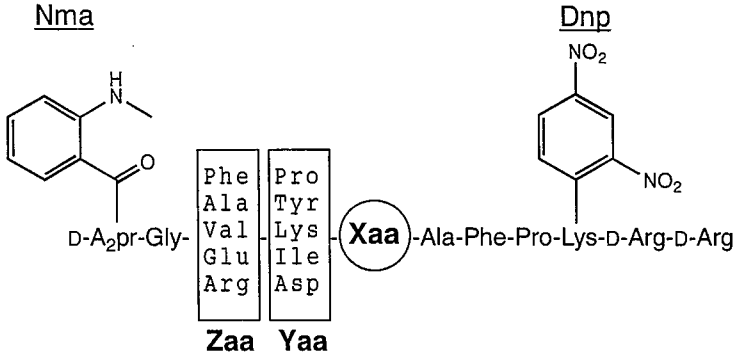
FRETS-25Xaa Series

* FRETS = Fluorescence Resonance Energy Transfer Substrates

Design of FRETS-25Xaa



Each substrate (#3701-v - #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-A2pr residue, which is efficiently quenched by a 2,4-dinitrophenyl (Dnp) group linked to the ε-amino function of Lys. Xaa represents a fixed position of each of the 19 natural amino acids excluding Cys (*noted in product name #3701-v - #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-A2pr(Nma) and Lys(Dnp) in the substrate, the fluorescence at λex = 340 nm and λem = 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 (#3701-v - #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 #3720-v and #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 λex = 340 nm and λem = 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)

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 stage 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 substrate, their analyses should be carried out by MS-MS sequencing and/or by Edman degradation.

