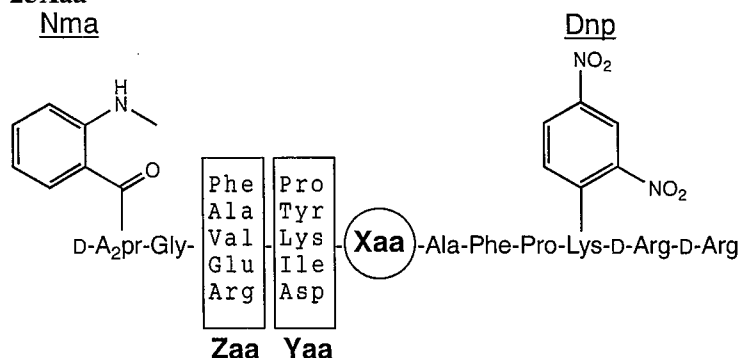# FRETS-25Xaa Series

* 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.

FRETS-25Ala	Average	Monoisotopic	FRETS-25Ala	Average	Monoisotopic	FRETS-25Ala	Average	Monoisotopic	FRETS-25Ala	Average	Monoisotopic
A2pr(Nma)G	294.31	294.1328	A2pr(Nma)GVDA	579.60	579.2653	A2pr(Nma)GEDAA	680.66	680.2766	A2pr(Nma)GAPAAFP	848.94	848.4181
A2pr(Nma)GA	365.38	365.1699	A2pr(Nma)GEY	586.59	586.2387	A2pr(Nma)GFPAA	680.75	680.3282	A2pr(Nma)GRIAAF	852.98	852.4606
A2pr(Nma)GV	393.44	393.2012	A2pr(Nma)GEPA	591.61	591.2653	A2pr(Nma)GRYA	684.74	684.3344	A2pr(Nma)GRDAAF	854.91	854.4035
A2pr(Nma)GE	423.42	423.1754	A2pr(Nma)GVKA	592.69	592.3333	A2pr(Nma)GRPAA	689.76	689.3609	A2pr(Nma)GFKAAF	858.98	858.4388
A2pr(Nma)GF	441.48	441.2012	A2pr(Nma)GAYA	599.64	599.2704	A2pr(Nma)GEKAA	693.75	693.3446	A2pr(Nma)GAIAAFP	864.99	864.4494
A2pr(Nma)GR	450.49	450.2339	A2pr(Nma)GFY	604.65	604.2645	A2pr(Nma)GFIAA	696.79	696.3595	A2pr(Nma)GADAAFP	866.92	866.3923
A2pr(Nma)GAP	462.50	462.2227	A2pr(Nma)GAPAA	604.66	604.2969	A2pr(Nma)GFDAA	698.72	698.3024	A2pr(Nma)GRKAAF	867.99	867.4715
A2pr(Nma)GAI	478.54	478.2540	A2pr(Nma)GEIA	607.66	607.2966	A2pr(Nma)GVYAA	698.77	698.3388	FPK(Dnp)rr	868.94	868.4304
A2pr(Nma)GAD	480.47	480.1969	A2pr(Nma)GEDA	609.59	609.2395	A2pr(Nma)GRIAA	705.81	705.3922	A2pr(Nma)GEYAAF	875.92	875.3814
A2pr(Nma)GVP	490.55	490.2540	A2pr(Nma)GFPA	609.67	609.2911	A2pr(Nma)GRDAA	707.74	707.3351	A2pr(Nma)GVPAAFP	877.00	876.4494
A2pr(Nma)GAK	493.56	493.2649	A2pr(Nma)GRY	613.67	613.2972	A2pr(Nma)GFKAA	711.81	711.3704	A2pr(Nma)GAKAAFP	880.00	879.4603
A2pr(Nma)GVI	506.60	506.2853	A2pr(Nma)GRPA	618.69	618.3238	A2pr(Nma)GRKAA	720.82	720.4031	A2pr(Nma)GVIAAFP	893.04	892.4807
A2pr(Nma)GVD	508.52	508.2282	A2pr(Nma)GAIAA	620.70	620.3282	PK(Dnp)rr	721.77	721.3620	A2pr(Nma)GFYAAF	893.98	893.4072
A2pr(Nma)GEP	520.54	520.2282	A2pr(Nma)GADAA	622.63	622.2711	A2pr(Nma)GEYAA	728.75	728.3130	A2pr(Nma)GVDAAFP	894.97	894.4236
