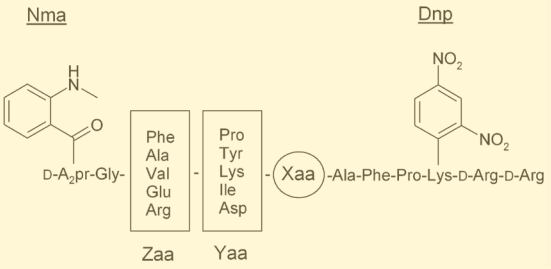
PEPTIDES INTERNATIONAL

Enzyme Inhibitors & Substrates

Frets

Fluorescence Resonance Energy Transfer Substrates 25Xaa Series



A_2pr(Nma) : N$^{\beta}$-[2-(N-Methylamino)benzoyl]-2,3-Diaminopropionic Acid
Lys(Dnp) : N$^{\varepsilon}$-2,4-Dinitrophenyl-Lysine

Each substrate (*SFA-3701-v* - *SFV-3719-v*) in the FRETS-25Xaa series contains a highly fluorescent 2-(*N*-methylamino)benzoyl (Nma) group linked to the side chain of the amino-terminal D-2,3-diamino proprionic acid (D-A_2pr) residue, which is efficiently quenched by a 2,4-dinitrophenyl (Dnp) group linked to the □-amino function of Lys. Xaa represents a fixed position of each of the 19 natural amino acids excluding Cys (noted in product name, code *SFA-3701-v* - *SFV-3719-v*). A mixture of 5 amino acid residues (P, Y, K, I, and D) is at the Yaa position along with a mixture of 5 amino acid residues (F, A, V, E, and R) at the Zaa position for each fixed Xaa. This provides a peptide mixture of 25 combinations of each Xaa series resulting in a combinatorial library totaling 475 peptide substrates. Both Nma and Dnp groups are linked to the side chain of the individual residues, allowing for the determination of the cleavage site by a specific enzyme, through mass spectrometric analysis and Edman degradation as well.

Principle

When an enzyme of interest cleaves any peptide bond between D-A_2pr(Nma) and Lys(Dnp) in the substrate, the fluorescence at □ex = 340 nm and □em = 440 nm increases in proportion to the release of the Nma fluorophore from the internal Dnp quencher.

Reagents

1) Each substrate stock solutions: each FRETS-25Xaa (Code *SFA-3701-v* - Code *SFV-3719-v*) in 1.0 ml of DMSO (1 mM, total of peptides)
2) Reference compounds stock solution: a 1:1 mixture of two solutions of Code *STD-3720-v* and Code *STD-3721-v*, each of which is reconstituted by dissolving peptides in 0.5 ml of DMSO at the concentration of 2 mM (1 mM, each reference compound)
3) Enzyme solution: an enzyme of interest in an appropriate buffer
4) Buffer

Procedure for the deduction of the substrate specificity of an enzyme with unidentified cleavage specificity

Choose the proper conditions for the measurement, such as substrate concentration and sensitivity setting, depending on the purpose of the experiment and the instrument available. Described here is one of the recommended procedures for determining the enzymatic cleavage site by the combination of the fluorometric analysis and liquid chromatography-mass spectrometry (LC-MS) analysis.

i) Primary screening: selection of the favored Xaa

- □ Substrate solution for primary screening (PS solution): Dilute 20 μl of each of the above substrate stock solution with 1980 μl of an appropriate buffer (10 μM)
- □ Reference compounds solution for primary screening (PR solution): Dilute 20 μl of the above reference compounds stock solution with 1980 μl of an appropriate buffer (10 μM)

1) Set a fluorescence spectrophotometer at □ex = 340 nm and □em = 440 nm
2) Mix one of the PS solution and the PR solution in ratios of 10/0, 9/1, 8/2, 5/5 and 0/10
3) Measure the fluorescence of the prepared solutions to obtain the calibration curve for the cleaved products
4) Pipette 200 μl each of all PS solutions into the cells and incubate them in the fluorescence spectrophotometer for 3 min (temperature equilibration)
5) Measure the fluorescence of each solution (initial fluorescence blank)
6) Add an appropriate volume of enzyme solution
7) Record the increase of the fluorescence intensity
8) Terminate the enzymatic reaction by using a proper inhibitor (leupeptin, E-64, pepstatin, EDTA and so on) or changing the pH of the reaction medium (using TCA, AcOH, NaOH and so on)
9) Choose the best Xaa-containing substrate for secondary screening

ii) Secondary screening: identification of the specificity of the enzyme (I)

- □ Substrate solution for secondary screening (SS solution): Dilute 200 μl of the stock solution of the best Xaa-containing substrate chosen by the above primary screening with 1800 μl of an appropriate buffer (100 μM)
- □ Reference compounds solution for secondary screening (SR solution): Dilute 200 μl of the above reference compounds stock solution with 1800 μl of an appropriate buffer (100 μM)

Fluorescence-Quenching Substrate Library
All library products have the general structure:
D-A_2pr(Nma)-Gly-[Phe/Ala/Val/Glu/Arg]-[Pro/Tyr/Lys/Ile/Asp]-Gly-Ala-Phe-Pro-Lys(Dnp)-D-Arg-D-Arg
where A_2pr(Nma) = N□-[2-(N-Methylamino)benzoyl]-2,3-Diaminopropionic Acid
All substrates are sold as trifluoroacetate form and contain 1 μmol of stated library.

