

## EnzChek® Protease Assay Kits

E6638 EnzChek® Protease Assay Kit \*green fluorescence\*  
E6639 EnzChek® Protease Assay Kit \*red fluorescence\*

### Quick Facts

#### Storage upon receipt:

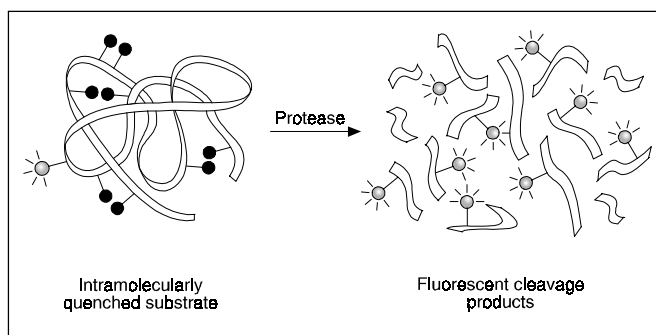
- -20°C
- Desiccate
- Protect from light

#### Ex/Em of Digestion Products:

- 505/513 nm for kit E6638
- 589/617 nm for kit E6639

#### Note:

- Ensure that the substrate is fully reconstituted before use
- Avoid freeze-thaw cycles after reconstituting



**Figure 1.** Principle of protease detection used in our EnzChek Protease Assay Kits (E6638, E6639).

a wide pH range. They can also be used to measure the total substrate turnover at a fixed time following addition of the enzyme. Furthermore, we have found that our protease assays are up to 100 times more sensitive and much easier to perform than the FTC-casein assay.<sup>2</sup>

In addition to their utility for detecting protease contamination of culture media and other experimental samples, BODIPY FL casein and BODIPY TR-X casein appear to have significant potential as general nontoxic, pH-insensitive markers for phagocytic cells in culture. Moreover, preliminary reports indicate that BODIPY FL casein is useful for monitoring proteolytic activity in frozen tissue sections with a fluorescence microplate reader.<sup>3</sup>

The BODIPY FL and BODIPY TR-X casein substrates can be used interchangeably, depending on whether green or red fluorescence is desired. The peptide hydrolysis products of BODIPY FL casein exhibit green fluorescence that is optimally excited by the argon-ion laser, permitting flow sorting of cells that have phagocytosed this reagent. The red-fluorescent BODIPY TR-X dye-labeled peptides, with excitation and emission spectra similar to those of the Texas Red® fluorophore, should be useful for multilabeling experiments or measurements in the presence of green autofluorescence.

## Introduction

Molecular Probes' EnzChek® Protease Assay Kits (E6638, E6639) are fast, simple and direct fluorescence-based assays for detecting metallo-, serine, acid and sulfhydryl proteases. Detecting low levels of protease activity is important in quality-control testing, high-throughput screening and basic research. However, current methods for detecting protease activity, such as the fluorescein thiocarbonyl (FTC)-casein protease assay, require extensive manipulation and are therefore prone to error. In the FTC-casein assay, unhydrolyzed protein must be precipitated with trichloroacetic acid, separated by centrifugation, transferred for measurement and then pH-adjusted for fluorescein signal enhancement.<sup>1</sup>

Our two EnzChek Protease Assay Kits contain casein derivatives that are heavily labeled with the pH-insensitive green-fluorescent BODIPY® FL (E6638) or red-fluorescent BODIPY® TR-X (E6639) dyes, resulting in almost total quenching of the conjugate's fluorescence. Protease-catalyzed hydrolysis releases highly fluorescent BODIPY FL or BODIPY TR-X dye-labeled peptides (Figure 1). The accompanying increase in fluorescence, which can be measured with a spectrofluorometer, minifluorometer or microplate reader, is proportional to protease activity.

