Novel QXL-Based Protease Substrates and Their Applications in Drug Discovery

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Abstract

QXL™ dyes are excellent dark quenchers that are individually optimized to pair with all of the popular fluorescent dyes such as fluoresceins and rhodamines. Our QXL™ series of nonfluorescent dyes cover the full visible spectrum with unusually high efficiency. QXL™ 520 has absorption maximum perfectly matching the emission of FAM while QXL™ 570 is proven to be the best quencher for TAMRA. We have demonstrated that QXL™ 670 and 680 are the most effective quenchers for Cy5 and Cy5-like fluorophores.

AnaSpec has used QXL dyes to develop a number of FRET peptide substrates for high throughput analysis of protease activities and screening of protease inhibitors. For example, HiLyte Fluor™ 488 (or FAM)/QXL™ 520-based HIV protease substrates have demonstrated significantly enhanced performance. Excellent performance has also been observed for our new HCV protease substrates that incorporate HiLyte Fluor™ 488 (or FAM) with QXL™ 520 as acceptor. We have also used QXL/HiLyte Fluor pairs to develop novel protease substrates for analysis of secretases, caspases and various MMP activities.

Introduction

Proteolytic cleavage of peptide bonds is one of the most important mechanisms affecting the properties and functions of proteins. Proteases are ubiquitously distributed in all tissues and biological fluids. They play essential roles in protein activation, cell regulation and signaling, as well as in the generation of amino acids for protein synthesis or utilization in other metabolic pathways.

Among the many methods that are used to analyze proteases present in solutions, cells or tissues, spectrophotometric method has been favored due to its high speed, great accuracy and ease of use. This method has been predominantly used in high throughput screening of protease activities and inhibitors. It is proven that the spectral and enzymatic properties of chromogenic and fluorogenic substrates play a critical role in the successful use of spectro-photometric methods for analyzing proteases. Although the spectral properties of chromogenic and fluorogenic protease substrates and their hydrolysis products are easily predictable, the utility of a given substrate for a specific enzyme depends on the kinetics of hydrolysis by the enzyme. The enzyme reaction depends on the substrate's concentration and amino acid sequence, as well as on the pH, temperature and presence of cofactors in the medium. For measurements in live cells, the suitability of a particular substrate also hinges on its accessibility to the enzyme and the cellular retention of the hydrolysis product. In addition to these factors, the chromophore or fluorophore conjugated to the substrate can influence its hydrolysis rate and specificity, as well as the permeability of the substrate and its hydrolysis product.

Although EDANS/DABCYL and MCA/DNP are used to develop a variety of FRET protease substrates, their short absorption wavelengths and low extinction coefficients have limited their use in the development of sensitive fluorogenic protease substrates. AnaSpec has developed QXL™ dyes to address these limitations.

The Perfect Spectral Overlap of QXL™ Quenchers with Classic FRET Donors

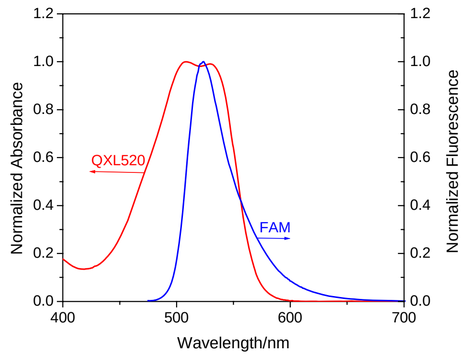

Figure 1. The spectral overlap of absorption spectrum of QXL™ 520 with emission spectrum of 5-FAM

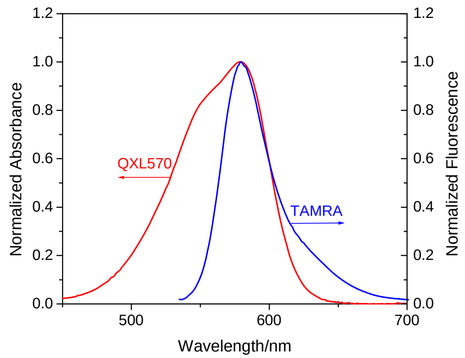

Figure 2. The spectral overlap of absorption spectrum of QXL™ 570 with emission spectrum of 5-TAMRA

The Application of QXL™ Quenchers in the Development of Ultra-sensitive Fluorogenic HCV protease Substrates

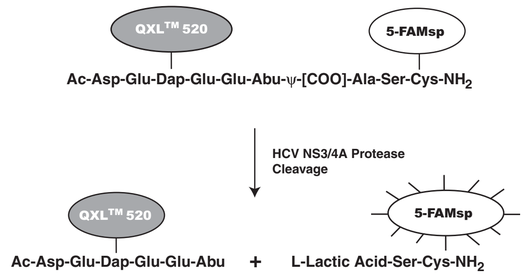

Figure 3. The enzymatic reaction scheme of HCV protease substrate that contains QXL™ 520-labelled FRET peptide.