Please refer to our complete product brochure for additional experimental details and protocol recommendations. Please contact our technical specialists or refer to our web site for additional information.

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1) Set a fluorescence spectrophotometer at λex = 340 nm and λem = 440 nm
2) Mix the SS solution and the SR solution in ratios of 100/0, 95/5, 90/10, 80/20, 50/50 and 0/100
3) Measure the fluorescence of the prepared solutions to obtain the calibration curve for the cleaved products
4) Pipette 200 μl of the SS solution into the cells and incubate them in the fluorescence spectrophotometer for 3 min (temperature equilibration)
5) Measure the fluorescence of each solution (initial fluorescence blank)
6) Add an appropriate volume of enzyme solution
7) Record the increase of the fluorescence intensity
8) Terminate the enzymatic reaction by using a proper inhibitor or changing the pH of the reaction medium upon completion of the reaction at the points of 0%, 5%, 10% and 20% of the total
9) Subject 100 μl aliquots to LC-MS

iii) LC-MS: identification of the specificity of the enzyme (II)
Analytical conditions
column: ODS
eluant: A) H_2O containing 0.05% TFA, B) CH_3CN containing 0.05% TFA
gradient: 10% to 40% B) in A) over 50 min
detection: UV at 220 nm and 400 nm or fluorescence

1) Inject 100 μl aliquots of each terminated solution at different stages of the reaction
2) Measure the MW of the cleaved product(s) in the peak(s) with the absorbance at 220 nm but not with 400 nm [identification of the N-terminal segment(s)]
3) Deduce their structure from the attached list of the theoretical MW for the cleaved products

** Comment 1: If the N-terminal segment has the identical retention time to the C-terminal segment or one of the starting uncleaved substrates, detection of the products by fluorescence is recommended.*
** Comment 2: In the accidental case where the two products with the same MW (ex. Zaa-Yaa=Phe-Asp and Val-Tyr, Glu-Asp and Phe-Pro) are generated from one of the substrates, their analyses should be carried out by MS-MS sequencing and/or by Edman degradation.*

1. K. Takada, M. Tsunemi, Y. Nishiuchi, and T. Kimura, A Fluorescence Resonance Energy Transfer Substrate (FRETS) Library for Determining Protease Specificity. [Peptide Revolution: Genomic, Proteomics & Therapeutics] (Proceedings of the 18th American Peptide Symposium) 327 (2003).
2. S. Tanskul, K. Oda, H. Oyama, N. Noparatnaraporn, M. Tsunemi, and K. Takada, Substrate specificity of alkaline serine proteinase isolated from photosynthetic bacterium, *Rubrivivax gelatinosus* KDDS1. *Biochem. Biophys. Res. Commun.*, **309,** 547 (2003).

Usefulness and limitation of FRETS-25Xaa series for screening of substrate specificities of proteases

We have confirmed that FRETS-25Xaa series are effectively used for the assay of numerous proteases such as trypsin, chymotyrpsin, elastase, thrombin, papain, calpain, pepsin and thermolysin. However, they did not work well for the assay of caspase-3 and furin, probably because they have only three changeable sites (Zaa-Yaa-Xaa) in each substrate (deficiency of P4 site). This fact implies that FRETS-25Xaa might not be applicable to the assay of an enzyme with wide range interacting sites with substrate.