In contrast to the FTC-casein assay, these EnzChek assays do not involve any separation steps and can be used to continuously measure the kinetics of a variety of exo- and endopeptidases over

## Materials

### Kit Contents

- **BODIPY FL casein or BODIPY TR-X casein** (Component A), five vials that each contain 200 µg substrate lyophilized from phosphate-buffered saline (PBS)
- **20X Digestion buffer** (Component B), 13 mL of 200 mM Tris-HCl, pH 7.8, containing 2 mM sodium azide

Each kit provides sufficient reagents for approximately 100 assays when using a standard fluorometer or 1000 assays when using a fluorescence microplate reader.

### Storage and Handling

Upon receipt, each kit should be stored frozen at  $-20^{\circ}\text{C}$ . Allow reagents to warm to room temperature before opening vials. When stored properly, these reagents are stable for six months to one year.

Reconstituted BODIPY casein substrates may be stored at  $4^{\circ}\text{C}$  for 2–4 weeks. We recommend the addition of sodium azide at a final concentration of 2 mM to act as a preservative. If longer storage is required, freeze at  $-20^{\circ}\text{C}$ . PROTECT FROM LIGHT. AVOID REPEATED FREEZING AND THAWING.

### Materials Required but Not Provided

- Deionized water ( $\text{dH}_2\text{O}$ )
- Phosphate-buffered saline (PBS) (E6638 only)
- 0.1 M sodium bicarbonate, pH 8.3 (E6639 only)
- Specific buffers for detection of enzymes requiring activation compounds or a unique pH environment, if applicable (see note A)
- An appropriate enzyme standard of known specific activity, if applicable (see step 2.1)

---

## Experimental Protocol

### Reagent Preparation

The solution volumes recommended in this section provide sufficient reagents for 20 assays using a fluorometer and standard 2.0 mL cuvettes, or 200 assays using a fluorescence microplate reader and 200  $\mu\text{L}$  per microplate well.

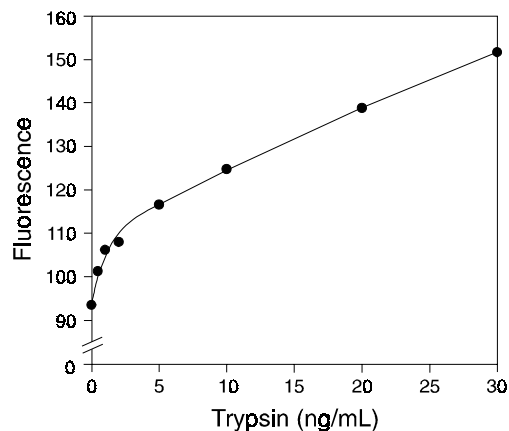
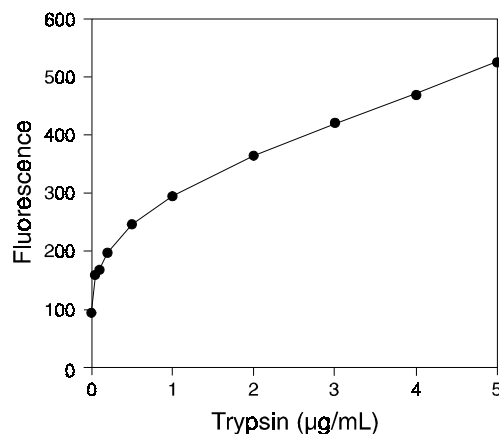
**1.1** If using the EnzChek Protease Assay Kit for green fluorescence (E6638), prepare a 1.0 mg/mL stock solution of the BODIPY FL casein by adding 0.2 mL of PBS directly to one of the vials containing the lyophilized substrate. If using the EnzChek Protease Assay Kit for red fluorescence (E6639), prepare a 1.0 mg/mL stock solution of the BODIPY TR-X casein by adding 0.2 mL of 0.1 M sodium bicarbonate, pH 8.3, to one of the vials containing the lyophilized substrate. In either case, mix well and allow sufficient time at room temperature for the substrate to dissolve fully.

