A Novel Fluorimetric Assay for the Detection of Calpain Activity Using a FRET-Based Substrate

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Introduction

The calpains are a family of intracellular cysteine proteases. The best characterized calpains are the μ- and m-isoforms, also known as calpain 1 and calpain 2, respectively. Calpains are calcium-dependent and function by modulating biological activities of many proteins through selective cleavage. Calpains are implicated in a variety of calcium-regulated cellular processes as well as in various pathological phenomena. It has been proposed that calpains represent potential therapeutic targets for drug development. Here we report the development of a novel fluorimetric assay for detecting calpain enzyme activity using fluorescence resonance energy transfer (FRET) techniques. We designed and synthesized a new calpain FRET substrate peptide labeled with a fluorophore, 5-carboxyfluorescein (5-FAM) and a quencher, QXL™ 520. Calpain cleaves the FRET substrate into two separate fragments resulting in the release of 5-FAM fluorescence, which can be monitored at excitation/emission= 490 nm/520 nm. Increase in 5-FAM fluorescence is proportional to calpain activity. The assay features a simple "add-mix-measure" protocol and has excellent sensitivity with a linear range of 32.5 ng/mL up to 5000 ng/mL for calpain 1. Two known calpain inhibitors, SJA6017 and B27-WT, yield IC_{50} of 3 nM and 3.9 nM, respectively. The assay can detect both calpain 1 (μ) and 2 (m) activities and can be used for enzyme kinetics study. The long wavelength fluorescence of 5-FAM is less interfered by the autofluorescence of small molecules compounds and cell components.

Assay Principle

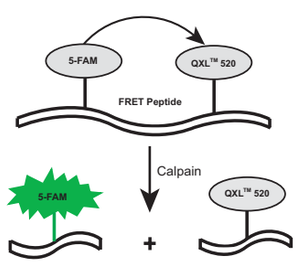

Figure 1. Proteolytic cleavage of 5-FAM/QXL™ 520 FRET peptide by Calpain. Fluorescence of 5-FAM is quenched by QXL™ 520 until this peptide is cleaved into two separate fragments by calpain. Upon cleavage, fluorescence of 5-FAM is recovered, and can be continuously monitored at Ex/Em = 488/520 nm.

Materials and Methods

- SensoLyte® 520 Calpain Activity Assay Kit (Cat# 72149)
  - √ Calpain (5-FAM)-peptide-(QXL™ 520) FRET Substrate - designed and synthesized by Fmoc solid phase peptide synthesis method (Ex/Em = 490/520 nm)
  - √ Calpain Assay Buffer
  - √ Calpain inhibitor: B27-WT
- Human Calpain 1 purified from erythrocytes, Rat Calpain 2-recombinant, Calpain inhibitor SJA6017 (Calbiochem, San Diego, CA)
- Calpain fluorogenic substrate Suc-LLVY-AMC, Ex/Em=354/442 nm upon cleavage (Sigma, St. Louis, MO)

SensoLyte® 520 Calpain Activity Assay Kit was used as recommended by the protocol. The reaction volumes were 40 μl of enzyme, 10 μl of test compound/buffer, 50 μl of substrate. Assays were done in 96-well black opaque plates.

Results

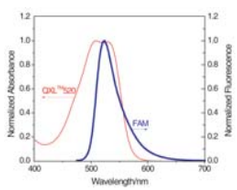

Figure 2. 5-FAM and QXL™ 520 is a new donor - acceptor pair for FRET substrates. QXL™ 520 is an excellent quencher when paired with 5-FAM. The absorption spectrum of QXL™ 520 overlaps with the emission spectrum of 5-FAM. The hydrophilic property of QXL™ 520 results in better solubility of the peptide substrate.

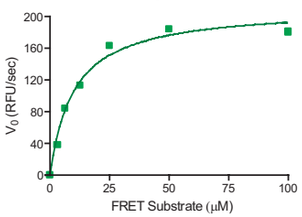

Figure 3. Michaelis-Menten plot. Initial velocities (Vo) of substrate hydrolysis by 500 ng of calpain 1 were plotted against substrate concentration. Km value obtained was 10 μM.

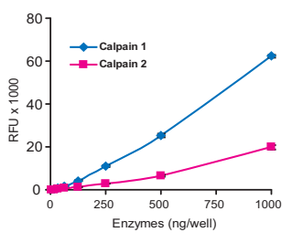

Figure 4. Validation of 5-FAM/QXL™ 520 FRET substrate with calpain isoforms. Fluorescence was measured at 30 min after incubation of FRET substrate with serial dilutions of calpain 1 and calpain 2.

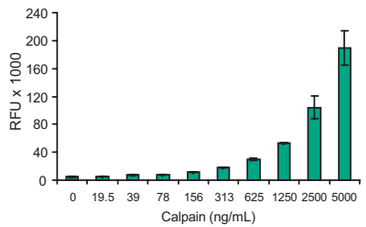

Figure 5. Calpain titration with 5-FAM/QXL™ 520 FRET substrate. Sensitivity of assay after a 30 min incubation is 32.5 ng/mL for calpain 1 enzyme (mean background+3SD).

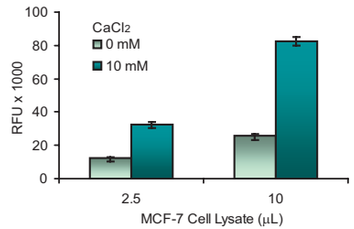

Figure 6. Validation of 5-FAM/QXL™ 520 FRET substrate with biological samples. MCF-7 (human breast adenocarcinoma cell line) lysate was incubated with calpain FRET substrate for 30 min. Addition of $CaCl_2$ increased activity of enzyme in the sample.

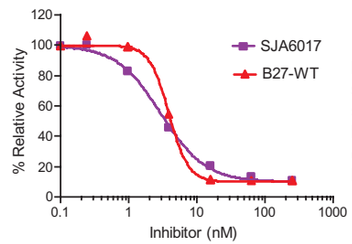

Figure 7. Inhibitor studies. To validate assay for inhibitor screening, 5-FAM/ QXL™ 520 substrate was incubated with 500 ng of enzyme in the presence of calpain inhibitors. Kinetic readings were taken every 5 min for 60 min. The calculated IC_{50} was 3 nM for SJA6017 and 3.9 nM for B27-WT.

Table 1. Comparison of the two calpain substrates.

Substrate	Ex/Em (nm)	Km (μM)	Signal/Background*	Sensitivity** (ng/mL)
5-FAM/QXL™ 520 FRET	490/520	10	41	32.5
Suc-LLVY-AMC	354/442	62.5	4.7	156

*With 5 ng/μl enzyme, 30 min incubation
**After 30 min incubation

Conclusions

- We have developed a new SensoLyte® 520 Calpain Assay Kit, based on a novel 5-FAM/QXL™ 520 FRET substrate. This assay detects subnanogram concentration of enzyme.
- The longer excitation and emission wavelengths of 5-FAM minimize interference from autofluorescence of test compounds and cell components.
- SensoLyte® 520 Calpain assay kit was validated for inhibitor screening and for use with biological samples