The Optimization of FRET Substrates for Detection of Cathepsins Activity

Deepa Mohan, Aneta Tawfik, Aimin Song, Jianjun He and Vera Rakhmanova

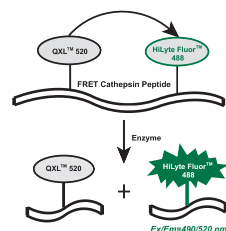
AnaSpec, Eurogentec Group, 34801 Campus Dr. Fremont, CA 94555, USA

Introduction

Cathepsins are a class of globular lysosomal proteases playing a vital role in mammalian cellular turnover and in multiple pathological processes, including cancer, inflammation, atherosclerosis, neuro- and autoimmune diseases. They degrade polypeptides and are distinguished by their substrate specificities. The use of FRET (Fluorescence Resonance Energy Transfer) and fluorogenic substrates has facilitated the assay of cathepsins. Previously described FRET substrates for cathepsins include Mca/Dnp, Abz/Dnp and EDANS/DABCYL donor acceptor pairs. The introduction of the longer wavelength 5-FAM/QXL™ 520 FRET based cathepsin substrates has resulted in minimizing interference from autofluorescence of test compounds and reaction components. In these FRET peptides, the fluorescence of 5-FAM is quenched by QXL™ 520. Upon cleavage of the peptide by active cathepsin, the fluorescence of 5-FAM is recovered, and can be continuously monitored at Ex/Em = 490/520 nm. Despite improved characteristics, these substrates still have limited sensitivity. The reason is that most of the cathepsins are lysosomal enzymes and require low pH for optimal activity, and 5-FAM shows decrease of signal in acidic environment. To further improve cathepsin assays, i.e. develop substrates for maximum sensitivity at low pH, we introduced a new pH independent fluorophore HiLyte Fluor™ 488 in place of 5-FAM. Besides uncompromised signal at low pH, HiLyte Fluor™ 488 has excellent brightness, high extinction coefficient (70,000 $M^{-1}cm^{-1}$), fluorescence quantum yield of 0.91, and Ex/Em = 497/525 nm. The absorption spectrum of QXL™ 520 overlaps with the emission spectrum of HiLyte Fluor™ 488, providing optimal quenching.

We developed a panel of FRET peptide substrates for cathepsin D, K, B and G. For each enzyme, we synthesized the optimized peptide sequence coupled with either 5-FAM/QXL™ 520 or HiLyte Fluor™ 488/QXL™ 520 FRET pair. For most cathepsins, HiLyte Fluor™ 488/QXL™ 520–based substrates provide better sensitivity and signal/background ratio compared with the same peptide sequences but having the 5-FAM/QXL™ 520 FRET pair. Using HiLyte Fluor™ 488, instead of 5-FAM, allowed an increase of signal/background ratio of least 1.5-fold for cathepsin K, 2-fold for cathepsin B and 10-fold for cathepsin D. HiLyte Fluor™ 488/QXL™ 520 FRET substrates have improved kinetic parameters and have been validated in assay for inhibitor screening. As well, cross-reaction between substrates for different cathepsins shows minimal interference.

Assay Principle



Scheme 1. Proteolytic cleavage of HiLyte Fluor™ 488/QXL™520 FRET peptide by cathepsins. Fluorescence of HiLyte Fluor™ 488 is quenched by QXL™ 520 in the intact FRETpeptide until it is cleaved into two separate fragments by cathepsin. Upon cleavage, the fluorescence of HiLyte Fluor™ 488 is recovered, and can be monitored continuously at Ex/Em = 490/520nm.

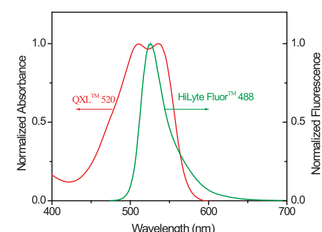


Figure 1. HiLyte Fluor™ 488 and QXL™ 520 is a new donor-acceptor pair for FRET substrates. The absorption spectrum of QXL™ 520 overlaps with the emission spectrum of HiLyte Fluor™ 488. Extinction coefficient of HiLyte Fluor™ 488 is 70,000 M^{-1} cm^{-1}.

Materials and Methods

- ➢ SensoLyte® 520 Cathepsin Assay Kits (Cathepsin B, D, G and K Assay Kits)
- √ Cathepsin (HiLyte Fluor™ 488)-peptide-(QXL™ 520) FRET substrates (Cathepsin B, D, G and K substrates) - designed and synthesized by Fmoc solid phase synthesis method. Ex/Em=490/520 nm upon cleavage
- ➢ Cathepsin (5-FAM)-peptide-(QXL™ 520) FRET substrates (Cathepsin B, D, G and K substrates) - designed and synthesized by Fmoc solid phase synthesis method. Ex/Em=490/520 nm upon cleavage.

SensoLyte® 520 Cathepsin Assay Kits were used as recommended by the protocol. Reaction volumes for these kits are 40 µl of enzyme, 10 µl of test compound/buffer, 50 µl of substrate. Assays were configured in 96-well black opaque plates.

