Revised: 30–August–2002

*RediPlate™ 96 EnzChek® Protease Assay Kit *red fluorescence* (R-22132)*

Quick Facts

Storage upon receipt:

- ≤ –20°C
- Desiccate
- Protect from light

Ex/Em of digestion product: 589/617 nm

Introduction

Molecular Probes' RediPlate™ 96 EnzChek® Protease Assay Kit for red fluorescence provides a fast, simple and direct fluorescence-based assay for detecting metallo-, serine, acid and sulfhydryl proteases and their corresponding modulators and inhibitors.[1] Unlike other microplate assays, this kit provides the necessary reagents already predispensed into a 96-well microplate. Simply dissolve the fluorescent substrate in the assay wells with buffer, add the desired sample to the wells, incubate and then quantitate the fluorescence in any standard fluorescence-based microplate reader.

The microplate included with the RediPlate 96 EnzChek Protease Assay Kit for red fluorescence is pre-loaded with a casein derivative that is heavily labeled with the pH-insensitive, red-fluorescent BODIPY® TR-X dye (excitation/emission ~589/617 nm, Figure 1). The heavy labeling of the casein results in almost total quenching of the conjugate's fluorescence[2]; however, protease-catalyzed hydrolysis relieves this quenching,

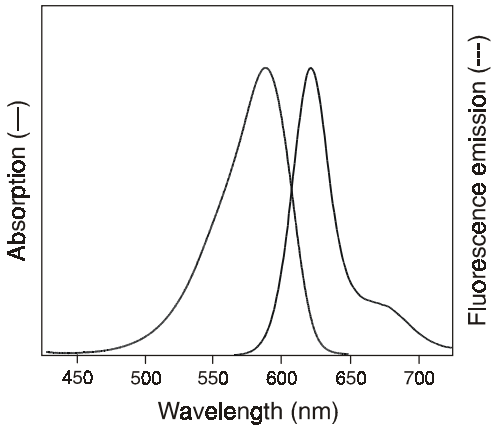

Figure 1. *Normalized absorption (—) and fluorescence emission (---) spectra of the BODIPY TR-X dye.*

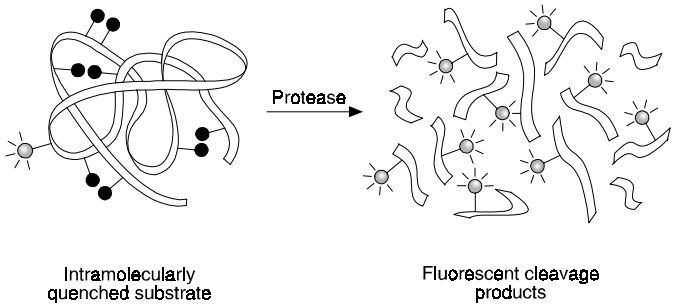

Figure 2. *Principle of protease detection via the disruption of intramolecular self-quenching. Protease-catalyzed hydrolysis of the heavily labeled and almost totally quenched substrate provided in our RediPlate 96 EnzChek Protease Assay Kit.*

releasing highly fluorescent BODIPY TR-X dye–labeled peptides (Figure 2). The resulting increase in fluorescence is proportional to protease activity (Figure 3). The RediPlate 96 EnzChek Protease Assay provides researchers with a sensitive and fast alternative to azocasein and fluorescein (FITC) casein end-point assays, which require an additional precipitation step for detection.[3,4] Protease inhibitors can be evaluated quantitatively in the assay for their effect on protease activity (Figure 4).

The RediPlate 96 EnzChek Protease Assay Kit includes one microplate and a bottle of reaction buffer. To ensure the integrity of the fluorogenic components, the microplate is contained in a resealable foil packet. The microplate consists of twelve removable strips, each with eight wells. Eleven of the strips (88 wells) are preloaded with the fluorogenic casein substrate. The remaining strip, marked with blackened tabs, contains a dilution series of BODIPY TR-X conjugated to glycine as a fluorescence reference standard.

Materials

Kit Contents

- **RediPlate 96 EnzChek protease assay microplate for red fluorescence** (Component A), one microplate
- **RediPlate protease reaction buffer** (Component B), 28 mL of 50 mM Tris-HCl, pH 7.5

Storage and Handling

Store the RediPlate 96 EnzChek Protease Assay Kit at –20°C or below, desiccated and protected from light. When stored properly, the kit components should remain stable for at least six months.

