FRET-Based Assays for the Detection of Amyloid Degrading Protease Activity

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Introduction

Amyloid degrading proteases (AβDPs) are major players in β-amyloid (Aβ) cleavage. The accumulation of Aβ plaques, a hallmark of Alzheimer's disease (AD) patient brain, has been hypothesized to be due to the imbalance between AβDPs activity and Aβ production, resulting in AD pathogenesis. In order to confirm the role(s) of AβDPs in neuronal degeneration diseases, reliable assays for AβDPs activity detection have to be developed.

We have designed a panel of assays to detect AβDP targets using FRET (Förster resonance energy transfer)-based peptide substrates. To develop assays for ADAM10, TACE (ADAM17, α-secretase), Neprilysin and IDE (insulin degrading enzyme), we synthesized FRET substrate peptides labeled with the fluorophore, 5-carboxyfluorescein (5-FAM) and the quencher, QXL® 520. The fluorescence of 5-FAM is quenched by QXL® 520 and recovered upon cleavage of the peptide by active AβDP. Fluorescence is then monitored at the excitation/emission wavelengths of 490/520 nm.

Many of AβDP targets require low pH for optimal activity, and 5-FAM shows decrease of signal in acidic environment. To optimize FRET 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 at the same wavelength as 5-FAM. The absorption spectrum of QXL® 520 overlaps with the emission spectrum of HiLyte™ Fluor 488, providing optimal quenching. Substrates with HiLyte™ Fluor 488/QXL® 520 were used for detection of β-secretase, cathepsins B and D, providing higher sensitivity and better assay window.

Some of AβDPs, such as MMP-2 and MMP-9, belong to matrix metalloproteases that are known for overlapping substrate specificities. To avoid non-specific substrate cleavage by multiple proteases, we utilized an immunocapture technique in addition to the FRET assay. Monoclonal antibody were applied to plate to pull down relevant MMP from biological sample and subsequent addition of MMP peptide substrate containing the 5-FAM/QXL® 520 FRET pair. These assays provided significant increase specificity of MMP-2 and MMP-9.

Materials and Methods

- SensoLyte® 520 ADAM10, TACE (ADAM17), Neprilysin and IDE Assay Kits
  - √ FRET 5-FAM/QXL® 520 substrate, Ex/Em=490/520 nm upon cleavage
- SensoLyte® 520 BACE, Cathepsin B and Cathepsin D Assay Kits
  - √ FRET HiLyte™ Fluor 488/QXL® 520 substrate, Ex/Em=490/520 nm upon cleavage
- SensoLyte® Plus 520 MMP-2 and MMP-9 Assay Kits
  - √ FRET 5-FAM/QXL® 520 substrates, Ex/Em=490/520 nm upon cleavage
  - √ Anti-MMP mAbs, recombinant MMPs and culture supernatants containing MMPs
- SensoLyte® 520 activity assay:
  - √ Protocol: Reaction volumes were 50 µL of purified enzyme sample and 50 µL of FRET substrate solution. Assays were performed in 96-well black plates.
- Immunocapture MMP Plus activity assay:
  - √ Protocol: Samples containing MMPs were activated with 1 mM APMA at 37ºC 1-2 h before/after adding to the 12 x 8 black strip plate pre-coated with antibody. After 2 h incubation at room temperature for immunocapture, plate was washed and 5-FAM/QXL® 520 FRET peptide substrate was added and incubated for 1-19 h at room temperature.
- Fluorescence measurement: FlexStation 384II (Molecular Devices, Sunny vale, CA)

Assay Principle

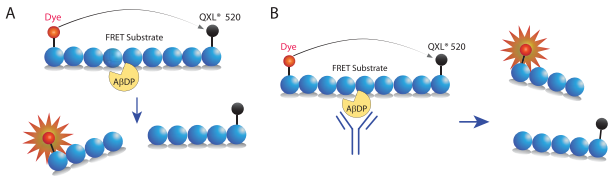


Figure 1. (A) Proteolytic cleavage of dye/QXL® 520 FRET peptide by AD proteases. Fluorescence of dye, such as HiLyte™ Fluor 488, 5-FAM, is quenched by QXL® 520 in the intact FRET substrate. Upon protease cleavage, the fluorescence of dye (HiLyte™ Fluor 488 or 5-FAM) is recovered because of its separation from QXL® 520. (B). Immunocapture FRET for AD proteases. MMP is captured by immobilized anti-MMP monoclonal antibody, and its proteolytic activity is measured using a 5-FAM/QXL® 520 FRET substrate.