Results

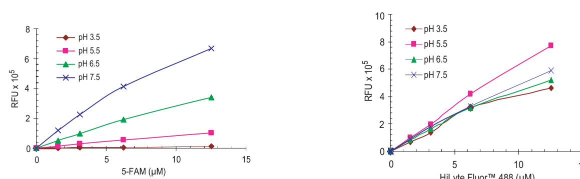


Figure 2. HiLyte Fluor™ 488 provides stable fluorescent signal even at low pH. Fluorescence of 5-FAM and HiLyte Fluor™ 488 was measured in 4 buffers with different pH.

Table 1. Km comparison of cathepsin substrates. Peptide sequences optimized for each cathepsin were coupled either with 5-FAM/QXL™ 520 or HiLyte Fluor™ 488/QXL™ 520 FRET pair.

	Km, µM			
FRET pair	Cathepsin B	Cathepsin D	Cathepsin G	Cathepsin K
5-FAM/QXL™ 520	12.6	12.5	7.3	15.6
HiLyte Fluor™ 488/QXL™ 520	6.25	6	2.5	6.2

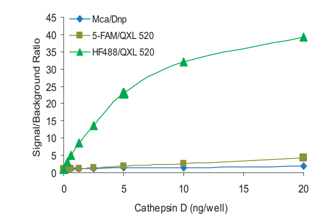


Figure 3. Titration of cathepsin D with peptide substrates with the same sequence, but different FRET pairs. Signal increases up to 40-fold after 1 hour of cathepsin D incubation with HiLyte Fluor™ 488/QXL™520 FRET substrate in comparison to only 2-fold signal increase observed for Mca/Dnp- based assay. A 4-fold increase is observed for the 5-FAM/QXL™ 520 FRET substrate.

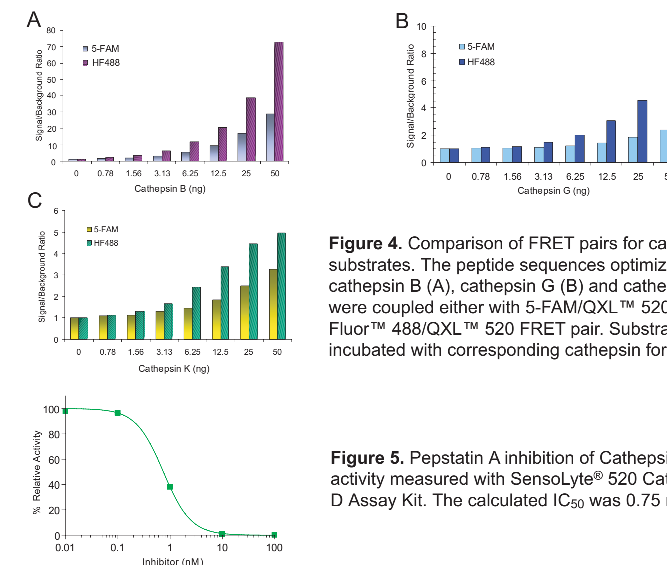


Figure 4. Comparison of FRET pairs for cathepsin substrates. The peptide sequences optimized for cathepsin B (A), cathepsin G (B) and cathepsin K (C) were coupled either with 5-FAM/QXL™ 520 or HiLyte Fluor™ 488/QXL™ 520 FRET pair. Substrates were incubated with corresponding cathepsin for 1 hour.

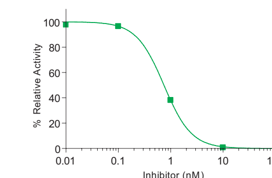


Figure 5. Pepstatin A inhibition of Cathepsin D activity measured with SensoLyte® 520 Cathepsin D Assay Kit. The calculated IC_{50} was 0.75 nM.

Table 2. Cross-reaction for cathepsin HiLyte Fluor™ 488/QXL™ 520 FRET substrates. Signal/background ratio was measured after 1 hour incubation of 10 ng each cathepsin with the panel of HiLyte Fluor™ 488/QXL™ 520 FRET substrates.

Enzyme \ Substrates	HF488 B FRET	HF488 D FRET	HF488 G FRET	HF488 K FRET
Cathepsin B	20	1	1	1
Cathepsin D	1	32	1.5	1
Cathepsin K	1	1	1.5	3
Cathepsin G	1	1	3	1

Conclusions

- ➢ We have introduced the new HiLyte Fluor™ 488/QXL™ 520 FRET pair for protease peptide substrates.
- ➢ HiLyte Fluor™488 provided stable fluorescent signal at low pH resulting in assay optimization for enzymes that require acidic pH for maximum activity.
- ➢ Highly sensitive SensoLyte® 520 Cathepsin assay kits based on a HiLyte Fluor™ 488/QXL™ 520 FRET substrates have been developed.
- ➢ HiLyte Fluor™ 488/QXL™ 520–based cathepsin substrates provided better sensitivity and signal/background ratio than the corresponding peptide sequences coupled with the 5-FAM/QXL™ 520 FRET pair.