

# Improved Protease Detection Using IRDye® 800RS Labeled Casein



Aerius® Automated Microplate Reader

Teresa Urlacher\*, Jiyan Chen, Chuck Prescott, Xinshe Xu, Xinzhan Peng, Dan Draney

LI-COR® Biosciences, 4647 Superior Street, Lincoln, NE USA 68504 USA

\* Corresponding author. E-mail: [teresa.urlacher@licor.com](mailto:teresa.urlacher@licor.com)

Odyssey® Infrared Imaging System



## ABSTRACT

We have developed a protease assay using a substrate, casein, that is over-labeled with LI-COR Biosciences IRDye® 800RS. Detection in the near-infrared provides for low interference from biological samples and low light scattering in biological assays. Target molecules labeled with LI-COR Biosciences near-infrared dyes provide a platform for biological assays that demonstrate superior sensitivity compared to visible dye systems. The system described here uses casein over-labeled with IRDye 800RS (emission at ~800 nm) that exhibits up to 20-fold fluorescence enhancement upon digestion with various proteases. The assay is performed in a microplate format and fluorescence enhancement is detected on a LI-COR Aerius® Automated Infrared Imaging System. The limits of detection for three proteases are significantly better compared to a visible dye system. This LI-COR Biosciences reagent and instrument system establishes a new benchmark in the field of protease detection.

## INTRODUCTION

Detection of protease activity is of great importance to establish protein purity, quality, and stability. For this reason, fluorescence quenching-based protease assays have been an attractive detection method due to their robustness, sensitivity, and versatility, not only in research laboratories, but also in the custom protein purification and drug development areas.

The use of near infrared (NIR) dyes provides a unique environment for biological assay detection because of the low interference from contaminating substances and the reduction of light-scattering effects. We used this to our advantage in developing an assay for detection of protease activity. Casein, a general protease substrate found in milk, was over-labeled with IRDye 800RS. In this over-labeled state, the fluorescence of 800RS Casein is quenched due to the proximity of the dye molecules to one another. Upon cleavage with proteases, smaller-labeled 800RS polypeptides are released. This decreases the intermolecular interactions of the dye, increasing the fluorescence emission of labeled IRDye 800RS peptides as shown in Figure 1. The low background fluorescence in the near IR spectral region provides for a large enhancement in signal due to the release of 800RS labeled polypeptides.

The NIR probe used in these studies is a proprietary infrared LI-COR dye, IRDye 800RS. As seen from the spectra (Figure 2), IRDye 800RS emits strongly at 786 nm and has a Stokes shift of 16-19 nm. The combination of these dye properties provides enhanced limits of detection and robustness for assays using IRDye 800RS.

There are commercial kits available that use the principle of fluorescence quenching to detect protease activity, but they all detect at visible wavelengths<sup>(1-4)</sup>. Detection in the visible spectrum reduces protease assay sensitivity due to autofluorescent properties of many biological molecules and contaminants, increased background from light-scattering effects, and microtiter plate interferences. The limitations encountered with visible dye conjugates for protease assays are overcome with NIR fluorescence technology, providing optimal protease activity detection with IRDye 800RS-labeled casein.

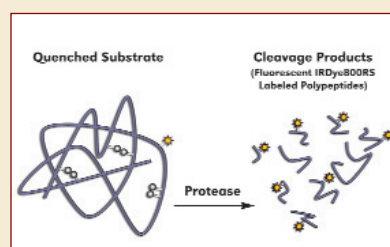


Figure 1. Schematic principle of fluorescence quenching protease assays.

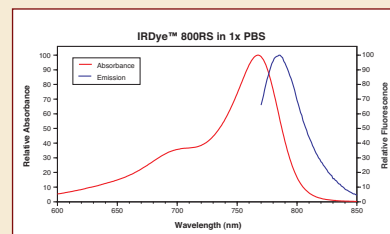


Figure 2. The absorbance and emission of LI-COR's proprietary NIR dye, IRDye 800RS, in 1X PBS.

## RESULTS & DISCUSSION

As Figure 3 shows, when the conjugated casein substrate is digested by trypsin, the fluorescence intensity significantly increases and the enhancement factor is correlated to the enzyme amount. The enhancement factor without enzyme to 5 µg/mL is consistently up to 20-fold, compared with only 5-fold enhancement from other commercial assays (Figure 4). The robustness of the assay to reject background fluorescence was tested by adding 100 nM fluorescein into our system. As shown in Figure 4, the result is almost identical to that in the absence of fluorescein.