A2pr(Nma)GVK	521.61	521.2962	A2pr(Nma)GEKA	622.67	622.3075	A2pr(Nma)GFYAA	746.81	746.3388	A2pr(Nma)GRYAAF	903.00	902.4399
A2pr(Nma)GAY	528.56	528.2332	K(Dnp)rr	624.65	624.3092	A2pr(Nma)GAPAAF	751.83	751.3653	A2pr(Nma)GEPAAFP	906.98	906.4236
A2pr(Nma)GAPA	533.58	533.2598	A2pr(Nma)GFIA	625.72	625.3224	A2pr(Nma)GRYAA	755.82	755.3715	A2pr(Nma)GVKAAFP	908.05	907.4916
A2pr(Nma)GEI	536.58	536.2595	A2pr(Nma)GFDA	627.65	627.2653	A2pr(Nma)GAIAAF	767.87	767.3966	A2pr(Nma)GAYAAFP	915.00	914.4287
A2pr(Nma)GED	538.51	538.2023	A2pr(Nma)GVYA	627.69	627.3017	A2pr(Nma)GADAAF	769.80	769.3395	A2pr(Nma)GEIAAFP	923.02	922.4549
A2pr(Nma)GFP	538.60	538.2540	A2pr(Nma)GVPAA	632.71	632.3282	A2pr(Nma)GVPAAF	779.88	779.3966	A2pr(Nma)GEDAAFP	924.95	924.3977
A2pr(Nma)GRP	547.61	547.2867	A2pr(Nma)GRIA	634.73	634.3551	A2pr(Nma)GAKAAF	782.89	782.4075	A2pr(Nma)GFPAAFP	925.04	924.4494
A2pr(Nma)GAIA	549.62	549.2911	A2pr(Nma)GAKAA	635.71	635.3391	A2pr(Nma)GVIAAF	795.92	795.4279	A2pr(Nma)GRPAAFP	934.05	933.4821
A2pr(Nma)GADA	551.55	551.2340	A2pr(Nma)GRDA	636.66	636.2980	A2pr(Nma)GVDAAF	797.85	797.3708	A2pr(Nma)GEKAAFP	938.04	937.4658
A2pr(Nma)GEK	551.59	551.2704	A2pr(Nma)GFKA	640.73	640.3333	A2pr(Nma)GEPAAF	809.87	809.3708	AFPK(Dnp)rr	940.02	939.4675
A2pr(Nma)GFI	554.64	554.2853	A2pr(Nma)GVIAA	648.75	648.3595	A2pr(Nma)GVKAAF	810.94	810.4388	A2pr(Nma)GFIAAFP	941.08	940.4807
A2pr(Nma)GFD	556.57	556.2282	A2pr(Nma)GRKA	649.74	649.3660	A2pr(Nma)GAYAAF	817.89	817.3759	A2pr(Nma)GFDAAFP	943.01	942.4236
A2pr(Nma)GVY	556.61	556.2645	A2pr(Nma)GVDAA	650.68	650.3024	A2pr(Nma)GEIAAF	825.91	825.4021	A2pr(Nma)GVYAAFP	943.06	942.4600
A2pr(Nma)GVPA	561.63	561.2911	A2pr(Nma)GEYA	657.67	657.2758	A2pr(Nma)GEDAAF	827.84	827.3450	A2pr(Nma)GRIAAFP	950.09	949.5134
A2pr(Nma)GRI	563.65	563.3180	A2pr(Nma)GEPAA	662.69	662.3024	A2pr(Nma)GFPAAF	827.93	827.3966	A2pr(Nma)GRDAAFP	952.02	951.4563