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 3713-v

FRETS-25Phe Component-masses

FRETS-25Phe	Average	Monoisotopic	FRETS-25Phe	Average	Monoisotopic	FRETS-25Phe	Average	Monoisotopic	FRETS-25Phe	Average	Monoisotopic
A2pr(Nma)G	294.31	294.1328	A2pr(Nma)GADF	627.65	627.2653	A2pr(Nma)GEIFA	754.83	754.3650	A2pr(Nma)GVYFAF	922.04	921.4385
A2pr(Nma)GA	365.38	365.1699	A2pr(Nma)GVPF	637.73	637.3224	A2pr(Nma)GEDFA	756.76	756.3079	A2pr(Nma)GAPFAFP	925.04	924.4494
A2pr(Nma)GV	393.44	393.2012	A2pr(Nma)GAKF	640.73	640.3333	A2pr(Nma)GFPFA	756.85	756.3595	A2pr(Nma)GRIFAF	929.08	928.4919
A2pr(Nma)GE	423.42	423.1754	A2pr(Nma)GVIF	653.77	653.3537	A2pr(Nma)GRYF	760.84	760.3657	A2pr(Nma)GRDFAF	931.01	930.4348
A2pr(Nma)GF	441.48	441.2012	A2pr(Nma)GVDF	655.70	655.2966	A2pr(Nma)GRPFA	765.86	765.3922	A2pr(Nma)GFKFAF	935.08	934.4701
A2pr(Nma)GR	450.49	450.2339	Ac-K(Dnp)rr	666.69	666.3198	A2pr(Nma)GEKFA	769.84	769.3759	AFPK(Dnp)rr	940.02	939.4675
A2pr(Nma)GAP	462.50	462.2227	A2pr(Nma)GEPF	667.71	667.2966	A2pr(Nma)GFIFA	772.89	772.3908	A2pr(Nma)GAIFAFP	941.08	940.4807
A2pr(Nma)GAI	478.54	478.2540	A2pr(Nma)GVKF	668.78	668.3646	A2pr(Nma)GFDFA	774.82	774.3337	A2pr(Nma)GADFAFP	943.01	942.4236
A2pr(Nma)GAD	480.47	480.1969	A2pr(Nma)GAYF	675.73	675.3017	A2pr(Nma)GVYFA	774.86	774.3701	A2pr(Nma)GRKFAF	944.09	943.5028
A2pr(Nma)GVP	490.55	490.2540	A2pr(Nma)GAPFA	680.75	680.3282	A2pr(Nma)GRIFA	781.90	781.4235	A2pr(Nma)GEYFAF	952.02	951.4127
A2pr(Nma)GAK	493.56	493.2649	A2pr(Nma)GEIF	683.75	683.3279	A2pr(Nma)GRDFA	783.83	783.3664	A2pr(Nma)GVPFAFP	953.09	952.4807
A2pr(Nma)GVI	506.60	506.2853	A2pr(Nma)GEDF	685.68	685.2708	A2pr(Nma)GFKFA	787.90	787.4017	A2pr(Nma)GAKFAFP	956.10	955.4916
A2pr(Nma)GVD	508.52	508.2282	A2pr(Nma)GFPF	685.77	685.3224	A2pr(Nma)GRKFA	796.92	796.4344	A2pr(Nma)GVIFAFP	969.14	968.5120
A2pr(Nma)GEP	520.54	520.2282	A2pr(Nma)GRPF	694.78	694.3551	A2pr(Nma)GEYFA	804.85	804.3443	A2pr(Nma)GFYFAF	970.08	969.4385
A2pr(Nma)GVK	521.61	521.2962	A2pr(Nma)GAIFA	696.79	696.3595	A2pr(Nma)GFYFA	822.91	822.3701	A2pr(Nma)GVDFAFP	971.07	970.4549
A2pr(Nma)GAY	528.56	528.2332	A2pr(Nma)GADFA	698.72	698.3024	A2pr(Nma)GAPFAF	827.93	827.3966	A2pr(Nma)GRYFAF	979.09	978.4712