Experimental Protocol

The following protocols describe the assay for protease or protease inhibitor activity in total volumes of 200 µL per microplate well. Each RediPlate 96 EnzChek Protease Assay Kit contains one 96-well microplate with 88 wells (11 strips) intended for assays and 8 wells (1 strip, with blackened tabs) for a fluorescence reference standard curve. Because each strip is removable, one can perform as many or as few assays as needed.

RediPlate 96 Microplate Preparation

1.1 Allow the kit components to warm to room temperature. Remove the RediPlate Kit from the freezer and allow it to warm to room temperature. DO NOT OPEN THE FOIL PACKET UNTIL IT IS WARM. The plate (Component A) will typically take ~20 minutes to warm. Because the reaction buffer (Component B) may take longer than 20 minutes to thaw at room temperature, place the vial of buffer in a warm water bath to accelerate thawing. After thawing, the buffer may be stored at 2–6°C, for convenience.

1.2 Remove any extra strips. Determine the number of strips required and carefully cut through the self-adhesive sealing film with a razor blade and remove any extra strips that are to be used at a later date. Return these to the protective foil bag. All of the strips, with the exception of the control strip with blackened tabs, contain equivalent amounts of the protease substrate. Empty strip holders from previously purchased RediPlate 96 kits are useful for storing extra assay strips.

Fluorescence Reference Standards

The fluorescence reference standards serve as controls for instrument-to-instrument variation, for day-to-day variation in single-instrument performance and for linearity of the fluorescence signal detection. Furthermore, the standard curve can also be used to convert fluorescence units obtained in an assay into BODIPY TR-X dye equivalents.

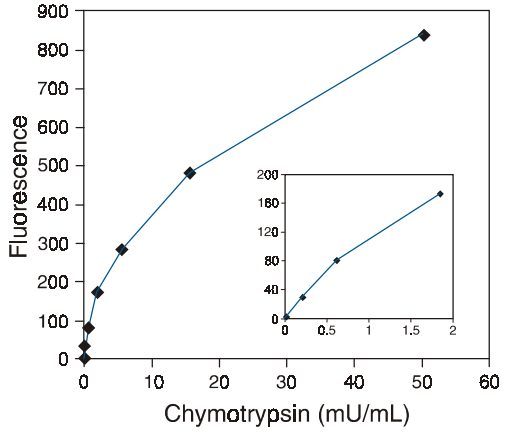

Figure 3. *Detection of proteolytic activity of chymotrypsin using the RediPlate 96 EnzChek Protease Assay Kit for red fluorescence. Each reaction contained BODIPY TR-X casein and the indicated concentration of chymotrypsin in the provided reaction buffer. Reactions were incubated at 37°C. After 60 minutes, fluorescence was measured in a fluorescence microplate reader using excitation at 590 ± 10 nm and fluorescence detection at 645 ± 20 nm. Background fluorescence (358 arbitrary units), determined for a no-chymotrypsin control reaction, has been subtracted from each value. The inset shows the sensitivity of the assay at very low levels of chymotrypsin.*

2.1 Prepare the fluorescence standards. Add 200 µL of the reaction buffer to the control strip of the RediPlate 96 microplate and mix by pipetting. Complete resuspension will take ~1 minute after the addition of buffer. Blackened tabs differentiate this strip from strips containing the BODIPY TR-X casein substrate. The control strip contains a twofold dilution series of the BODIPY TR-X glycine reference standard (Table 1 and Figure 5). Well A has the highest concentration of the reference standard (2.5 µM); well H contains no reference dye.

2.2 Measure the fluorescence. The fluorescence standard samples are typically measured for fluorescence along with the samples from the protease or protease-inhibitor assays.

2.3 Proceed to step 3.1 for the protease assay or step 4.1 for the protease-inhibitor assay.

Table 1. *Reference standard for the RediPlate 96 Protease Assay Kit for red fluorescence.*

Well	Concentration of BODIPY TR-X glycine in 200 µL
A	2.50 µM
B	1.25 µM
C	0.62 µM
D	0.31 µM
E	0.16 µM
F	0.08 µM
G	0.04 µM
H	0 µM

Protease Assay

3.1 Add 100 µL of the reaction buffer to the assay wells. Add the provided reaction buffer (Component B) or another appropriate buffer to as many assay wells as will be needed, and then mix by pipetting. It is important to fully solubilize the protease substrate in the microplate wells before adding the protease sample. Complete resuspension of the dye-conjugate will take ~1 minute after the addition of buffer. To minimize background, rehydrate the substrate immediately before the assay.