A2pr(Nma)GAKA	564.63	564.3020	A2pr(Nma)GVKAA	663.77	663.3704	A2pr(Nma)GRPAAF	836.94	836.4293	A2pr(Nma)GFKAAFP	956.10	955.4916
A2pr(Nma)GRD	565.58	565.2609	Ac-K(Dnp)rr	666.69	666.3198	A2pr(Nma)GEKAAF	840.92	840.4130	A2pr(Nma)GRKAAFP	965.11	964.5243
A2pr(Nma)GFK	569.65	569.2962	A2pr(Nma)GAYAA	670.71	670.3075	A2pr(Nma)GFIAAF	843.97	843.4279	A2pr(Nma)GEYAAFP	973.04	972.4341
A2pr(Nma)GVIA	577.67	577.3224	A2pr(Nma)GFYA	675.73	675.3017	A2pr(Nma)GFDAAF	845.90	845.3708	A2pr(Nma)GFYAAFP	991.10	990.4600
A2pr(Nma)GRK	578.66	578.3289	A2pr(Nma)GEIAA	678.73	678.3337	A2pr(Nma)GVYAAF	845.94	845.4072	A2pr(Nma)GRYAAFP	1000.11	999.4926

FRETS-25Ala	Average	Monoisotopic	FRETS-25Ala	Average	Monoisotopic	FRETS-25Ala	Average	Monoisotopic	FRETS-25Ala	Average	Monoisotopic
AAFPK(Dnp)rr	1011.09	1010.5046	A2pr(Nma)GRIAAFPK(Dnp)	1244.36	1243.6098	A2pr(Nma)GAIAAFPK(Dnp)r	1315.44	1314.6469	A2pr(Nma)GRKAAFPK(Dnp)r	1415.56	1414.7218
PAAFPK(Dnp)rr	1108.21	1107.5574	AYAAFPK(Dnp)rr	1245.35	1244.6051	A2pr(Nma)GADAAFPK(Dnp)r	1317.37	1316.5898	A2pr(Nma)GEYAAFPK(Dnp)r	1423.49	1422.6317
IAAFPK(Dnp)rr	1124.25	1123.5887	A2pr(Nma)GRDAAFPK(Dnp)	1246.29	1245.5527	FYAAFPK(Dnp)rr	1321.44	1320.6364	A2pr(Nma)GFYAAFPK(Dnp)r	1441.55	1440.6575
DAAFPK(Dnp)rr	1126.18	1125.5316	A2pr(Nma)GFKAAFPK(Dnp)	1250.36	1249.5880	GRPAAFPK(Dnp)rr	1321.45	1320.6800	A2pr(Nma)GRYAAFPK(Dnp)r	1450.56	1449.6902
KAAFPK(Dnp)rr	1139.27	1138.5996	GAIAAFPK(Dnp)rr	1252.38	1251.6473	GEKAAFPK(Dnp)rr	1325.43	1324.6636	A2pr(Nma)GAPAAFPK(Dnp)rr	1455.58	1454.7167
A2pr(Nma)GAPAAFPK(Dnp)	1143.21	1142.5145	EIAAFPK(Dnp)rr	1253.37	1252.6313	A2pr(Nma)GVPAAFPK(Dnp)r	1327.45	1326.6469	A2pr(Nma)GAIAAFPK(Dnp)rr	1471.62	1470.7480
A2pr(Nma)GAIAAFPK(Dnp)	1159.25	1158.5458	GADAAFPK(Dnp)rr	1254.31	1253.5901	GFIAAFPK(Dnp)rr	1328.48	1327.6786	A2pr(Nma)GADAAFPK(Dnp)rr	1473.55	1472.6909
A2pr(Nma)GADAAFPK(Dnp)	1161.18	1160.4887	EDAAFPK(Dnp)rr	1255.30	1254.5741	GFDAAFPK(Dnp)rr	1330.41	1329.6214	A2pr(Nma)GVPAAFPK(Dnp)rr	1483.63	1482.7480