A2pr(Nma)GEI	536.58	536.2595	A2pr(Nma)GEKF	698.77	698.3388	A2pr(Nma)GRYFA	831.92	831.4028	A2pr(Nma)GEPFAFP	983.08	982.4549
A2pr(Nma)GED	538.51	538.2023	A2pr(Nma)GFIF	701.81	701.3537	A2pr(Nma)GAIFAF	843.97	843.4279	A2pr(Nma)GVKFAFP	984.15	983.5229
A2pr(Nma)GFP	538.60	538.2540	A2pr(Nma)GFDF	703.74	703.2966	A2pr(Nma)GADFAF	845.90	845.3708	A2pr(Nma)GAYFAFP	991.10	990.4600
A2pr(Nma)GRP	547.61	547.2867	A2pr(Nma)GVYF	703.78	703.3330	A2pr(Nma)GVPFAF	855.98	855.4279	A2pr(Nma)GEIFAFP	999.12	998.4862
A2pr(Nma)GEK	551.59	551.2704	A2pr(Nma)GVPFA	708.80	708.3595	A2pr(Nma)GAKFAF	858.98	858.4388	A2pr(Nma)GEDFAFP	1001.05	1000.4290
A2pr(Nma)GFI	554.64	554.2853	A2pr(Nma)GRIF	710.82	710.3864	FPK(Dnp)rr	868.94	868.4304	A2pr(Nma)GFPFAFP	1001.14	1000.4807
A2pr(Nma)GFD	556.57	556.2282	A2pr(Nma)GAKFA	711.81	711.3704	A2pr(Nma)GVIFAF	872.02	871.4592	A2pr(Nma)GRPFAFP	1010.15	1009.5134
A2pr(Nma)GVY	556.61	556.2645	A2pr(Nma)GRDF	712.75	712.3293	A2pr(Nma)GVDFAF	873.95	873.4021	A2pr(Nma)GEKFAFP	1014.13	1013.4971
A2pr(Nma)GRI	563.65	563.3180	A2pr(Nma)GFKF	716.83	716.3646	A2pr(Nma)GEPFAF	885.96	885.4021	A2pr(Nma)GFIFAFP	1017.18	1016.5120
A2pr(Nma)GRD	565.58	565.2609	PK(Dnp)rr	721.77	721.3620	A2pr(Nma)GVKFAF	887.04	886.4701	A2pr(Nma)GFDFAFP	1019.11	1018.4549
A2pr(Nma)GFK	569.65	569.2962	A2pr(Nma)GVIFA	724.85	724.3908	A2pr(Nma)GAYFAF	893.98	893.4072	A2pr(Nma)GVYFAFP	1019.15	1018.4913
A2pr(Nma)GRK	578.66	578.3289	A2pr(Nma)GRKF	725.84	725.3973	A2pr(Nma)GEIFAF	902.00	901.4334	A2pr(Nma)GRIFAFP	1026.19	1025.5447
A2pr(Nma)GEY	586.59	586.2387	A2pr(Nma)GVDFA	726.78	726.3337	A2pr(Nma)GEDFAF	903.93	903.3763	A2pr(Nma)GRDFAFP	1028.12	1027.4876
A2pr(Nma)GFY	604.65	604.2645	A2pr(Nma)GEYF	733.77	733.3071	A2pr(Nma)GFPFAF	904.02	903.4279	A2pr(Nma)GFKFAFP	1032.19	1031.5229
A2pr(Nma)GAPF	609.67	609.2911	A2pr(Nma)GEPFA	738.79	738.3337	A2pr(Nma)GRPFAF	913.03	912.4606	A2pr(Nma)GRKFAFP	1041.21	1040.5556
A2pr(Nma)GRY	613.67	613.2972	A2pr(Nma)GVKFA	739.86	739.4017	A2pr(Nma)GEKFAF	917.02	916.4443	A2pr(Nma)GEYFAFP	1049.13	1048.4654
K(Dnp)rr	624.65	624.3092	A2pr(Nma)GAYFA	746.81	746.3388	A2pr(Nma)GFIFAF	920.06	919.4592	A2pr(Nma)GFYFAFP	1067.19	1066.4913
A2pr(Nma)GAIF	625.72	625.3224	A2pr(Nma)GFYF	751.83	751.3330	A2pr(Nma)GFDFAF	921.99	921.4021	A2pr(Nma)GRYFAFP	1076.21	1075.5240