3.2 Prepare a no-protease control. Include a negative control by adding an additional 100 µL of reaction buffer to any well containing the reconstituted substrate (prepared in step 3.1), and mix by pipetting.

3.3 Dilute the protease-containing samples. Dilute the samples in the provided reaction buffer or another appropriate buffer. A volume of 100 µL will be used for each reaction. The dilution factor required depends on the total amount of protease present in the sample. In the first trial, the samples should be serially diluted to determine the optimal amount of sample for the assay. Please note that these protease samples will be further diluted in the assay by a factor of two.

3.4 *(Optional)* Prepare a plus-enzyme positive control. Generate a positive control by diluting an appropriate enzyme standard (e.g., trypsin) of known activity in the reaction buffer. A 100 µL

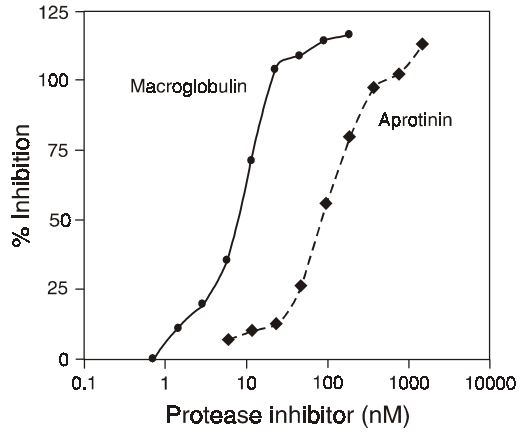

Figure 4. *Detection of aprotinin and a2 macroglobulin inhibition of chymotrypsin using the RediPlate 96 EnzChek Protease Assay Kit for red fluorescence. Each reaction contained BODIPY TR-X casein, 25 mU/mL chymotrypsin and the indicated concentration of aprotinin (□) or a2 macroglobulin (●) in the provided reaction buffer. Reactions were incubated at 37°C. After 30 minutes, fluorescence was measured in a fluorescence microplate reader using excitation at 590 ± 10 nm and fluorescence detection at 645 ± 20 nm.*

volume will be used. This plus-protease control will demonstrate hydrolytic activity of the enzyme on the fluorogenic substrate.

3.5 Begin the reactions. Pipet 100 µL of the protease-containing samples, including any positive control samples, into the wells of the microplate containing reconstituted substrate (prepared in step 3.1), and mix well.

3.6 Incubate the reactions. Incubate at the optimal temperature for the protease, protected from light. The exact interval will have to be determined experimentally. A suggested starting range is 30–60 minutes. Incubate for up to 24 hours to increase sensitivity. Because the assay is continuous (not terminated), fluorescence can be measured at multiple time points to follow the kinetics of the reactions.

3.7 Measure the fluorescence. Use a fluorescence microplate reader equipped with appropriate filters. BODIPY TR-X dye–labeled peptides have excitation/emission maxima of approximately 589/617 nm (see Figure 1). Filters appropriate for Texas Red® dye (e.g., excitation = 590 ± 10 nm, emission = 645 ± 20 nm) can be used to detect the red-fluorescent BODIPY TR-X dye–labeled peptides.

3.8 Correct for background fluorescence. For each point, subtract the value derived from the no-protease control.

Protease-Inhibitor Assay

The following provides one possible protocol for measuring protease inhibition with the RediPlate 96 EnzChek Protease Assay; other protocols can also be devised. Please note that the spectral properties of the protease-inhibitor alone may require evaluation prior to the experiment to see if the compound absorbs or fluoresces at wavelengths that might interfere with the BODIPY TR-X dye's fluorescence. Intrinsic fluorescence or, to a lesser extent, absorbance of the inhibitor can complicate the interpretation of fluorescence observed in the reactions. Thus, it may be necessary to perform additional controls not specified in the following protocol.

4.1 Dilute the protease inhibitor in reaction buffer. A volume of 100 µL will be used for each reaction. A variable dilution will be required depending on the potency of the inhibitor and the total amount of protease present in the sample. Please note that these inhibitor samples will be further diluted in the assay by a factor of two.

4.2 Add the protease inhibitor–containing samples to the assay. Pipet 100 µL of the protease inhibitor–containing samples into as many assay wells as will be needed, and then mix by pipetting. It is important to fully solubilize the protease substrate before adding the protease sample (see step 4.6, below).