**1.2** Prepare 1X digestion buffer. Dilute 2.5 mL of the 20X digestion buffer with  $\text{dH}_2\text{O}$  to a final volume of 50 mL (note A).

**1.3** Prepare a 10  $\mu\text{g}/\text{mL}$  working solution of the BODIPY casein. Add 0.2 mL of the stock solution prepared in step 1.1 to 19.8 mL of the 1X digestion buffer prepared in step 1.2.

### Protease Activity Standard Curve

**2.1** When quantitating the activity of purified protease preparations, generate a protease activity standard curve. If possible, use an appropriate enzyme standard of known specific activity that closely matches the protease activity being determined. A standard curve may not be relevant for samples containing one or more unknown proteases. In this case, protease activity may be expressed as fluorescence change per unit sample. Also, for



**Figure 2.** Sample standard curve obtained with the EnzChek Protease Assay Kit (E-6638). The top panel shows fluorescence versus trypsin concentration ( $\mu\text{g}/\text{mL}$ ) measured with a filter fluorometer (excitation  $485 \pm 10$  nm, emission  $530 \pm 12.5$  nm). The bottom panel displays a standard curve at lower trypsin concentrations ( $\text{ng}/\text{mL}$ ), obtained using the same fluorometer but with a higher gain setting.

simple detection of protease activity or contamination, a standard curve may not be necessary; proceed to step 3.3.

**2.2** Determine the range of enzyme response. Titrate at least four concentrations of enzyme and one buffer-only control in 1.0 mL (or 100  $\mu\text{L}$  for microplate assays) of the 1X digestion buffer prepared in step 1.2. Add 1.0 mL (or 100  $\mu\text{L}$  for microplate assays) of the BODIPY casein working solution prepared in step 1.3.

**2.3** Incubate the samples for one hour, protected from light (notes B, C).

**2.4** Read the fluorescence in a fluorometer or fluorescence microplate reader. BODIPY FL and BODIPY TR-X dye-labeled peptides have excitation/emission maxima of approximately 505/513 nm and 589/617 nm, respectively. We have found that standard fluorescein filters (e.g., excitation =  $485 \pm 12.5$  nm, emission =  $530 \pm 15$  nm) can be used to detect BODIPY FL dye-labeled peptides, whereas longer wavelength filters (e.g., excitation =  $590 \pm 10$  nm, emission =  $645 \pm 20$  nm) can be used to detect BODIPY TR-X dye-labeled peptides.

**2.5** Plot the data to show fluorescence versus protease concentration (Figure 2) (note **D**).

### Sample Analysis

**3.1** To detect enzyme activity in a sample, dilute the sample to 1.0 mL (or 100  $\mu$ L for microplate assays) in 1X digestion buffer prepared in 1.2. Add 1.0 mL (or 100  $\mu$ L for microplate assays) of the BODIPY casein working solution prepared in step 1.3.

**3.2** Incubate sample for one hour, protected from light (notes **B**, **C**).

**3.3** Read the fluorescence in a fluorometer or fluorescence microplate reader. BODIPY FL and BODIPY TR-X dye-labeled peptides have excitation/emission maxima of approximately 505/513 nm and 589/617 nm, respectively. We have found that standard fluorescein filters (e.g., excitation =  $485 \pm 12.5$  nm, emission =  $530 \pm 15$  nm) can be used to detect BODIPY FL dye-labeled peptides, whereas longer wavelength filters (e.g., excitation =  $590 \pm 10$  nm, emission =  $645 \pm 20$  nm) can be used to detect BODIPY TR-X dye-labeled peptides.

**3.4** If the protease sample has a high fluorescence background, prepare an additional control without the BODIPY casein prepared in step 1.3. Then, subtract the fluorescence background of the substrate-free control from the sample containing the substrate to determine the true fluorescence increase due to protease activity.

