Design of a Novel FRET Substrate with a Long Wavelength Fluorophore for Detection of Matrix Metalloproteinases

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Introduction

Matrix metalloproteinases (MMPs) belong to a family of secreted or membrane-associated zinc endopeptidases involved in both normal and disease related tissue remodeling. MMPs are capable of degrading extracellular matrix (ECM) proteins and processing a number of bioactive molecules. MMPs are key players in normal and pathological processes, including embryogenesis, wound healing, inflammation, arthritis and cancer. The use of FRET technology has facilitated MMP assay development. Previously described FRET donor acceptor pairs include Mca/Dnp and EDANS/DABCYL. The introduction of longer wavelength 5-FAM/QXL™ 520 FRET based MMP substrates increased assay sensitivity, and signal to background ratio. To further improve MMP FRET assays, i.e. minimize autofluorescence from reaction components, we developed a new series of MMP substrates containing the 5-TAMRA/QXL™ 570 FRET pair. In these FRET peptides, the fluorescence of 5-TAMRA is quenched by QXL™ 570 and recovered upon cleavage of the peptide by active MMP enzyme. Fluorescence is then monitored at excitation/emission wavelengths = 540 /575 nm. After screening several 5-TAMRA/QXL™ 570 substrates, one sequence was identified to be cleaved by most of the MMPs. This substrate was chosen for the development of the SensoLyte® 570 Generic MMP Assay Kit. This kit provides high sensitivity and accuracy and can detect MMP-1, 2, 7, 8, 9, 12, 13, and 14. It is ideal for detecting of enzyme activity in samples containing multiple MMPs or for high throughput screening (HTS) of MMP inhibitors using purified enzymes.

Materials and Methods

- SensoLyte® 570 Generic MMP Assay Kit (Cat# 72101)
  - √ MMP generic 5-TAMRA/QXL™ 570 FRET Substrate - designed and synthesized by Fmoc solid phase peptide synthesis method.
  - √ APMA, 4-aminophenylmercuric acetate
  - √ Assay Buffer
- Galardin is a broad-spectrum inhibitor of matrix metalloproteinases (Calbiochem, San Diego, CA)
- Human recombinant MMP-1, 2, 7, 8, 9, 12, 13 and 14

SensoLyte® 570 Generic MMP Assay Kit was used as recommended by the protocol. The reaction volumes for this kit are 40 µl of enzyme, 10 µl of test compound/buffer, 50 µl of substrate. Assays were done in 96-well black opaque plates. Fluorescence was measured using FlexStation 384II (Molecular Devices, Sunnyvale, CA).

Assay Principle

The SensoLyte® 570 Generic MMP Assay is based on FRET (Fluorescence or Förster resonance energy transfer) principle.

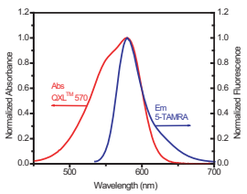

Figure 1. 5-TAMRA and QXL™ 570 is a novel FRET donor - acceptor pair for peptides. The absorption spectrum of QXL™ 570 overlaps with the emission spectrum of 5-TAMRA. QXL™ 570 serves as an excellent quencher for 5-TAMRA in FRET substrates.

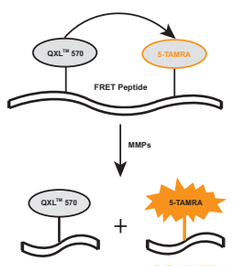

Figure 2. Proteolytic cleavage of 5-TAMRA/QXL™ 570 FRET peptide by MMPs. In intact FRET peptide, the fluorescence of 5-TAMRA is quenched by QXL™ 570. When MMPs cleave the substrate, the dye and quencher become separated and fluorescence of TAMRA is released, which can be monitored at Ex/Em = 540/575 nm.

Results

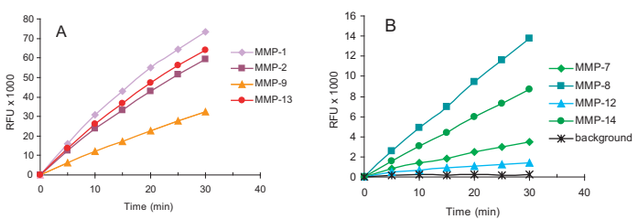

Figure 3. Kinetics of 5-TAMRA/QXL™ 570 FRET substrate hydrolysis by MMP enzymes. MMP generic substrate was cleaved by MMP-1, 2, 9, and 13 (A), and by MMP-7, 8, 12 and 14 (B). Fluorescence signal was continuously monitored at Ex/Em=540/575 nm for 30 min.

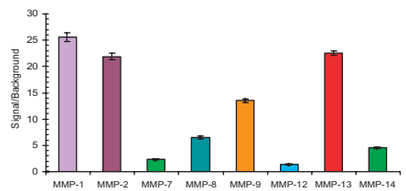

Figure 4. Comparison of signal/background ratio for various MMPs. APMA-activated MMPs, 30 ng each, were mixed with the 5-TAMRA/QXL™ 570 FRET peptide substrate and fluorescence monitored for 1 h. Endpoint signals were compared to background (FRET substrate without enzyme) to demonstrate MMP activities.

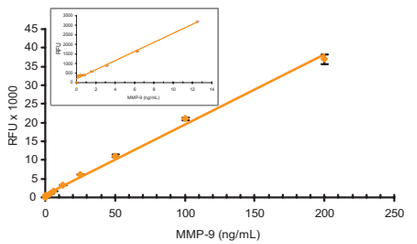

Figure 5. MMP-9 titration with 5-TAMRA/QXL™ 570 FRET substrate. Sensitivity of assay at 1 hour incubation was 0.78 ng/mL of MMP-9 enzyme. The insert shows detection at low MMP-9 concentrations.

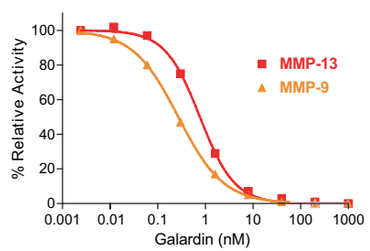

Figure 6. Inhibitor studies. 5-TAMRA/QXL™ 570 substrate was incubated with 10 ng of each of recombinant human MMP-9 and MMP-13 enzymes in the presence of a broad spectrum matrix metalloproteinases inhibitor, Galardin. The calculated IC_{50} were 0.27 nM and 0.77 nM for MMP-9 and MMP-13, respectively.

Conclusions

- We used a MMP peptide substrate incorporating the novel FRET pair, 5-TAMRA/QXL™ 570, to develop the SensoLyte® 570 Generic MMP Assay Kit. This substrate can detect a majority of the human MMPs tested.
- The red-shifted excitation and emission wavelengths of 5-TAMRA show minimal interference from autofluorescence of test compounds.
- The new 5-TAMRA /QXL™ 570 FRET substrate is highly sensitive and can detect nanogram range of MMPs tested.
- The SensoLyte® 570 Generic MMP Kit is very conducive for kinetic measurements due to its continuous, homogeneous assay format.
- The SensoLyte® 570 Generic MMP Assay Kit is validated for inhibitor screening using broad-spectrum matrix metalloproteinases inhibitors.