low relative to the range of dilutions used for the standard curve	Prepare and use additional standard dilutions to ensure that the full range of assay sensitivity is utilized.
	Sample protease activity is low relative to the protease used to make standard curve	Prepare a standard curve using the known amounts of the specific protease being tested
Plotted standard curve is not linear	Linear rather than logarithmic scale used for the X-axis	Use a logarithmic scale for the X-axis (protease enzyme concentration)
Sample ΔA_{450} values do not fall in the linear range of the assay curve	Linear rather than logarithmic scale used for the X-axis	Use a logarithmic scale for the X-axis (protease enzyme concentration)
	Logarithmic scale used, but the range of standard dilutions exceeds the range of assay detection	Test more concentrated sample (assuming blank absorbance values remain < 0.5) in an attempt to obtain values within the useable assay range

Additional Information

Graphs of example data are instructive in understanding the behavior of the Pierce Protease Assay and the solutions suggested in the Troubleshooting Section. Results from the Pierce Assay are best plotted using a logarithmic scale rather than a linear scale (Figure 1). A linear scale may be adequate when using only a very narrow assay range.

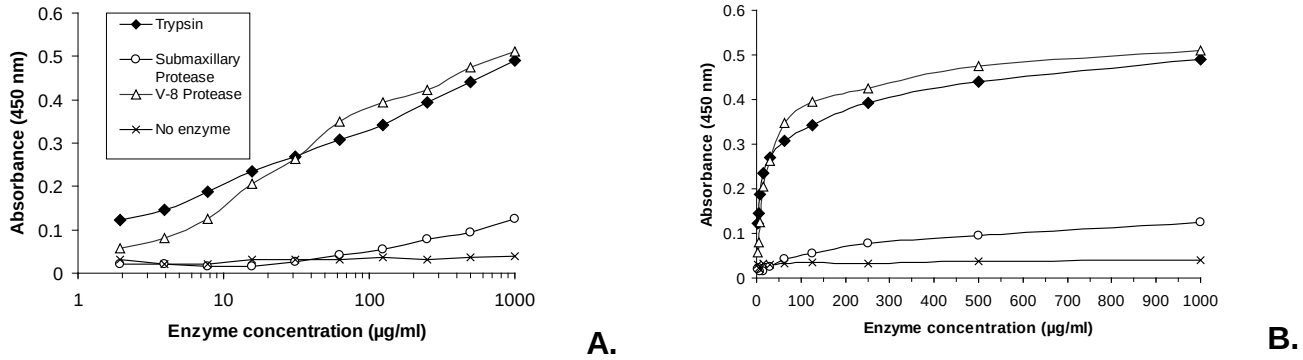

Figure 1. Thermo Scientific Pierce Protease Assay standard curves for three enzymes. Two-fold dilution series of TPCK-Trypsin (kit standard), Submaxillary Protease (Product No. 20199) and V-8 *Staphylococcus aureus* protease (Product No. 20195) were assayed using the default kit procedure. Net absorbance values are plotted using both logarithmic (A) and linear (B) scales.

Comparison of complete standard curves for different proteases reveal that equivalent concentrations of different proteases do not produce the same level of activity with respect to digestion of the succinylated casein substrate and default borate buffer used in the default assay method (Figure 1a). For example, if Submaxillary Protease (Product No. 20199) were being tested using the default buffer conditions, then trypsin would not be a very appropriate standard because 1000µg/mL submaxillary proteins apparently has activity equal to ~ 2µg/mL trypsin.

The lower limits of assay detection for different proteases are exemplified in Figure 2. In the default procedure, the lower limit of detection for purified TPCK Trypsin is ~50ng/mL (Figure 2a), and the upper limit is > 1.0mg/mL (Figure 1a). For purified pronase, the lower limit of detection is apparently < 10ng/mL (Figure 2b).

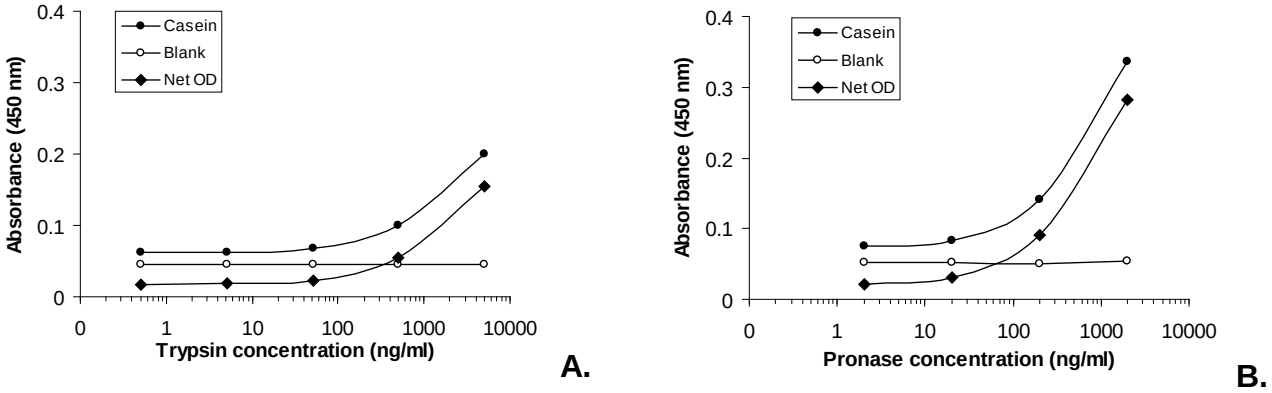

Figure 2. Lower limits of detection for trypsin (A) and Pronase™ Protease (B). When plotted on a logarithmic scale, a flattening trend line (at either high or low end) is indicative of the assay limits. With careful measurements and use of replicates, trypsin concentrations as low as 50ng/mL can be statistically distinguished from zero; for Pronase Protease, values < 10ng/mL are significant.

Related Thermo Scientific Products

15041	**Pierce 96-Well Plates**, corner notched, clear, 100/pkg
15031	**Pierce 8-Well Strip Plates**, corner notched, clear, 100/pkg
15082	**Microtube Racked System**, 10 rack of 96 individual 1.1mL tubes per rack
72288	**PCC-54™ Detergent Concentrate**, 3L
20233	**TPCK Trypsin**, 50mg
28997	**TNBSA**, 5% w/v methanol solution, 100mL
23266	**Pierce Fluorescent Protease Assay Kit**, for FRET and fluorescent polarization applications

Cited References

1. Hatakeyama, T., *et al*. (1992). A microassay for proteases using succinylcasein as a substrate. *Anal Biochem* **204:**181-4.
2. Bubnis, W.A. and Ofner, III, C.M. (1992). The determination of e-amino groups in soluble and poorly soluble proteinaceous materials by a spectrophotometric method using trinitrobenzenesulfonic acid. *Anal Biochem* **207:**129-33.
3. Habeeb, A.F.S.A. (1966). Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. *Anal Biochem* **14:**328-36.

Product References

Rao, S.K., *et al.* (1997). A versatile microassay for elastase using succinylated elastin. *Anal Biochem* **150:**222-7.

Tian, M., *et al.* (2004). A Kazal-like extracellular serine protease inhibitor from Phytophthora infestans targets the tomato pathogenesis-related protease P69B. *J Biol Chem* **297(25):**26370-7.

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