



A Sensitive Fluorimetric Assay for Detection of HIV-1 Protease Using a Novel FRET Peptide Substrate

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Introduction

The 10~12 kD aspartic protease of human immunodeficiency virus-1 (HIV-1) is required for the post-translational cleavage of the precursor polyproteins, Pr⁹⁸ and Pr⁹⁵.¹ These cleavages are essential for the maturation of HIV infectious particles. Thus, the protease becomes one of the key targets for developing anti-AIDS drugs.

Although the EDANS/DABCYL FRET pair has been widely used in the fluorimetric assay for the detection of HIV-1 protease, this pair has relatively weak fluorescence signal with short wavelength. We designed a novel HiLyte FluorTM488/QXLTM520 pair that are used to develop more sensitive HIV-1 substrates for detecting HIV-1 protease activity.

In the FRET peptide, the fluorescence of HiLyte FluorTM488 is quenched by QXLTM520 until the peptide is cleaved by HIV-1 protease. With excellent fluorescence quantum yield and longer excitation and emission wavelength, the fluorescence signal of HiLyte FluorTM488 is less interfered by the autofluorescence of cell components and test compounds.

Results

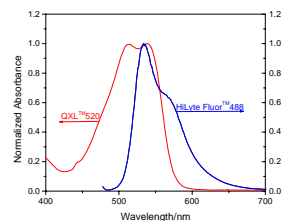


Figure 1. The absorption spectrum of QXLTM520 perfectly overlaps with the emission spectrum of HiLyte FluorTM488.

HiLyte FluorTM488 is a new fluorophore we designed. Its extinction coefficient is 92,400 M⁻¹cm⁻¹ which is 3-fold higher than that of EDANS. The excitation and emission wavelengths of HiLyte FluorTM488 are 490 nm and 520 nm, respectively.

These wavelengths are longer than those of EDANS (Ex = 340 nm and Em = 490 nm), thus fluorescence of HiLyte FluorTM488 is less interfered by the short wavelength autofluorescence of drug candidates. Additionally, HiLyte FluorTM488 is much brighter and less sensitive to the environment than EDANS. In addition, we also developed a quencher QXLTM520. Its absorption spectrum perfectly overlaps with the emission spectrum of HiLyte FluorTM488, and QXLTM520 is a hydrophilic compound unlike DABCYL, which is hydrophobic. This property of QXLTM520 increases the solubility of the peptide substrate. Thus, these characteristics of HiLyte FluorTM488 and QXLTM520 prompted us to design a more sensitive HiLyte FluorTM488/QXLTM520 FRET peptide substrate for HIV-1 protease.

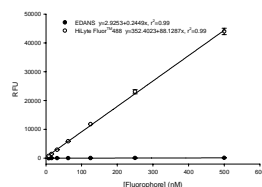
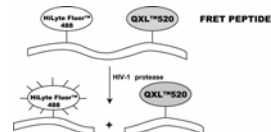


Figure 2. Proteolytic cleavage of HiLyte FluorTM488/QXLTM520 FRET peptide by HIV-1 protease

At the same concentration, HiLyte FluorTM488 has much stronger fluorescence intensity than EDANS (Figure 3). Thus, HiLyte FluorTM488-based FRET peptide is potentially more sensitive than EDANS-based FRET peptide.

Figure 3. Compare the fluorescence intensity of HiLyte FluorTM488 and EDANS

HiLyte FluorTM488/QXLTM520 FRET peptide (Figure 2) based on the sequence of EDANS/DABCYL FRET peptide. We changed the donor and quencher to HiLyte FluorTM488 and QXLTM520, respectively. The fluorescence of HiLyte FluorTM488 is quenched by QXLTM520 until the peptide is cleaved by HIV-1 protease. Upon cleavage, the fluorescence of HiLyte FluorTM488 is recovered and can be continuously monitored at Excitation/Emission=490 nm/520 nm over time.

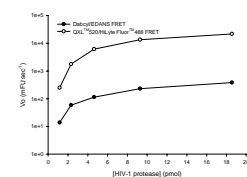


Figure 4a. The initial velocities (V_o) at each enzyme concentration were calculated and plotted against the HIV-1 molecules. The initial velocities are expressed at mFU/sec.

Although at the same enzyme concentration, the HIV-1 protease cleaves the EDANS/DABCYL peptide a slightly faster than HiLyte FluorTM488/QXLTM520 peptide (Fig 4b), the relative fluorescence unit (RFU) generated in the assay using HiLyte FluorTM488/QXLTM520 peptide was much higher than that in the assay using EDANS/DABCYL peptide (Figure 4a), since the fluorescence intensity of HiLyte FluorTM488 is stronger than EDANS (Figure 3). The enzyme detection dynamic range of HiLyte FluorTM488/QXLTM520 FRET peptide is almost same as EDANS/DABCYL FRET peptide. However, figure 4a also demonstrates that the HiLyte FluorTM488/QXLTM520 FRET peptide is eight times more sensitive than EDANS/DABCYL FRET peptide.

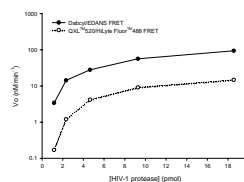


Figure 4b. The initial velocities (V_o) at each enzyme concentration were calculated and plotted against the HIV-1 molecules. The initial velocities are expressed at nFU/min.

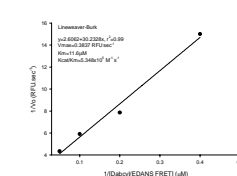


Figure 5a. Double-reciprocal plot of the initial hydrolysis velocity versus EDANS/DABCYL FRET peptide concentration.

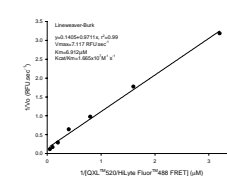


Figure 5b. Double-reciprocal plot of the initial hydrolysis velocity versus HiLyte FluorTM488/QXLTM520 FRET peptide concentration.

Table 1. The comparison of kinetic parameters of two FRET substrates.*

	V_{max} (RFU/sec)	K_m (μM)	K_{cat}/K_m (M ⁻¹ s ⁻¹)
HiLyte Fluor TM 488/QXL TM 520 FRET peptide	7.12	6.912	1.7x10 ⁵
EDANS/DABCYL FRET peptide	0.38	11.6	5.3x10 ³

HiLyte FluorTM488/QXLTM520 FRET peptide has smaller K_m and higher K_{cat}/K_m value compared to EDANS/DABCYL FRET peptide.

Discussion and Conclusion

We have developed a highly sensitive FRET substrate for monitoring HIV-1 protease activity. The K_{cat}/K_m of HiLyte FluorTM488/QXLTM520 FRET peptide is 32-fold higher than that of the corresponding EDANS/DABCYL equivalent. This HiLyte FluorTM488/QXLTM520 FRET peptide is more sensitive than its EDANS/DABCYL equivalent. Compared to EDANS, HiLyte FluorTM488's longer excitation and emission wavelength can minimize the interference from the autofluorescence emitted by test compounds.

In conclusion, this HiLyte FluorTM488/QXLTM520 FRET peptide has improved enzyme kinetic parameters and assay sensitivity compared to the existing EDANS/DABCYL peptide. It can be applied to the high throughput screening of anti-HIV-1 protease drugs.

Reference:

1. S. Seelmeier, H. et al. *Proc.Natl.Acad.Sci.U.S.A* 85, 6612-6616 (1988).