CODE	DESCRIPTION	CODE	DESCRIPTION
SFA-3701-v	FRETS-25Ala	SFK-3711-v	FRETS-25Lys
SFR-3702-v	FRETS-25Arg	SFM-3712-v	FRETS-25Met
SFN-3703-v	FRETS-25Asn	SFF-3713-v	FRETS-25Phe
SFD-3704-v	FRETS-25Asp	SFP-3714-v	FRETS-25Pro
SFQ-3705-v	FRETS-25Gln	SFS-3715-v	FRETS-25Ser
SFE-3706-v	FRETS-25Glu	SFT-3716-v	FRETS-25Thr
SFG-3707-v	FRETS-25Gly	SFW-3717-v	FRETS-25Trp
SFH-3708-v	FRETS-25His	SFY-3718-v	FRETS-25Tyr
SFI-3709-v	FRETS-25Ile	SFV-3719-v	FRETS-25Val
SFL-3710-v	FRETS-25Leu	All vials contain 1 μmol of library Price 106 USD/vial	
STD-3720-v Price 25 USD vial	FRETS-25-STD1 Lyophilized from DMSO containing aqueous HCl D-A_2pr(Nma)-Gly where A_2pr(Nma)= N^{β}-[2-(N-Methylamino)benzoyl]-2,3-Diaminopropionic Acid (M.W. 294.31) $C_{13}H_{18}N_4O_4$ Standard Compound 1 for Fluorescence-Quenching Substrate Library FRETS-25 Xaa Series		
STD-3721-v Price 25 USD vial	FRETS-25-STD2 (Trifluoroacetate Form) Ala-Phe-Pro-Lys(Dnp)-D-Arg-D-Arg Lys(Dnp) = N^{β}-2,4-Dinitrophenyl-Lysine (M.W. 940.02) $C_{41}H_{61}N_{15}O_{11}$ Standard Compound 2 for Fluorescence-Quenching Substrate Library FRETS-25 Xaa Series		

CODE	PRODUCT	QTY
	FRETS (Peptide) Library .	
SFA-3701-v	**FRETS-25Ala** (Trifluoroacetate Form) D-A_2pr(Nma)-Gly-[Phe/Ala/Val/Glu/Arg]-[Pro/Tyr/Lys/Ile/Asp]-Ala-Ala-Phe-Pro-Lys(Dnp)-D-Arg-D-Arg *Fluorescence-Quenching Substrate Library*	1 μmol
SFR-3702-v	**FRETS-25Arg** (Trifluoroacetate Form) D-A_2pr(Nma)-Gly-[Phe/Ala/Val/Glu/Arg]-[Pro/Tyr/Lys/Ile/Asp]-Arg-Ala-Phe-Pro-Lys(Dnp)-D-Arg-D-Arg *Fluorescence-Quenching Substrate Library*	1 μmol

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CODE	PRODUCT	QTY
SFN-3703-v	**FRETS-25Asn** (Trifluoroacetate Form) D-A_2pr(Nma)-Gly-[Phe/Ala/Val/Glu/Arg]-[Pro/Tyr/Lys/Ile/Asp]-Asn-Ala-Phe-Pro-Lys(Dnp)-D-Arg-D-Arg *Fluorescence-Quenching Substrate Library*	1 µmol
SFD-3704-v	**FRETS-25Asp** (Trifluoroacetate Form) D-A_2pr(Nma)-Gly-[Phe/Ala/Val/Glu/Arg]-[Pro/Tyr/Lys/Ile/Asp]-Asp-Ala-Phe-Pro-Lys(Dnp)-D-Arg-D-Arg *Fluorescence-Quenching Substrate Library*	1 µmol
SFQ-3705-v	**FRETS-25Gln** (Trifluoroacetate Form) D-A_2pr(Nma)-Gly-[Phe/Ala/Val/Glu/Arg]-[Pro/Tyr/Lys/Ile/Asp]-Gln-Ala-Phe-Pro-Lys(Dnp)-D-Arg-D-Arg *Fluorescence-Quenching Substrate Library*	1 µmol
SFE-3706-v	**FRETS-25Glu** (Trifluoroacetate Form) D-A_2pr(Nma)-Gly-[Phe/Ala/Val/Glu/Arg]-[Pro/Tyr/Lys/Ile/Asp]-Glu-Ala-Phe-Pro-Lys(Dnp)-D-Arg-D-Arg *Fluorescence-Quenching Substrate Library*	1 µmol
SFG-3707-v	**FRETS-25Gly** (Trifluoroacetate Form) D-A_2pr(Nma)-Gly-[Phe/Ala/Val/Glu/Arg]-[Pro/Tyr/Lys/Ile/Asp]-Gly-Ala-Phe-Pro-Lys(Dnp)-D-Arg-D-Arg *Fluorescence-Quenching Substrate Library*	1 µmol
SFH-3708-v	**FRETS-25His** (Trifluoroacetate Form) D-A_2pr(Nma)-Gly-[Phe/Ala/Val/Glu/Arg]-[Pro/Tyr/Lys/Ile/Asp]-His-Ala-Phe-Pro-Lys(Dnp)-D-Arg-D-Arg *Fluorescence-Quenching Substrate Library*	1 µmol
SFI-3709-v	**FRETS-25Ile** (Trifluoroacetate Form) D-A_2pr(Nma)-Gly-[Phe/Ala/Val/Glu/Arg]-[Pro/Tyr/Lys/Ile/Asp]-Ile-Ala-Phe-Pro-Lys(Dnp)-D-Arg-D-Arg *Fluorescence-Quenching Substrate Library*	1 µmol
SFL-3710-v	**FRETS-25Leu** (Trifluoroacetate Form) D-A_2pr(Nma)-Gly-[Phe/Ala/Val/Glu/Arg]-[Pro/Tyr/Lys/Ile/Asp]-Leu-Ala-Phe-Pro-Lys(Dnp)-D-Arg-D-Arg *Fluorescence-Quenching Substrate Library*	1 µmol
SFK-3711-v	**FRETS-25Lys** (Trifluoroacetate Form) D-A_2pr(Nma)-Gly-[Phe/Ala/Val/Glu/Arg]-[Pro/Tyr/Lys/Ile/Asp]-Lys-Ala-Phe-Pro-Lys(Dnp)-D-Arg-D-Arg *Fluorescence-Quenching Substrate Library*	1 µmol
SFM-3712-v	**FRETS-25Met** (Trifluoroacetate Form) D-A_2pr(Nma)-Gly-[Phe/Ala/Val/Glu/Arg]-[Pro/Tyr/Lys/Ile/Asp]-Met-Ala-Phe-Pro-Lys(Dnp)-D-Arg-D-Arg *Fluorescence-Quenching Substrate Library*	1 µmol