Figure 5 shows the integrated intensity response to low nanogram amounts of trypsin. The response is linear under a limited range, but not at saturating enzyme or low enzyme amounts that approach signals near the background.

The data in Table 1 shows a 2-30-fold improvement in the limit of detection (LOD) for three unique proteases with 800RS Casein. From responses in the linear range, we determined the LOD using two distinct methods. The first method defines LOD by the amount of enzyme required to cause a 20% change in fluorescence compared to the background samples. The second method uses the minimal response of samples containing ultra low amounts of enzyme to define the LOD. The threshold value considered to be above the minimal response was determined by adding the mean of the minimal response samples ( $\mu_{MR}$ ) to the standard deviation of the minimal response values ( $\sigma_{MR}$ ) expressed as the equation

$$\text{Detection Level}_{MR} = \mu_{MR} + \sigma_{MR}^{(5)}$$

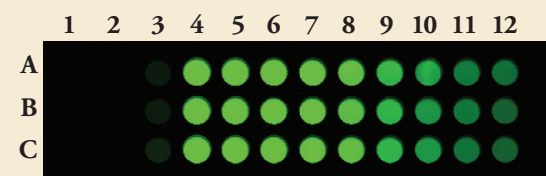


Figure 3. A, B, and C are three replicates. Column 1: buffer only; column 2: trypsin only; and column 3: the substrate only, 800RS Casein. A two-fold dilution series of trypsin from 5 µg/mL to 19.7 ng/mL is in columns 4-12 respectively, at a substrate concentration of 20 nM.

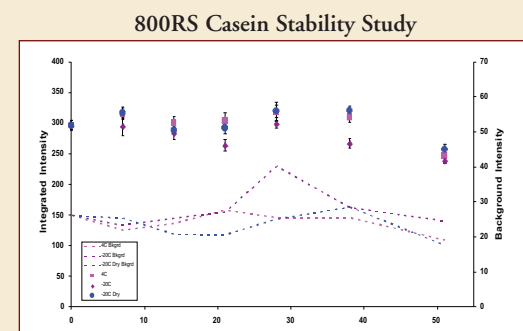


Figure 6. 800RS Casein was stored lyophilized at -20 °C, in solution at -20 °C, or in solution at 4 °C and monitored over 50 days. The background (dashed lines) was determined in the absence of enzyme. The maximum integrated intensity (solid points) was measured after cleavage of the 800RS casein with saturating amounts of trypsin.

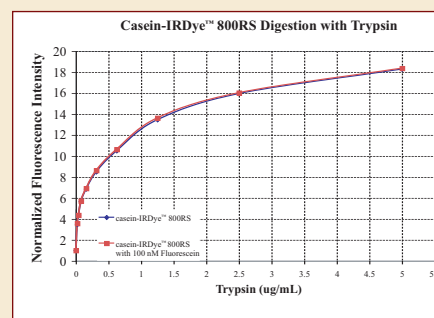


Figure 4. Normalized IRDye® 800RS fluorescence intensity reading on Aerius of reactions incubated with trypsin in the presence or absence of fluorescein.

800RS-Casein			
LIMIT OF DETECTION COMPARISONS			
Enzyme (Source)	Class	LI-COR 20% Above Blank LOD <sup>a</sup> (Units x10 <sup>3</sup> )	LI-COR 'Rigorous' LOD <sup>b</sup> (Units x10 <sup>3</sup> )
Trypsin (porcine pancreas)	Serine Protease	82	270
Thermolysin (B. proteolyticus rokkii)	Acid Protease	0.68	1.9
Papain (papaya latex)	Sulphydryl Protease	0.62	0.61

<sup>a</sup>Limit of detection is defined as the enzyme amount required to cause a 20% change in fluorescence signal compared to the background samples.  
<sup>b</sup>Molecular Probes EnzoChem Protease Assay Kit product information. Limit of detection defined as the amount of enzyme required to cause a 10-20% change in fluorescence compared to the control sample at 22 °C.  
<sup>c</sup>A more rigorous definition of limit of detection is defined by the amount of enzyme required to observe fluorescence signal that is above the minimal response (MR) detection level. This is defined by the following equation:  $\text{Detection Level}_{MR} = \mu_{MR} + 3\sigma_{MR}$ , where  $\mu_{MR}$  is the mean of the minimal response and  $\sigma_{MR}$  is the standard deviation of the minimal response.

