INSTRUCTIONS

Pierce Fluorescent Protease Assay Kit

(For FRET and Fluorescence Polarization Applications)

23266 23267

1284.5

Number	Description
23266	**Pierce Fluorescent Protease Assay Kit,** sufficient FTC-Casein to assay > 2000 samples (including standards) in 96-well plates
	Kit Contents:
	FTC-Casein, 2.5mg, lyophilized, FRET/FP-certified Grade
	TPCK Trypsin, 50 mg (for use as a standard)
	BupH™ Tris Buffered Saline, 1 pack, makes a 25mM Tris (pH 7.2), 150mM NaCl when dissolved in 500mL of ultrapure water
23267	**FTC-Casein**, 2.5mg, lyophilized, FRET/FP Certified Grade
	Storage: Upon receipt store FTC-Casein and TPCK Trypsin at 4°C. Product shipped at ambient temperature.

Introduction

The Thermo Scientific™ Pierce™ Fluorescent Protease Assay Kit includes fluorescein-labeled casein for use as a substrate for assessing protease activity in a sample by either fluorescence resonance energy transfer (FRET) with a standard fluorometer or fluorescence polarization (FP) with capable instrumentation. FTC-Casein is native casein that has been labeled using a large molar excess of fluorescein isothiocyanate (FITC). Fluorescence properties of this heavily-labeled, intact protein substrate change dramatically upon digestion by proteases, resulting in a measurable indication of proteolysis (Figure 1).

FRET-based measurement detects the decrease in fluorescence quenching (= increased total fluorescence) that occurs as the FTC-Casein substrate is digested into smaller fluorescein-labeled fragments. Classical FRET involves electron energy transfer between two different fluorophores, one acting as an energy donor and the other as the energy acceptor. In the Fluorescent Assay, FRET events occur as a result of fluorescence homotransfer in which fluorescein is acting both as the energy “donor” and energy “acceptor”.[1]

FP-based measurement detects the increased rate of molecular rotation (= decreased total fluorescence polarization) that occurs as the FTC-Casein substrate is digested into smaller fluorescein-labeled fragments. fluorescein excitation with plane-polarized light results initially in similarly plane-polarized fluorescence emission. Compared to smaller fluorescent molecules, a large molecule such as FTC-Casein has emission that remains highly-polarized for more time because it takes longer for the molecule to rotate out of the excitation plane. Intact FTC-Casein has a high polarization value (typically measured in millipolarization units, mP), whereas a digested FTC-Casein has a lower polarization value. Polarization units can be mathematically converted to anisotropy units. (Polarization and anisotropy, are used frequently in the literature to quantitate the same event.) Fluorescent polarization requires more costly instrumentation than FRET but has the advantage of being insensitive to optically absorptive compounds that detract from the excitation or emission of the fluorescein label.

Both assay formats use standard fluorescein excitation/emission filters (485/538nm). TPCK Trypsin is provided in the kit as a general protease calibrator so that the results can be compared to a reference protease. The assay can be performed in either microplate or test tube format. Less than 400pg of protease can be detected with the Pierce Fluorescent Protease Assay Kit in a microplate format.

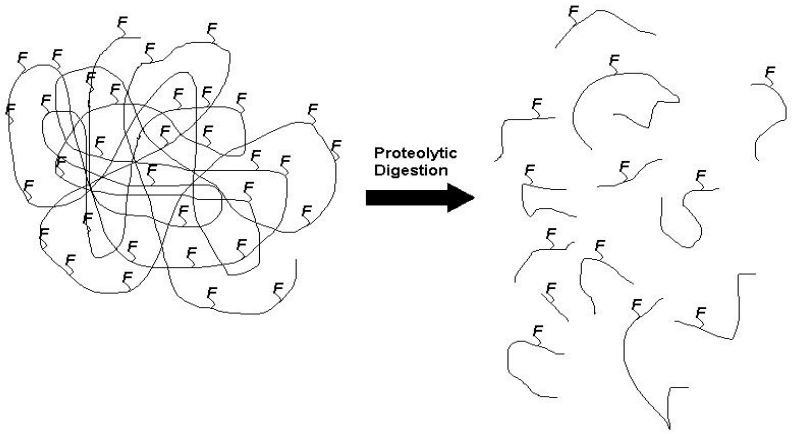

Figure 1. Basis of the Thermo Scientific Pierce Fluorescent Protease Assay. Digestion of fluorescein-labeled casein into smaller, labeled fragments results in measurable change in fluorescence properties.