Code 3713-v

FRETS-25Phe Component-masses

FRETS-25Phe	Average	Monoisotopic	FRETS-25Phe	Average	Monoisotopic	FRETS-25Phe	Average	Monoisotopic	FRETS-25Phe	Average	Monoisotopic
FAFPK(Dnp)rr	1087.19	1086.5359	A2pr(Nma)GRIFAFPK(Dnp)	1320.45	1319.6411	A2pr(Nma)GAIFAFPK(Dnp)r	1391.53	1390.6782	A2pr(Nma)GRKFAFPK(Dnp)r	1491.65	1490.7531
PFAFPK(Dnp)rr	1184.31	1183.5887	AYFAFPK(Dnp)rr	1321.44	1320.6364	A2pr(Nma)GADFAFPK(Dnp)r	1393.46	1392.6211	A2pr(Nma)GEYFAFPK(Dnp)r	1499.58	1498.6630
IFAFPK(Dnp)rr	1200.35	1199.6200	A2pr(Nma)GRDFAFPK(Dnp)	1322.38	1321.5840	FYFAFPK(Dnp)rr	1397.54	1396.6677	A2pr(Nma)GFYFAFPK(Dnp)r	1517.64	1516.6888
DFAFPK(Dnp)rr	1202.28	1201.5629	A2pr(Nma)GFKFAFPK(Dnp)	1326.46	1325.6193	GRPFAFPK(Dnp)rr	1397.54	1396.7113	A2pr(Nma)GRYFAFPK(Dnp)r	1526.66	1525.7215
KFAFPK(Dnp)rr	1215.36	1214.6309	GAIFAFPK(Dnp)rr	1328.48	1327.6786	GEKFAFPK(Dnp)rr	1401.53	1400.6949	A2pr(Nma)GAPFAFPK(Dnp)rr	1531.68	1530.7480
A2pr(Nma)GAPFAFPK(Dnp)	1219.30	1218.5458	EIFAFPK(Dnp)rr	1329.46	1328.6626	A2pr(Nma)GVPFAFPK(Dnp)r	1403.54	1402.6782	A2pr(Nma)GAIFAFPK(Dnp)rr	1547.72	1546.7793
A2pr(Nma)GAIFAFPK(Dnp)	1235.35	1234.5771	GADFAFPK(Dnp)rr	1330.41	1329.6214	GFIFAFPK(Dnp)rr	1404.57	1403.7099	A2pr(Nma)GADFAFPK(Dnp)rr	1549.65	1548.7222
A2pr(Nma)GADFAFPK(Dnp)	1237.28	1236.5200	EDFAFPK(Dnp)rr	1331.39	1330.6054	GFDFAFPK(Dnp)rr	1406.50	1405.6527	A2pr(Nma)GVPFAFPK(Dnp)rr	1559.73	1558.7793
A2pr(Nma)GVPFAFPK(Dnp)	1247.36	1246.5771	FPFAFPK(Dnp)rr	1331.48	1330.6571	A2pr(Nma)GAKFAFPK(Dnp)r	1406.55	1405.6891	A2pr(Nma)GAKFAFPK(Dnp)rr	1562.73	1561.7902
A2pr(Nma)GAKFAFPK(Dnp)	1250.36	1249.5880	A2pr(Nma)GRKFAFPK(Dnp)	1335.47	1334.6520	GVYFAFPK(Dnp)rr	1406.55	1405.6891	A2pr(Nma)GVIFAFPK(Dnp)rr	1575.77	1574.8106
YFAFPK(Dnp)rr	1250.36	1249.5992	GVPFAFPK(Dnp)rr	1340.49	1339.6786	RYFAFPK(Dnp)rr	1406.55	1405.7004	A2pr(Nma)GVDFAFPK(Dnp)rr	1577.70	1576.7535
APFAFPK(Dnp)rr	1255.38	1254.6258	RPFAFPK(Dnp)rr	1340.49	1339.6898	GRIFAFPK(Dnp)rr	1413.59	1412.7426	A2pr(Nma)GEPFAFPK(Dnp)rr	1589.71	1588.7535
A2pr(Nma)GVIFAFPK(Dnp)	1263.40	1262.6084	A2pr(Nma)GEYFAFPK(Dnp)	1343.40	1342.5619	GRDFAFPK(Dnp)rr	1415.52	1414.6854	A2pr(Nma)GVKFAFPK(Dnp)rr	1590.79	1589.8215
A2pr(Nma)GVDFAFPK(Dnp)	1265.33	1264.5513	GAKFAFPK(Dnp)rr	1343.49	1342.6895	A2pr(Nma)GVIFAFPK(Dnp)r	1419.58	1418.7095	A2pr(Nma)GAYFAFPK(Dnp)rr	1597.73	1596.7586
AIFAFPK(Dnp)rr	1271.43	1270.6571	EKFAFPK(Dnp)rr	1344.48	1343.6735	GFKFAFPK(Dnp)rr	1419.59	1418.7208	A2pr(Nma)GEIFAFPK(Dnp)rr	1605.75	1604.7848
ADFAFPK(Dnp)rr	1273.36	1272.6000	FIFAFPK(Dnp)rr	1347.52	1346.6884	A2pr(Nma)GVDFAFPK(Dnp)r	1421.51	1420.6524	A2pr(Nma)GEDFAFPK(Dnp)rr	1607.68	1606.7277