Assay Sensitivity

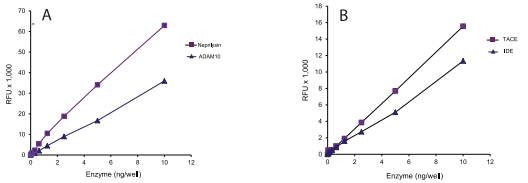


Figure 2. Sensitivity of SensoLyte® 520 ADAM10, Neprilysin, TACE and IDE Assays. Fluorescence was measured at 1h after incubation of FRET substrates using serial dilutions of enzymes. Sensitivity of assays at these conditions: 0.39 ng/mL for ADAM10, 3.1 ng/mL for TACE, 0.78 ng/mL for IDE and Neprilysin. (n=3, mean±SD)

FRET Pair Optimization at Low pH

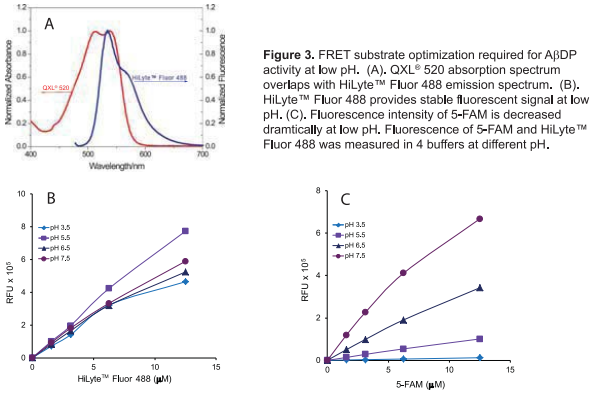


Figure 3. FRET substrate optimization required for AβDP activity at low pH. (A). QXL® 520 absorption spectrum overlaps with HiLyte™ Fluor 488 emission spectrum. (B). HiLyte™ Fluor 488 provides stable fluorescent signal at low pH. (C). Fluorescence intensity of 5-FAM is decreased dramtically at low pH. Fluorescence of 5-FAM and HiLyte™ Fluor 488 was measured in 4 buffers at different pH.

AβDP Activity Detection at Low pH

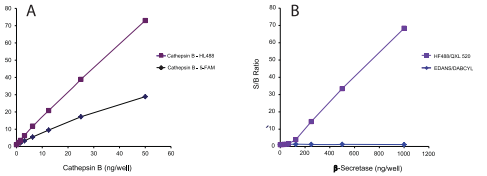


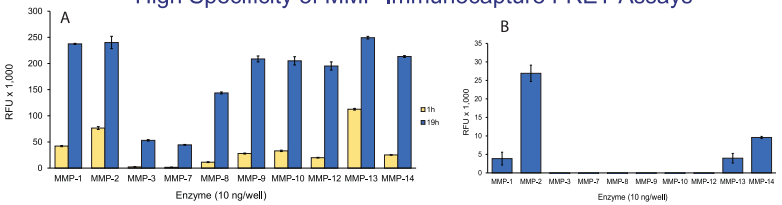
Figure 4. Comparison of substrates with identical sequences but different FRET pairs at acidic pH. (A). Cathepsin B assay was performed at pH 5; (B). β-secretase assay at pH 3.75.

AβDP Cross Reactivity

Table 1. Cross reaction of AβDPs FRET substrates. Signal/background ratio was measured after 1h incubation of protease with panel of FRET substrates. Enzyme concentration: 10ng/well, except for β-secretase: 100 ng/ well. (n=3).

Substrate \ Enzyme	ADAM10	TACE	BACE	IDE	Neprilysin
ADAM 10	**10.65**	1.34	1.09	1.53	1.78
TACE	2.11	**5.84**	1.15	1.60	5.97
BACE	1.50	1.44	**3.25**	1.77	1.43
IDE	1.32	1.36	1.43	**6.71**	1.55
Neprilysin	1.07	1.06	1.06	1.11	**42.62**

High Specificity of MMP Immunocapture FRET Assays



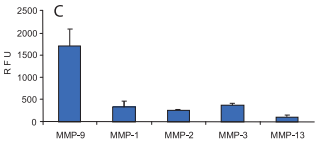


Figure 5. MMPs activity detection by FRET and immunocapture MMPs Plus assay. (A). MMPs activity detected by FRET assay using MMP-2 substrate at 1h and 19h. Cross reactivity with MMP-1 and MMP-13 was detected at 1h incubation. Other MMPs activities were also detectable at 19h incubation. (B). MMP activity detected by Immunocapture MMP-2 Plus assay. Specificity of MMP-2 activity is significantly improved by FRET-Immunocapture method at 19h incubation. (C). Specificity of Immunocapture MMP-9 Plus assay with biological samples. CHO cells were transfected with MMP-1, 2, 3, 9, or 13 plasmid individually. Culture supernatants were collected 24-48 h after transfection and tested by Immunocapture MMP-9 Plus assay.

Conclusions

➢ We have developed a series of FRET-based assays to detect the activity of AβDPs , such as ADAM10, TACE, β-secretase, IDE, Neprilysin , Cathepsins B and D, MMPs-2 and -9. These assays are capable of measuring sub-nanogram range of enzymes.

➢ High sensitivity of assays was achieved using proprietary FRET pairs, 5-FAM/QXL® 520 and HiLyte™ Fluor 488/QXL® 520. The latter FRET pair provided stable fluorescent signal at acidic pH resulting in assay optimization for cathepsins and β-secretase that require low pH for maximum activity.

➢ To differentiate between proteases activities in biological samples, we combined FRET and ELISA principles. These FRET-based immunocapture assays enables detection of specific matrix metalloproteases, such as MMP-2 and MMP-9, which are involved in amyloid degradation.