### Protease Detection Limits

Using the EnzChek protease assay with a fluorescence microplate reader, we have determined the protease detection limits for a number of proteases. In these assays, 200  $\mu$ L reaction mixtures were incubated in a 96-well microplate for one hour at room temperature, protected from light. The fluorescence was then measured in a fluorescence microplate reader, using excitation and emission filters of  $485 \pm 12.5$  nm and  $530 \pm 15$  nm, respectively, for detection of BODIPY FL dye-labeled peptides and  $590 \pm 10$  nm and  $645 \pm 20$  nm for detection of BODIPY TR-X dye-labeled peptides. Table 1 shows the approximate detection limits for a variety of enzymes when assayed at 22°C (note **E**).

### Notes

**[A]** The digestion buffer provided (pH 7.8) is recommended for detecting the protease activity of most proteolytic enzymes with activity optima from pH 7.4 to 8.0. However, if you are working with an enzyme that requires activation compounds or a unique pH environment, then prepare the specific buffer required in place of the digestion buffer.

### References

1. Anal Biochem 143, 30 (1984); 2. Anal Biochem 251, 144 (1997); 3. Derek Winslow, "Measurement of Proteolytic Activity in Whole Tissue Sections using Quenched Fluorescent Substrates: An Analogue of *In-Situ* Zymography?" Focus on Fluorescence: Industrial Applications Symposium, Leiden, The Netherlands, November 17, 1997.

**Table 1.** Detection limits of the EnzChek protease assay.

Enzyme (Source)	Class	Detection Limit (Units)	Buffer Conditions
Elastase, Type IV (porcine pancreas)	Serine Protease	$2.2 \times 10^{-3}$	10 mM Tris-HCl, pH 8.8
Chymotrypsin, Type II (bovine pancreas)	Serine Protease	$5.0 \times 10^{-5}$	10 mM Tris-HCl, pH 7.8
Thermolysin ( <i>B. proteolyticus rokko</i> )	Acid Protease	$4.4 \times 10^{-5}$	10 mM Tris-HCl, pH 7.8
Trypsin, Type IX (porcine pancreas)	Serine Protease	$1.3 \times 10^{-2}$	10 mM Tris-HCl, pH 7.8
Papain (papaya latex)	Sulfhydryl Protease	$2.1 \times 10^{-4}$	10 mM MES, pH 6.2
Pepsin (porcine stomach mucosa)	Acid Protease	$2.1 \times 10^{-3}$	10 mM HCl, pH 2.0
Elastase ( <i>Pseudomonas aeruginosa</i> )	Metalloprotease	$1.0 \times 10^{-3}$	20 mM sodium phosphate, pH 8.0
Cathepsin D	Acid Protease	$2.0 \times 10^{-4}$	20 mM sodium citrate, pH 5.0
Elastase (human leukocyte)	Serine Protease	$1.0 \times 10^{-3}$	10 mM Tris, HCl, pH 7.5

The detection limit is defined as the amount of enzyme required to cause a 10–20% change in fluorescence compared to the control sample at 22°C. Enzyme unit definitions are standard definitions for each individual enzyme. Detection limits were determined with BODIPY FL casein and with BODIPY TR-X casein; both substrates yielded similar results. Detection limits may vary with instrumentation.

**[B]** Sensitivity may be increased by incubating for up to 24 hours.

**[C]** The exact time interval is not critical. However, it is important that all reactions, experimental samples and controls be incubated for approximately the same time. For consistent incubation periods, it may be desirable to initiate the reactions with offset starting times to allow sufficient time for reading the fluorescence of each at the end of the reaction.

**[D]** Standard curves will vary with enzyme type.

**[E]** Enzyme activity may vary depending on incubation buffers and temperature, as well as the storage conditions and number of freeze-thaw cycles to which the enzyme preparation has been subjected.