A2pr(Nma)GEPFAFPK(Dnp)	1277.34	1276.5513	FDFAFPK(Dnp)rr	1349.45	1348.6313	GRKFAFPK(Dnp)rr	1428.60	1427.7534	A2pr(Nma)GFPFAFPK(Dnp)rr	1607.77	1606.7793
A2pr(Nma)GVKFAFPK(Dnp)	1278.41	1277.6193	VYFAFPK(Dnp)rr	1349.50	1348.6677	A2pr(Nma)GEPFAFPK(Dnp)r	1433.53	1432.6524	A2pr(Nma)GRPFAFPK(Dnp)rr	1616.78	1615.8120
VPFAFPK(Dnp)rr	1283.44	1282.6571	GVIFAFPK(Dnp)rr	1356.53	1355.7099	A2pr(Nma)GVKFAFPK(Dnp)r	1434.60	1433.7204	A2pr(Nma)GEKFAFPK(Dnp)rr	1620.77	1619.7957
A2pr(Nma)GAYFAFPK(Dnp)	1285.36	1284.5564	RIFAFPK(Dnp)rr	1356.53	1355.7211	GEYFAFPK(Dnp)rr	1436.53	1435.6633	A2pr(Nma)GFIFAFPK(Dnp)rr	1623.81	1622.8106
AKFAFPK(Dnp)rr	1286.44	1285.6680	GVDFAFPK(Dnp)rr	1358.46	1357.6527	A2pr(Nma)GAYFAFPK(Dnp)r	1441.55	1440.6575	A2pr(Nma)GFDFAFPK(Dnp)rr	1625.74	1624.7535
A2pr(Nma)GEIFAFPK(Dnp)	1293.38	1292.5826	RDFAFPK(Dnp)rr	1358.46	1357.6640	A2pr(Nma)GEIFAFPK(Dnp)r	1449.57	1448.6837	A2pr(Nma)GVYFAFPK(Dnp)rr	1625.79	1624.7899
A2pr(Nma)GEDFAFPK(Dnp)	1295.31	1294.5255	A2pr(Nma)GFYFAFPK(Dnp)	1361.46	1360.5877	A2pr(Nma)GEDFAFPK(Dnp)r	1451.50	1450.6266	A2pr(Nma)GRIFAFPK(Dnp)rr	1632.83	1631.8433
A2pr(Nma)GFPFAFPK(Dnp)	1295.40	1294.5771	FKFAFPK(Dnp)rr	1362.54	1361.6993	A2pr(Nma)GFPFAFPK(Dnp)r	1451.59	1450.6782	A2pr(Nma)GRDFAFPK(Dnp)rr	1634.76	1633.7862
VIFAFPK(Dnp)rr	1299.48	1298.6884	A2pr(Nma)GRYFAFPK(Dnp)	1370.47	1369.6204	GFYFAFPK(Dnp)rr	1454.59	1453.6891	A2pr(Nma)GFKFAFPK(Dnp)rr	1638.83	1637.8215
VDFAFPK(Dnp)rr	1301.41	1300.6313	GEPFAFPK(Dnp)rr	1370.47	1369.6527	A2pr(Nma)GRPFAFPK(Dnp)r	1460.60	1459.7109	A2pr(Nma)GRKFAFPK(Dnp)rr	1647.84	1646.8542
A2pr(Nma)GRPFAFPK(Dnp)	1304.41	1303.6098	GVKFAFPK(Dnp)rr	1371.55	1370.7208	GRYFAFPK(Dnp)rr	1463.60	1462.7218	A2pr(Nma)GEYFAFPK(Dnp)rr	1655.77	1654.7641
A2pr(Nma)GEKFAFPK(Dnp)	1308.40	1307.5935	RKFAFPK(Dnp)rr	1371.55	1370.7320	A2pr(Nma)GEKFAFPK(Dnp)r	1464.58	1463.6946	A2pr(Nma)GFYFAFPK(Dnp)rr	1673.83	1672.7899
A2pr(Nma)GFIFAFPK(Dnp)	1311.44	1310.6084	A2pr(Nma)GAPFAFPK(Dnp)r	1375.49	1374.6469	A2pr(Nma)GFIFAFPK(Dnp)r	1467.63	1466.7095	A2pr(Nma)GRYFAFPK(Dnp)rr	1682.84	1681.8226
GAPFAFPK(Dnp)rr	1312.44	1311.6473	GAYFAFPK(Dnp)rr	1378.49	1377.6578	A2pr(Nma)GFDFAFPK(Dnp)r	1469.56	1468.6524			
A2pr(Nma)GFDFAFPK(Dnp)	1313.37	1312.5513	EYFAFPK(Dnp)rr	1379.48	1378.6418	A2pr(Nma)GVYFAFPK(Dnp)r	1469.60	1468.6888			
A2pr(Nma)GVYFAFPK(Dnp)	1313.41	1312.5877	GEIFAFPK(Dnp)rr	1386.51	1385.6840	A2pr(Nma)GRIFAFPK(Dnp)r	1476.64	1475.7422			
EPFAFPK(Dnp)rr	1313.42	1312.6313	GEDFAFPK(Dnp)rr	1388.44	1387.6269	A2pr(Nma)GRDFAFPK(Dnp)r	1478.57	1477.6851			
VKFAFPK(Dnp)rr	1314.49	1313.6993	GFPFAFPK(Dnp)rr	1388.53	1387.6786	A2pr(Nma)GFKFAFPK(Dnp)r	1482.64	1481.7204			