A2pr(Nma)GVPAAFPK(Dnp)	1171.26	1170.5458	FPAAFPK(Dnp)rr	1255.38	1254.6258	A2pr(Nma)GAKAAFPK(Dnp)r	1330.45	1329.6578	A2pr(Nma)GAKAAFPK(Dnp)rr	1486.64	1485.7589
A2pr(Nma)GAKAAFPK(Dnp)	1174.26	1173.5567	A2pr(Nma)GRKAAFPK(Dnp)	1259.37	1258.6207	GVYAAFPK(Dnp)rr	1330.45	1329.6578	A2pr(Nma)GVIAAFPK(Dnp)rr	1499.67	1498.7793
YAAFPK(Dnp)rr	1174.27	1173.5679	GVPAAFPK(Dnp)rr	1264.39	1263.6473	RYAAFPK(Dnp)rr	1330.45	1329.6691	A2pr(Nma)GVDAAFPK(Dnp)rr	1501.60	1500.7222
APAAFPK(Dnp)rr	1179.29	1178.5945	RPAAFPK(Dnp)rr	1264.40	1263.6585	GRIAAFPK(Dnp)rr	1337.49	1336.7113	A2pr(Nma)GEPAAFPK(Dnp)rr	1513.62	1512.7222
A2pr(Nma)GVIAAFPK(Dnp)	1187.30	1186.5771	A2pr(Nma)GEYAAFPK(Dnp)	1267.30	1266.5306	GRDAAFPK(Dnp)rr	1339.42	1338.6541	A2pr(Nma)GVKAAFPK(Dnp)rr	1514.69	1513.7902
A2pr(Nma)GVDAAFPK(Dnp)	1189.23	1188.5200	GAKAAFPK(Dnp)rr	1267.40	1266.6582	A2pr(Nma)GVIAAFPK(Dnp)r	1343.49	1342.6782	A2pr(Nma)GAYAAFPK(Dnp)rr	1521.64	1520.7273
AIAAFPK(Dnp)rr	1195.33	1194.6258	EKAAFPK(Dnp)rr	1268.38	1267.6422	GFKAAFPK(Dnp)rr	1343.49	1342.6895	A2pr(Nma)GEIAAFPK(Dnp)rr	1529.66	1528.7535
ADAAFPK(Dnp)rr	1197.26	1196.5687	FIAAFPK(Dnp)rr	1271.43	1270.6571	A2pr(Nma)GVDAAFPK(Dnp)r	1345.42	1344.6211	A2pr(Nma)GEDAAFPK(Dnp)rr	1531.59	1530.6964
A2pr(Nma)GEPAAFPK(Dnp)	1201.24	1200.5200	FDAAFPK(Dnp)rr	1273.36	1272.6000	GRKAAFPK(Dnp)rr	1352.50	1351.7221	A2pr(Nma)GFPAAFPK(Dnp)rr	1531.68	1530.7480
A2pr(Nma)GVKAAFPK(Dnp)	1202.32	1201.5880	VYAAFPK(Dnp)rr	1273.40	1272.6364	A2pr(Nma)GEPAAFPK(Dnp)r	1357.43	1356.6211	A2pr(Nma)GRPAAFPK(Dnp)rr	1540.69	1539.7807
VPAAFPK(Dnp)rr	1207.34	1206.6258	GVIAAFPK(Dnp)rr	1280.43	1279.6786	A2pr(Nma)GVKAAFPK(Dnp)r	1358.50	1357.6891	A2pr(Nma)GEKAAFPK(Dnp)rr	1544.67	1543.7644
A2pr(Nma)GAYAAFPK(Dnp)	1209.27	1208.5251	RIAAFPK(Dnp)rr	1280.44	1279.6898	GEYAAFPK(Dnp)rr	1360.43	1359.6320	A2pr(Nma)GFIAAFPK(Dnp)rr	1547.72	1546.7793