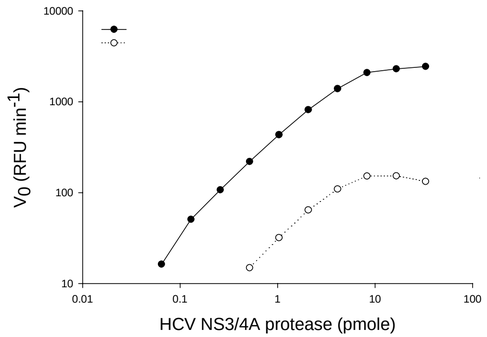

Figure 4. The sensitivity comparison of 5-FAM/QXL™520 FRET peptide (solid curve) and EDANS/DABCYL FRET peptide (dash curve). The enzyme detection dynamic range of 5-FAM/QXL™520 FRET peptide is from 8.27 to 0.064 pmole, while that of EDANS/DABCYL FRET peptide is from 8.27 to 0.52 pmole.

In conclusion, 5-FAM/QXL™520 FRET peptide is 8-fold more sensitive than EDANS/DABCYL FRET peptide.

The Application of QXL™ Quenchers in the Development of Ultra-Sensitive MMP Assays

QXL520 TM -γ-Abu-Pro-Cha-Abu-Smc-His-Ala-Dab(5-FAM)-Ala-Lys-NH_2

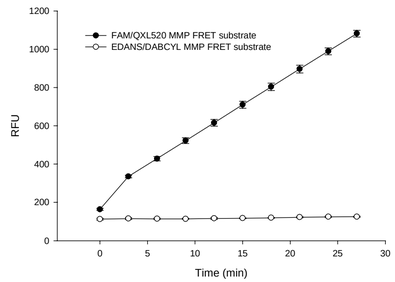

Figure 5. MMP-1 kinetic comparison of MMP peptide substrates that have the same peptide sequence, but are labeled respectively with FRET pairs of FAM/QXL™ 520 *vs* EDANS/DABCYL. These two FRET peptides are tested with incubation of MMP-1 at pH 7.5 using same concentrations of the enzyme and substrates. It is apparent that FAM/QXL™ 520-labeled peptide is a much more sensitive fluorescent indicator for MMP-1 activity.

The use of fluorogenic FRET peptide substrates facilitates the development of MMP activity assays. Several FRET pairs have been incorporated into MMP sensitive peptide sequences as fluorogenic MMP substrates. Among them, Mca/Dnp and Dabcyl/Edans are the most common ones. However, these existing FRET pairs have a few limitations such as lower sensitivity and shorter wavelengths. We developed MMP substrates incorporating 5-FAM (donor) and QXLTM 520 (quencher). This design offers the following advantages: a) The system gives a fluorescence signal, which can be continuously monitored at Ex/Em = 490nm/520nm. b) 5-FAM has stronger absorption and fluorescent signals than Mca and Edans. c) 5-FAM has longer absorption and emission wavelengths than Mca and Edans. 5-FAM/QXLTM 520 FRET peptides are more readily adapted to high throughput screening since this pair is less interfered by autofluorescence of test compounds and cellular components.

Among the sixteen 5-FAM/QXL 520TM FRET peptides screened, one peptide showed the highest proteolytic kinetics to all the MMPs tested. Submicromolar of this 5-FAM/QXL 520TM FRET peptide is adequate to detect picomolar level of MMPs.

Conclusions

In summary, our QXL™ dyes have the following advantages:

- *Most Powerful:* enable you to maximize the FRET potentials
- *Versatile Reactive Forms:* convenient for self-constructing your desired FRET biomolecules
- *A Complete Set of Non-fluorescent Dyes:* to perfectly match your desired fluorescent donors
- *Enhanced Value:* competitive price with the best performance