#P325

Tide Quencher™-Based FRET Protease Substrates and Their Applications In Drug Discovery and Disease Diagnosis

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Introduction

Proteases 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. Although EDANS/DABCYL and MCA/DNP are widely 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 protease assays. We have developed a new class of non-fluorescent dyes called Tide Quencher™ to eliminate these limitations.

Tide Quencher™ (TQ) dyes are excellent dark quenchers that are individually optimized to pair with all of the popular fluorescent dyes such as fluoresceins and rhodamines. Our TQ series of non-fluorescent dyes cover the full visible spectrum with unusually high quenching efficiency. For example, Tide Quencher™ 2 (TQ2) has absorption maximum perfectly matching the emission of FAM while Tide Quencher™ 3 (TQ3) is proven to be the best quencher for TAMRA.

We have used TQ dyes to develop new FRET peptide substrates for high throughput analysis of protease activities and screening of protease inhibitors. Our TQ2-based HIV protease substrates have demonstrated significantly enhanced assay window. Excellent performance has also been observed for our new MMP protease substrates that incorporate TQ2 or TQ3 as acceptor. We have also used TQ2 and TQ3 to develop novel protease substrates for analysis of secretases, HDAC, HCV, HIV and caspases.

Materials and Methods

All the peptides were synthesized using the standard FMOC chemistry by American Peptide Company. Some of the TQ peptides were prepared using FMOC-Lys(TQ)-OH, FMOC-Asp(TQ)-OH or FMOC-Glu(TQ)-OH amino acids. For the post-labeling of the premade peptides, TF NHS esters were used for labeling N-terminal amino or ε–amino group of lysine residue. TQ maleimides were used for labeling the SH group of cysteine residues.

TQ1 acid, TQ2 acid, TQ3 acid, TQ1 NHS ester, TQ2 NHS ester, TQ3 NHS ester, TQ1 maleimide, TQ2 maleimide, TQ3 maleimide, FMOC-Lys(TQ1)-OH, FMOC-Lys(TQ2)-OH, FMOC-Lys(TQ3)-OH, FMOC-Asp(TQ1)-OH, FMOC-Asp(TQ2)-OH, FMOC-Asp(TQ3)-OH, FMOC-Glu(TQ1)-OH, FMOC-Glu(TQ2)-OH and FMOC-Glu(TQ3)-OH are now commercially available from ABD Bioquest.

All the enzyme assays were done with purified enzymes that are commercially available from Sigma, R&D Systems or EMD Chemicals. Absorption spectra were taken with Hitachi U-3010. Endpoint fluorescence assays were run on either BMG NovoStar or Gemini SpectraMax (Molecular Devices). Enzyme kinetic assays were run on FlexStation (Molecular Devices).

FRET & Spectral Properties of Tide Quenchers (TQ)

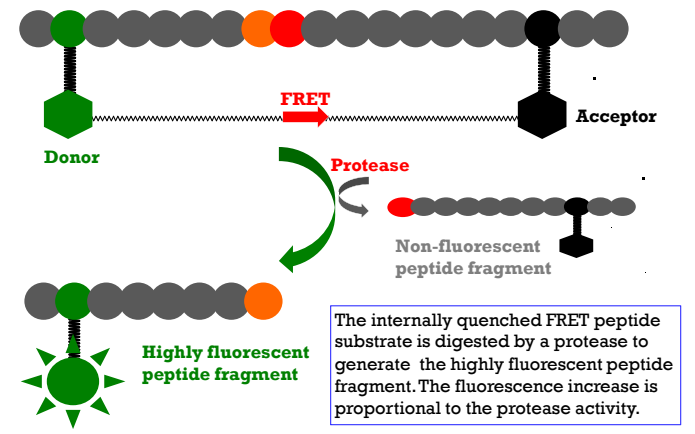
Tide Quencher™ (TQ) dyes are excellent dark quenchers that are individually optimized to pair with all of the popular fluorescent dyes such as fluoresceins, rhodamines and Alexa Fluor dyes. Our TQ series of non-fluorescent dyes cover the full visible spectrum with unusually high quenching efficiency as shown below:

FRET Acceptors / FRET Donors	Tide Quenchers				
	TQ1	TQ2	TQ3	TQ4	TQ5
	λ_{Max} = ~490 nm	λ_{Max} = ~520 nm	λ_{Max} = ~570 nm	λ_{Max} = ~610 nm	λ_{Max} = ~670 nm
EDANS/MCA/TF1	+ + + + +	+ + +			
FAM/FITC/TF2/AF488	+ + +	+ + + + +	+ + +		
Cy2/6-HEX/6-TET/6-JOE		+ + +	+ + + + +	+ + +	
Cy3/TAMRA/TF3/AF555		+ + +	+ + + + +	+ + +	
ROX/Texas Red®/TF4/AF594			+ + +	+ + + + +	+ + +
Cy5/TF5/Cy5.5/AF680				+ + +	+ + + + +

AF= Alexa Fluor® and Texas Red® (Invitrogen)

Legends: + + + + + Best to use + + + OK to use

Assay Principle of FRET-Based Protease Substrates



The internally quenched FRET peptide substrate is digested by a protease to generate the highly fluorescent peptide fragment. The fluorescence increase is proportional to the protease activity.

Detection of HIV Protease Activities Using TQ2-Based FRET Substrates

Inhibition of HIV-1 protease represents an important avenue for AIDS therapy. Currently combination chemotherapy of reverse transcriptase inhibitors and protease inhibitors have shown to suppress the replication of HIV-1 and extend the life expectancy of HIV-1-infected individuals. Robust HIV protease assays are still needed for developing new HIV protease inhibitors. The following TQ2-containing FRET peptides were screened for developing a HTS-friendly HIV protease assay. As shown below Substrates #1 and 2 are excellent HTS-compatible fuorogenic HIV protease substrates.

#Sub	Peptide Sequence
1	TQ2-Gaba-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-5-FAM
2	Arg-Glu(5-FAM)-Val-Ser-Phe-Asn-Phe-Pro-Gln-Ile-Thr-Lys(TQ2)-Arg
3	Arg-Glu(5-FAM)-Ser-Gln-Asn-Tyr-Ile-Val-Gln-Lys(TQ2)-Arg
4	Arg-Arg-Glu(5-FAM)-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-Lys(5-FAM)-Arg-Arg
5	Ac-Arg-Glu(5-FAM)-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-Lys(5-FAM)-Arg-NH_2

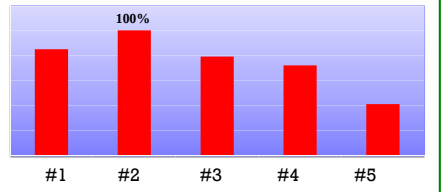


Figure 1. HIV Protease cleavage of TQ2-derived FRET peptides. All the substrates were used @10 uM. The reaction rate is set @ 100% with Sub #2.

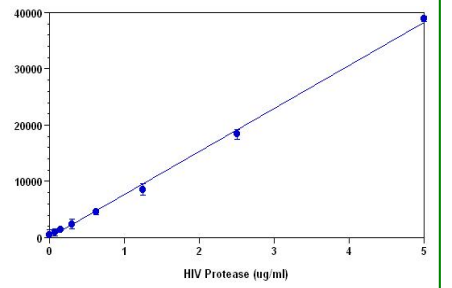


Figure 2. HIV Protease cleavage of Substrate #2. The substrate is incubated with HIV protease . Upon HIV protease cleavage, the fluorescence of 5-FAM is recovered, and monitored with excitation/emission = 490 nm/520 nm.