4.3 Prepare no-inhibitor control. For controls, add 100 µL of reaction buffer, alone, to two assay wells and mix by pipetting. Add 100 µL to an empty assay well. The first well will serve as a no-inhibitor/no-protease control; the other will serve as a no-inhibitor/plus-protease control.

4.4 Dilute the protease in reaction buffer. A volume of 100 µL will be used for each reaction. An appropriate dilution will be required depending on the total amount of protease inhibitor in the sample. The optimal protease concentration for the protease-inhibitor must be determined in advance.

4.5 Prepare a no-inhibitor/no-protease control. Add an additional 100 µL of reaction buffer to the no-inhibitor/no-protease assay well (prepared in step 4.3) and mix by pipetting.

4.6 Begin the reactions. Pipet 100 µL volumes of the protease into each inhibitor-containing well. In addition, pipet 100 µL of the protease into the no-inhibitor/plus-protease control well (prepared in step 4.3).

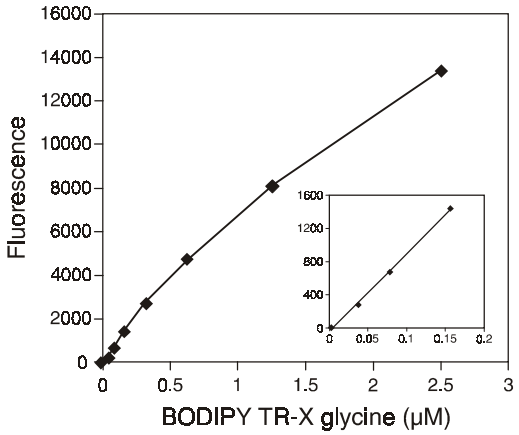

Figure 5. *Fluorescence reference standards of the RediPlate 96 EnzChek Protease Assay Kit for red-fluorescence. A volume of 200 µL of the provided reaction buffer was added to each well containing the BODIPY TR-X glycine reference standard. Fluorescence was measured in a fluorescence-based microplate reader using excitation at 590 ± 10 nm, and fluorescence detection at 645 ± 20 nm. Background fluorescence (12 arbitrary units), determined for well H, has been subtracted from each value. The inset shows an enlargement of the results obtained with BODIPY TR-X glycine concentrations between 0 and 0.16 µM.*

4.7 Incubate the reactions. Incubate at the optimal temperature for the protease, protected from light. The optimal time interval will have to be determined experimentally. A suggested starting range is 30–60 minutes. Because the assay is continuous (not terminated), fluorescence can be measured at multiple time points to follow the kinetics of the reactions.

4.8 Measure the fluorescence. Use a fluorescence microplate reader equipped with appropriate filters. BODIPY TR-X dye–labeled peptides have excitation/emission maxima of approximately 589/617 nm (see Figure 1). Filters appropriate for Texas Red® dye (e.g., excitation = 590 ± 10 nm, emission = 645 ± 20 nm) can be used to detect the red-fluorescent BODIPY TR-X dye–labeled peptides.

4.9 Correct for background fluorescence. For each point, subtract the value derived from the no-inhibitor/no-protease control.

4.10 Report the change in fluorescence. Inhibition can be reported either directly as corrected fluorescence (from step 4.9) or as percent inhibition. Percent inhibition is calculated by using the following formula:

$$\% \text{ inhibition } = \left(1 - \frac{F_{\text{inhibitor}}}{F_{\text{control}}}\right) \times 100\%$$

where $F_{\text{inhibitor}}$ is the corrected fluorescence of the inhibitor-containing sample and F_{control} is the corrected fuorescence of the no-inhibitor/plus-protease control (see Figure 4).

References

1. J Histochem Cytochem 49, 1473 (2001); **2.** Anal Biochem 251, 144 (1997); **3.** Anal Biochem 254, 144 (1997); **4.** Anal Biochem 279, 170 (2000).

Product List

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

Cat #	Product Name	Unit Size
R-22130	RediPlate™ 96 EnzChek® Protease Assay Kit *green fluorescence* *one 96-well microplate*	1 kit
R-22132	RediPlate™ 96 EnzChek® Protease Assay Kit *red fluorescence* *one 96-well microplate*	1 kit
R-22131	RediPlate™ 384 EnzChek® Protease Assay Kit *green fluorescence* *one 384-well microplate*	1 kit
R-22133	RediPlate™ 384 EnzChek® Protease Assay Kit *red fluorescence* *one 384-well microplate*	1 kit

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