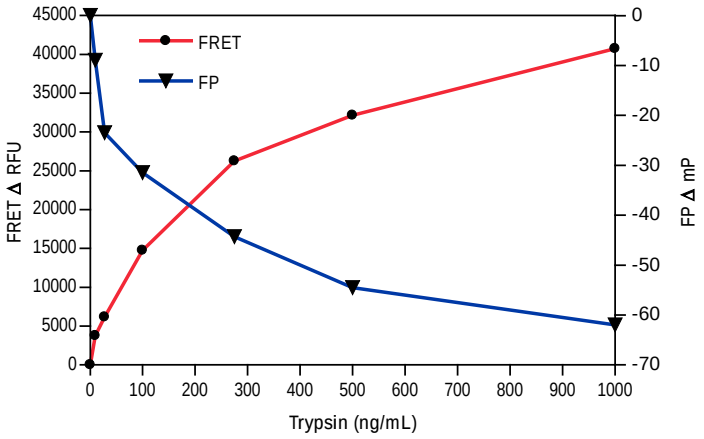

Figure 2. Example response curves of the Thermo Scientific Pierce Fluorescent Protease Assay. FRET (circles) is graphed as the change in relative fluorescence units (ΔRFU, left Y axis). FP (triangles) is graphed as the change in millipolarization units (mP, right Y axis).

Important Product Information

- The following example procedure is suitable for analysis of many proteases including trypsin, elastase, chymotrypsin, pronase and others. However, particular proteases will have specific pH and temperature optima, and the procedure may have to be optimized with respect to incubation time, volumes, buffers and dynamic working range.
- A variety of buffers (e.g. Tris, borate or phosphate) may be used for the assay. However, fluorescein has pH-dependent fluorescence: maximum fluorescence occurs at pH 8.5 and is effectively no fluorescence occurs at pH < 6.0. Therefore, to assay acid proteases, see the section “Suggestions for Non-homogenous Assay”.
- Typical laboratory lighting will not photobleach the fluorophore. Nevertheless, avoid directly exposing the FTC-Casein to intense light and protect it from light during long-term storage, especially after reconstitution.

Example Homogeneous Protease Assay Procedure

A. Materials Required

- Pipettors capable of accurately dispensing 10µL, 100µL and 5mL. An 8-channel pipettor is very convenient and well-suited for large numbers of samples.
- Microplate(s). Use white or black 96-well or 384-well 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.
- Fluorometer or FP-capable instrument fitted with a typical fluorescein filter set (i.e., 485/538nm excitation/emission maxima). The reaction can be performed successfully in 96- and 384-well microplates, as well as in cuvettes. If a cuvette-based instrument will be used, increase volumes proportionately to accommodate the cuvette.

B. Material Preparation

TBS (Assay Buffer)	Prepare by dissolving contents of the BupH TBS Pack in 500mL ultrapure water. After reconstitution, the composition of this buffer will be 25mM Tris, 0.15M NaCl, pH 7.2. **Note:** Other buffers may be used, provided that the pH is > 6.0. For proteases requiring digestion at pH < 6.0, use a non-homogeneous assay method (See subsequent Procedure section).
FTC-Casein Stock Solution	Dissolve 2.5mg of FTC-Casein with 500µL of ultrapure water to make 5mg/mL stock solution. Prepare small (20-40µL) aliquots of this stock and store frozen (-20° or -80°C). Thaw a new aliquot each time an assay is performed; do not refreeze a thawed aliquot.
FTC-Casein Working Reagent (WR)	Thaw one aliquot of FTC-Casein Stock Solution and dilute 1:500 in TBS. Prepare 10mL of WR for use with a 96-well microplate; prepare 15mL of WR for use with a 384-well microplate.
Trypsin Stock Solution	Dissolve the lyophilized TPCK Trypsin in 1mL of either ultrapure water or TBS 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 0.5µg/mL in TBS. Serially dilute this solution to yield 6-8 standards that can be used to construct a standard curve for the assay. **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.

C. Microplate Assay Protocol

For best results, use white plates to measure assay in FRET mode, and use black plates to measure in FP mode. The ratios of WR to sample (or standard) may be varied to suit particular assay needs. Smaller volumes of sample and WR than those suggested in the procedure can be used, but thorough mixing will be more difficult and reproducibility may decrease.