---

**Product List** *Current prices may be obtained from our Web site or from our Customer Service Department.*

Cat #	Product Name	Unit Size
E6638	EnzChek® Protease Assay Kit *green fluorescence* *100-1000 assays* .....	1 kit
E6639	EnzChek® Protease Assay Kit *red fluorescence* *100-1000 assays* .....	1 kit

---

**Contact Information**

Further information on Molecular Probes products, including product bibliographies, is available from your local distributor or directly from Molecular Probes. Customers in Europe, Africa and the Middle East should contact our office in Paisley, United Kingdom. All others should contact our Technical Assistance Department in Eugene, Oregon.

Please visit our Web site — [www.probes.com](http://www.probes.com) — for the most up-to-date information

**Molecular Probes, Inc.**

29851 Willow Creek Road, Eugene, OR 97402  
Phone: (541) 465-8300 • Fax: (541) 335-0504

**Customer Service:** 6:00 am to 4:30 pm (Pacific Time)  
Phone: (541) 335-0338 • Fax: (541) 335-0305 • [order@probes.com](mailto:order@probes.com)

**Toll-Free Ordering for USA and Canada:**  
Order Phone: (800) 438-2209 • Order Fax: (800) 438-0228

**Technical Assistance:** 8:00 am to 4:00 pm (Pacific Time)  
Phone: (541) 335-0353 • Toll-Free: (800) 438-2209  
Fax: (541) 335-0238 • [tech@probes.com](mailto:tech@probes.com)

**Invitrogen European Headquarters**

Invitrogen, Ltd.  
3 Fountain Drive  
Inchinnan Business Park  
Paisley PA4 9RF, UK  
Phone: +44(0) 141 814 6100 • Fax: +44(0) 141 814 6260  
Email: [euroinfo@invitrogen.com](mailto:euroinfo@invitrogen.com)  
Technical Services: [eurotech@invitrogen.com](mailto:eurotech@invitrogen.com)

*Molecular Probes products are high-quality reagents and materials intended for research purposes only. These products must be used by, or directly under the supervision of, a technically qualified individual experienced in handling potentially hazardous chemicals. Please read the Material Safety Data Sheet provided for each product; other regulatory considerations may apply.*

**Limited Use Label License No. 193: BODIPY® Dye**

The manufacture, use, sale or import of this product is subject to one or more of US Patent Nos. 4,774,339, 5,187,288, 5,248,782, 5,274,113, 5,338,854, 5,433,896, 5,451,663, 6,005,113 and corresponding foreign equivalents, owned by Invitrogen Corp. The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the use of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) to not transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. Invitrogen Corporation will not assert a claim against the buyer of infringement of the above patents based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that neither this product nor any of its components was used in the manufacture of such product. If the purchaser is not willing to accept the limitations of this limited use statement, Invitrogen is willing to accept return of the product with a full refund. For information on purchasing a license to this product for purposes other than research, contact Molecular Probes, Inc., Business Development, 29851 Willow Creek Road, Eugene, OR 97402. Tel: (541)465-8300. Fax: (541)335-0504.

**Limited Use Label License No. 203: EnzChek® Technology**

The manufacture, use, sale or import of this product is subject to U.S. Patent No. 5,719,031, owned by Invitrogen Corp. The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for profit entity). The buyer cannot sell or otherwise transfer (a) this product, (b) its components, or (c) materials made by the employment of this product or its components to a third party or otherwise use this product or its components or materials made by the employment of this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the employment of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) not to transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic

purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. Invitrogen Corporation will not assert a claim against the buyer of infringement of the above patents based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that none of this product or any of its components was used in the manufacture of such product. If the purchaser is not willing to accept the limitations of this limited use statement, Invitrogen is willing to accept return of the product with a full refund. For information on purchasing a license to this product for purposes other than research, contact Molecular Probes, Inc., Business Development, 29851 Willow Creek Road, Eugene, OR 97402. Tel: (541)465-8300. Fax: (541)335-0504.

All names containing the designation ® are registered with the U.S. Patent and Trademark Office.

Copyright 2004, Molecular Probes, Inc. All rights reserved. This information is subject to change without notice.