CODE	PRODUCT	QTY
SFF-3713-v	**FRETS-25Phe*** (Trifluoroacetate Form) D-A_2pr(Nma)-Gly-[Phe/Ala/Val/Glu/Arg]-[Pro/Tyr/Lys/Ile/Asp]-Phe-Ala-Phe-Pro-Lys(Dnp)-D-Arg-D-Arg *Fluorescence-Quenching Substrate Library*	1 µmol
SFP-3714-v	**FRETS-25Pro** (Trifluoroacetate Form) D-A_2pr(Nma)-Gly-[Phe/Ala/Val/Glu/Arg]-[Pro/Tyr/Lys/Ile/Asp]-Pro-Ala-Phe-Pro-Lys(Dnp)-D-Arg-D-Arg *Fluorescence-Quenching Substrate Library*	1 µmol
SFS-3715-v	**FRETS-25Ser** (Trifluoroacetate Form) D-A_2pr(Nma)-Gly-[Phe/Ala/Val/Glu/Arg]-[Pro/Tyr/Lys/Ile/Asp]-Ser-Ala-Phe-Pro-Lys(Dnp)-D-Arg-D-Arg *Fluorescence-Quenching Substrate Library*	1 µmol
SFT-3716-v	**FRETS-25Thr*** (Trifluoroacetate Form) D-A_2pr(Nma)-Gly-[Phe/Ala/Val/Glu/Arg]-[Pro/Tyr/Lys/Ile/Asp]-Thr-Ala-Phe-Pro-Lys(Dnp)-D-Arg-D-Arg *Fluorescence-Quenching Substrate Library*	1 µmol
SFW-3717-v	**FRETS-25Trp*** (Trifluoroacetate Form) D-A_2pr(Nma)-Gly-[Phe/Ala/Val/Glu/Arg]-[Pro/Tyr/Lys/Ile/Asp]-Trp-Ala-Phe-Pro-Lys(Dnp)-D-Arg-D-Arg *Fluorescence-Quenching Substrate Library*	1 µmol
SFY-3718-v	**FRETS-25Tyr*** (Trifluoroacetate Form) D-A_2pr(Nma)-Gly-[Phe/Ala/Val/Glu/Arg]-[Pro/Tyr/Lys/Ile/Asp]-Tyr-Ala-Phe-Pro-Lys(Dnp)-D-Arg-D-Arg *Fluorescence-Quenching Substrate Library*	1 µmol
SFV-3719-v	**FRETS-25Val*** (Trifluoroacetate Form) D-A_2pr(Nma)-Gly-[Phe/Ala/Val/Glu/Arg]-[Pro/Tyr/Lys/Ile/Asp]-Val-Ala-Phe-Pro-Lys(Dnp)-D-Arg-D-Arg *Fluorescence-Quenching Substrate Library*	1 µmol
STD-3720-v	**FRETS-25-STD1** Lyophilized from DMSO containing aqueous HCl D-A_2pr(Nma)-Gly (M.W. 294.31) $C_{13}H_{18}N_4O_4$ *Standard Compound 1 for Fluorescence-Quenching Substrate Library FRETS-25 Xaa Series*	1 µmol
STD-3721-v	**FRETS-25-STD2** (Trifluoroacetate Form) Ala-Phe-Pro-Lys(Dnp)-D-Arg-D-Arg (M.W. 940.02) $C_{41}H_{61}N_{15}O_{11}$ *Standard Compound 2 for Fluorescence-Quenching Substrate Library FRETS-25 Xaa Series*	1 µmol