AKAAFPK(Dnp)rr	1210.35	1209.6367	GVDAAFPK(Dnp)rr	1282.36	1281.6214	A2pr(Nma)GAYAAFPK(Dnp)r	1365.45	1364.6262	A2pr(Nma)GFDAAFPK(Dnp)rr	1549.65	1548.7222
A2pr(Nma)GEIAAFPK(Dnp)	1217.29	1216.5513	RDAAFPK(Dnp)rr	1282.37	1281.6327	A2pr(Nma)GEIAAFPK(Dnp)r	1373.47	1372.6524	A2pr(Nma)GVYAAFPK(Dnp)rr	1549.69	1548.7586
A2pr(Nma)GEDAAFPK(Dnp)	1219.22	1218.4942	A2pr(Nma)GFYAAFPK(Dnp)	1285.36	1284.5564	A2pr(Nma)GEDAAFPK(Dnp)r	1375.40	1374.5953	A2pr(Nma)GRIAAFPK(Dnp)rr	1556.73	1555.8120
A2pr(Nma)GFPAAFPK(Dnp)	1219.30	1218.5458	FKAAFPK(Dnp)rr	1286.44	1285.6680	A2pr(Nma)GFPAAFPK(Dnp)r	1375.49	1374.6469	A2pr(Nma)GRDAAFPK(Dnp)rr	1558.66	1557.7549
VIAAFPK(Dnp)rr	1223.38	1222.6571	A2pr(Nma)GRYAAFPK(Dnp)	1294.37	1293.5891	GFYAAFPK(Dnp)rr	1378.49	1377.6578	A2pr(Nma)GFKAAFPK(Dnp)rr	1562.73	1561.7902
VDAAFPK(Dnp)rr	1225.31	1224.6000	GEPAAFPK(Dnp)rr	1294.38	1293.6214	A2pr(Nma)GRPAAFPK(Dnp)r	1384.50	1383.6796	A2pr(Nma)GRKAAFPK(Dnp)rr	1571.74	1570.8229
A2pr(Nma)GRPAAFPK(Dnp)	1228.32	1227.5785	GVKAAFPK(Dnp)rr	1295.45	1294.6895	GRYAAFPK(Dnp)rr	1387.51	1386.6905	A2pr(Nma)GEYAAFPK(Dnp)rr	1579.67	1578.7328
A2pr(Nma)GEKAAFPK(Dnp)	1232.30	1231.5622	RKAAFPK(Dnp)rr	1295.45	1294.7007	A2pr(Nma)GEKAAFPK(Dnp)r	1388.49	1387.6633	A2pr(Nma)GFYAAFPK(Dnp)rr	1597.73	1596.7586
A2pr(Nma)GFIAAFPK(Dnp)	1235.35	1234.5771	A2pr(Nma)GAPAAFPK(Dnp)r	1299.39	1298.6156	A2pr(Nma)GFIAAFPK(Dnp)r	1391.53	1390.6782	A2pr(Nma)GRYAAFPK(Dnp)rr	1606.74	1605.7913
GAPAAFPK(Dnp)rr	1236.34	1235.6160	GAYAAFPK(Dnp)rr	1302.40	1301.6265	A2pr(Nma)GFDAAFPK(Dnp)r	1393.46	1392.6211			
A2pr(Nma)GFDAAFPK(Dnp)	1237.28	1236.5200	EYAAFPK(Dnp)rr	1303.38	1302.6105	A2pr(Nma)GVYAAFPK(Dnp)r	1393.50	1392.6575			
A2pr(Nma)GVYAAFPK(Dnp)	1237.32	1236.5564	GEIAAFPK(Dnp)rr	1310.42	1309.6527	A2pr(Nma)GRIAAFPK(Dnp)r	1400.54	1399.7109			
EPAAFPK(Dnp)rr	1237.32	1236.6000	GEDAAFPK(Dnp)rr	1312.35	1311.5956	A2pr(Nma)GRDAAFPK(Dnp)r	1402.47	1401.6538			
VKAAFPK(Dnp)rr	1238.40	1237.6680	GFPAAFPK(Dnp)rr	1312.44	1311.6473	A2pr(Nma)GFKAAFPK(Dnp)r	1406.55	1405.6891			