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, Pr^{gag} and $Pr^{gag\text{-}pol}$.[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 Fluor™488/QXL™520 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 Fluor™488 is quenched by QXL™520 until this peptide is cleaved into two separate fragments by HIV-1 protease. With excellent fluorescence quantum yield and longer excitation and emission wavelength, the fluorescence signal of HiLyte Fluor™488 is less interfered by the autofluorescence of cell components and test compounds.

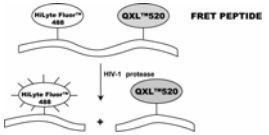

Figure 2. Proteolytic cleavage of HiLyte Fluor™488/QXL™520 FRET peptide by HIV-1 protease

At the same concentration, HiLyte Fluor™488 has much stronger fluorescence intensity than EDANS (Figure 3). Thus, HiLyte Fluor™488-based FRET peptide is potentially more sensitive than EDANS-based FRET peptide.

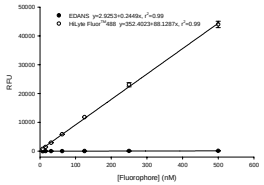

Figure 3. Compare the fluorescence intensity of HiLyte Fluor™488 and EDANS

HiLyte Fluor™488/QXL™520 FRET peptide (Figure 2) based on the sequence of EDANS/DABCYL FRET peptide. We changed the donor and quencher to HiLyte Fluor™488 and QXL™520, respectively. The fluorescence of HiLyte Fluor™488 is quenched by QXL™520 until the peptide is cleaved by HIV-1 protease. Upon cleavage, the fluorescence of HiLyte Fluor™488 is recovered and can be continuously monitored at Excitation/Emission=490 nm/520 nm over time.

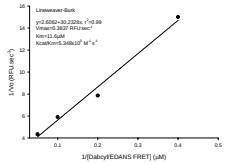

Figure 5a. Double-reciprocal plot of the initial hydrolysis velocity versus EDANS/DABCYL FRET peptide concentration.

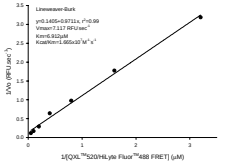

Figure 5b. Double-reciprocal plot of the initial hydrolysis velocity versus HiLyte Fluor™488/QXL™520 FRET peptide concentration.

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™488/QXL™520 FRET peptide	7.12	6.912	$1.7x10^7$
EDANS/DABCYL FRET peptide	0.38	11.6	$5.3x10^5$

HiLyte Fluor™488/QXL™520 FRET peptide has smaller *Km* and higher *Kcat/Km* value compared to EDANS/DABCYL FRET peptide.

Results

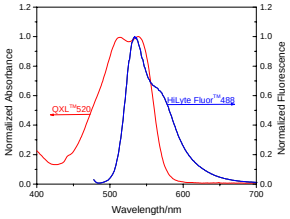

Figure 1. The absorption spectrum of QXL™520 perfectly overlaps with the emission spectrum of HiLyte Fluor™488.

HiLyte Fluor™488 is a new flurophore we designed. Its extinction coefficient is 92,400 $M^{-1}cm^{-1}$which is 3-fold higher than that of EDANS. The excitation and emission wavelengths of HiLyte Fluor™488 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 Fluor™488 is less interfered by the short wavelength auto-fluorescence of drug candidates. Additionally, HiLyte Fluor™488 is much brighter and less sensitive to the environment than EDANS. In addition, we also developed a quencher QXL™520. Its absorption spectrum perfectly overlaps with the emission spectrum of HiLyte Fluor™488, and QXL™520 is a hydrophilic compound unlike DABCYL which is hydrophobic. This property of QXL™520 increases the solubility of the peptide substrate. Thus, these characteristics of HiLyte Fluor™488 and QXL™520 prompted us to design a more sensitive HiLyte Fluor™488/ QXL™520 FRET peptide substrate for HIV-1 protease.

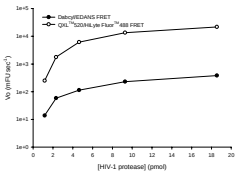

Figure 4a. The initial velocities (Vo) at each enzyme concentration were calculated and plotted against the HIV-1 molecules. The initial velocities are expressed at mFU/sec.

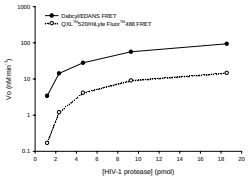

Figure 4b. The initial velocities (Vo) at each enzyme concentration were calculated and plotted against the HIV-1 molecules. The initial velocities are expressed at nM/min.

Although at the same enzyme concentration, the HIV-1 protease cleaves the EDANS/DABCYL peptide a slightly faster than HiLyte Fluor™488/QXL520 peptide (Fig 4b), the relative fluorescence unit (RFU) generated in the assay using HiLyte Fluor™488/QXL520 peptide was much higher than that in the assay using EDANS/DABCYL peptide (Figure 4a), since the fluorescence intensity of HiLyte Fluor™488 is stronger than EDANS (Figure 3). The enzyme detection dynamic range of HiLyte Fluor™488/QXL™520 FRET peptide is almost same as EDANS/DABCYL FRET peptide. However, fiugure 4a also demonstrates that the HiLyte Fluor™488/QXL™520 FRET peptide is eight times more sensitive than EDANS/DABCYL FRET peptide.

Discussion and Conclusion

We have developed a highly sensitive FRET substrate for monitoring HIV-1 protease acitivity. The *Kcat/Km* of HiLyte Fluor™488/ QXL™520 FRET peptide is 32-fold higher than that of the corresponding EDANS/DABCYL equivalent. This HiLyte Fluor™488/ QXL™520 FRET peptide is more sensitive than its EDANS/DABCYL equivalent. Compared to EDANS, HiLyte Fluor™488's longer excitation and emission wavelength can minimize the interference from the autofluorescence emitted by test compounds.

In conclusion, this HiLyte Fluor™488/QXL™ 520 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).