FRET-Based Assays for the Detection of Amyloid Degrading Protease Activity
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Introduction
Amyloid degrading proteases (ADPs) are major players in β-amyloid (Aβ) clearance. The accumulation of Aβ plaques, a hallmark of Alzheimer’s disease (AD) patient brain, has been hypothesized to be due to the imbalance between ADPs activity and Aβ production, resulting in Aβ pathogenesis. In order to confirm the role of ADPs in neuronal degeneration diseases, reliable assays for ADPs activity detection have to be developed.

We have designed a panel of assays to detect ADP targets using FRET (Fluorescence resonance energy transfer) based peptide substrates. To develop assays for AβM19, TACE (ADAM17), α-secretase, Neprilysin and IDE (γ-secretase degrading enzyme), we synthesized FRET substrate peptides labeled with the fluorophore, 5-carboxyfluorescein (FAM) and the quencher, QXL-520. The fluorescence of FAM is quenched by QXL-520 and restored upon cleavage of the peptide by active ADP. Fluorescence is then monitored at the excitation/emission wavelengths of 490/520 nm.

Many of ADPs target 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 pKR independent Baesium, HLLTP [FLUOR 488] in place of F520. Besides unquenched signal at low pH, HLLTP [FLUOR 488] has excellent brightness at the same wavelength as 5-FAM. The absorbance spectrum of QXL-520 overlaps with the emission spectrum of HLLTP [FLUOR 488], providing optimal co-washing. Substrates with HLLTP [FLUOR 488] are used for detection of 5-APNase, cathepsin B and D, providing higher sensitivity and better assay window.

Some of ADPs, such as MMP-2 and MMP-9, belong to matrix metalloproteinases that are known for overlapping substrate specificities. To avoid non-specific substrate cleavage by multiple proteases, we utilized an Immuno capture technique in addition to the FRET assay. Monoclonal antibody was applied to pull down relevant MMP from biological sample and subsequent addition of MMP-9 peptide substrate containing the 5-FAMQXL-520 FRET pair. These assays provided significant increase specificity of MMP-2 and MMP-9.

Materials and Methods

- Sensory® 520 AβM19, TACE (ADAM17), Neprilysin and IDE Assay Kits
- FRET FAMAX™ QXL 520 substrate, Exc/Em=490/520 nm upon dequenching
- Sensory® 520 BAEC, Cathespin B and Cathespin D Assay Kits
- FRET HLLTP [FLUOR 488]QXL 520 substrate, Exc/Em=490/520 nm upon dequenching
- Sensory® 520 MPP-2 and MMP-9 Assay Kits
- FRET FAMAX™ QXL 520 substrate, Exc/Em=490/520 nm upon dequenching
- AnnexVAP® Mice, recombinant MMP4 and MMP6 superantigens containing MMPs
- Sensory® 520 AβM19 activity assay:
  - Protocol fixation volumes were 50 μl of purified enzyme sample and 50 μl of FRET substrate solution. Assays were performed in 96-well black plate.
- Immuno capture MMP Plus activity assay:
  - Protocol fixation volumes were 1 ml MMPs at 37°C 1 h. Before adding to the 12 x 8 wells strip plate preincubated antibody, After 2 h incubation at room temperature for immunocapture, plate was washed and 5-FAMAX™ QXL 520 FRET peptide substrate was added and incubated for 1-1.5 h at room temperature.
- Fluorescence measurement: FlexStation 3844 (Molecular Devices, Sunnyvale, CA)

Conclusions

- We have developed a series of FRET-based assays to detect the activity of ADPs, such as AβM19, TACE, α-secretase, IDE, Neprilysin, Cathepsin B and D, MMP-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-FAMAX™ QXL 520 and HLLTP [FLUOR 488]QXL 520. The latter FRET pair provided stable fluorescent signal at acidic pH resulting in assay optimization for cathespin and protease that require low pH for maximum activity.
- To differentiate between proteases activities in biological samples, we combined FRET and ELISA principles. These FRET-based immuno capture assays enables detection of specific matrix metalloproteinase, such as MMP-2 and MMP-9, which are involved in amyloid degradation.

Assay Principle

Figure 1: (A) Prototypic cleavage of dynamin QXL 520 FRET peptide by ADP proteases. Fluorescence of dynamin, 5-FAM, is quenched by QXL-520 in the intact FRET substrates. Upon protease cleavage, the fluorescence of dynamin (5-FAM) [FLUOR 488] is recovered because of the separation from QXL-520 [QXL 520], (B) Immuno capture FRET for ADP proteases, MMP is captured by immobilized anti-MMP monoclonal antibody, and its proteolytic activity is measured using 5-FAMAX™ QXL 520 FRET substrate.

Assay Sensitivity

Figure 2: Sensitivity of Sensory®, 520 AβM19, Neprilysin, TACE and IDE Assays. IC50 concn was measured at the end of incubation of FRET substrate using serial dilutions of enzymes. Sensitivity of assay at these conditions; 0.38 ng/ml for AβM19, 3.7 ng/ml for TACE, 0.78 ng/ml for IDE and Neprilysin (n=3, means±SD).

High Specificity of MMP Immuno capture FRET Assays

Figure 3: FRET substrate optimization required for ADP activity at low pH, (A) QXL 520 absorption spectrum overlaps with HLLTP [FLUOR 488] emission spectrum, (B) HLLTP [FLUOR 488] provides stable fluorescent signal at low pH (C) Fluorescence intensity of FAM is decreased essentially at low pH. Fluorescence of QXL-520 and HLLTP [FLUOR 488] was measured in 4 buffers at different pH

Figure 4: Comparison of activities with identical sequences but different FRET pairs at acidic pH. (A) Cathespin B assay was performed at pH 5.5. The 5-secanone assay at pH 7.5

Table 1: Cross-reactivity of ADP’s FRET substrates. Signal (background ratio) was measured after 1 h incubation of proteases with FRET substrates, Enzyme concentration: 10 ng/ml except for Neprilysin (100 ng/ml).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>AβM19</th>
<th>TACE</th>
<th>BACE</th>
<th>IDE</th>
<th>Neprilysin</th>
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<tr>
<td>Sensory® 520</td>
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<td>Neprilysin</td>
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