1. Add 100µL sample (or standard) to each well of a 96-well plate (use 37.5µL/well for 384-well plate). In addition to the zero-standard, prepare a blank using a buffer similar to that used for the protease sample.
2. Add 100µL of WR to all wells of a 96-well plate (use 37.5µL/well for 384-well plate).
3. Incubate for 5-60 minutes at room temperature.
4. Measure fluorescence in a plate reader using a fluorescein excitation/emission filter set.
5. Subtract the blank from each sample and standard measurement and then prepare a standard curve:
   - For FRET, plot the change in relative fluorescence units (RFU) of the standards vs. protease concentration.
   - For FP, plot the change in millipolarization units (mP) of the standards vs. protease concentration.

D. Cuvette/Test Tube Assay Protocol

1. Combine 1 part sample (or standard) with one part WR (e.g., 2mL sample + 2mL WR). In addition to the zero-standard, prepare a blank using a buffer similar to that used for the protease sample.
2. Mix cuvette/tube contents and incubate for 5-60 minutes at room temperature.

   Note: Kinetic results can be obtained by measuring fluorescence at multiple time-points during incubation.
3. Measure fluorescence in a fluorometer using a fluorescein excitation/emission filter set.
4. Subtract the blank from each sample and standard measurement and then prepare a standard curve:
   - For FRET, plot the change in relative fluorescence units (RFU) of the standards vs. protease concentration.
   - For FP, plot the change in millipolarization units (mP) of the standards vs. protease concentration.

Suggestions for Non-homogeneous Assay

The original method of Twining may be used to acid protease activities that require buffer pH values lower than that permissible for detection of the pH-dependent emission of the fluorescein label. In this method, samples are reacted with FTC-Casein Working Reagent in buffer followed by addition of a two-fold volume excess of 5-10% trichloroacetic acid solution. After a 15-minute incubation, the solutions are centrifuged and the supernatant removed. The acidic supernatant containing the low molecular weight fluorescein-peptide fragments is then made alkaline by addition of a three-fold volume excess of 0.5M Tris, pH 8.5-9.0. The resulting solution can then be quantitated by FP or standard fluorescence techniques, and it can also be quantitated by optical absorbance at 485nm, provided that sufficient digestion has occurred.[2]

Troubleshooting

A. No change in signal occurs.

Ensure that the instrument gain setting is sufficiently low to avoid saturating the instrument detector. For fluorescence readers containing multiple excitation/emission positions, ensure that "top/top" is selected for excitation/emission settings.

B. Negative mP units are observed.

Although the instrument can be calibrated to the standardized mP value defined by free fluorescein in solution, the observed change in polarization is sufficient for generation of a standard curve or assessment of relative activity.

C. K factor (G-factor) calibration.

FP instruments typically will calculate a "correction" factor to account for optical variations associated with the instrument at a particular gain setting. Use the K (or G) factor from a well containing a high level of digestion, such as the highest concentration on a standard curve, and apply that factor to all samples.

D. Poor CVs and/or assay reproducibility is observed.

Variations in pipetting can directly contribute to assay error, especially in FRET-mode, because the WR itself has some intrinsic background fluorescence. Use reverse-pipetting or other technique to prevent the introduction of small air bubbles into the plate wells.

In FP-mode, if the sample matrix contains very high amounts of interfering fluorescent material, better quality results may be obtained by subtraction of buffer blanks from the data used for mP calculation. In addition, FP detection is very sensitive to the position of the optical head in relation to the center of the well with respect to its X and Y coordinates, especially with 384-well plates. This position-artifact may be identified as reproducible patterned data by positioning the plate for a second reading 180 degrees relative to the first reading. Ensure that the instrument read positioning settings are appropriate for the type of plate chosen.

Related Thermo Scientific Products

15042	**Pierce White Opaque 96-Well Plates**, corner notched, 25 per package
15082	**MicroTube Racked System**, 10 rack of 96 individual 1.1mL tubes per rack
20233	**TPCK Trypsin**, 50mg (activity > 14,000 BAEE units/mg)
23263	**Pierce Colorimetric Protease Assay Kit**

Cited References

1. Runnels, L.W. and Scarlatta, S.F. (1995). Theory and application of fluorescence homotransfer to melittin oligomerization. *Biophys J* **69(4):**1569-83.
2. Twining, S.S. (1984). Fluorescein isothiocyanate-labeled casein assay for proteolytic enzymes. *Anal Biochem* **143:**30-4.

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