Table 1. Limit of detection comparisons of 800RS Casein to those of a competitor.

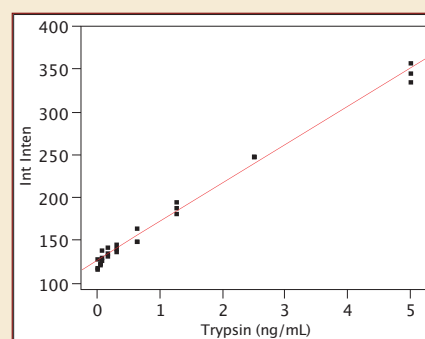


Figure 5. The integrated intensity of the IRDye 800RS fluorescence on Aerius in response to the trypsin amounts when the enzyme is in the range of a few nanograms.

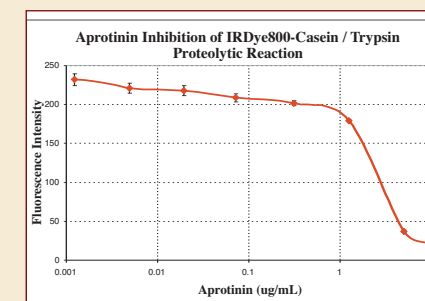


Figure 7. Aprotinin inhibition of 800RS Casein digestion with trypsin.

## MATERIALS AND METHODS

**Reagents.** IRDye 800RS NHS ester and its proprietary protein labeling and purification protocols are from LI-COR Biosciences. Casein was purchased from Calbiochem. Trypsin, thermolysin, papain, and aprotinin were all purchased from Sigma-Aldrich.

**Instrument.** The Aerius Automated Microplate Reader is a two-color fluorescence imager for solution and surface-based fluorescent assays performed in a microtiter plate. A scanning optical assembly carries two laser diodes that generate excitation light at 680 nm and 780 nm, with simultaneous detection of emitted light in the two distinct wavelength bands at approximately 710 nm and 810 nm. Under normal operating conditions, there is virtually no cross-talk between the two channels. The instrument detects IRDye 680 and IRDye 700DX in the 700 channel and both IRDye 800RS and IRDye 800CW in the 800 channel. The optical capabilities of the Odyssey® scanner are similar to those of the Aerius Automated Microplate Reader.

**Protease Assays.** The proteolytic reactions are carried out in black 96-well plates with optically clear bottoms. 800RS Casein is resuspended in 50 mM TRIS-HCl, pH 7.8, and 0.01% sodium azide and used at a final concentration of 20 nM in a total volume of 150 µL. Trypsin reactions are incubated at 37 °C for 1 hour in Reaction Buffer (50 mM TRIS-HCl, pH 7.8, 0.05% tween-20, and 0.01% sodium azide). Thermolysin reactions were performed in Reaction Buffer supplemented with 125 µM calcium acetate and 30 µM zinc acetate and incubated at 70 °C for 1 hour. Papain reactions contain 10 mM MES, pH 6.2, 20 mM cysteine, 0.05% tween-20 and 0.01% sodium azide and are incubated for 1 hour at 25 °C. Following the appropriate incubation condition, the sealed plates are centrifuged to remove condensation and the fluorescence response in each well is determined by the Aerius.

**Inhibitor Assay.** 800RS Casein concentration is 20 nM, trypsin concentration is 5 µg/mL, and aprotinin concentration varies from 10 µg/mL to 1.2 ng/mL. All inhibitor reactions are incubated with trypsin for 1 hour at 37 °C prior to reading the fluorescence on the Aerius.

## CONCLUSIONS

Fluorescence quenching-based protease assays are sensitive, versatile, convenient, and robust. However, with NIR detection, the fluorescence quenching assays provide the following advantages for protease detection:

1. 800RS Casein provides superior sensitivity, stability, and enhanced dynamic range at 1/10th the concentration of substrate recommended in current protease assays.
2. The limits of detection determination for three unique proteases show a 2-30-fold improvement in sensitivity.
3. The IRDye 800RS-based NIR protease assay demonstrates robustness in rejecting fluorescence background.

We expect this assay to impact both basic research and drug development areas.

## REFERENCES

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