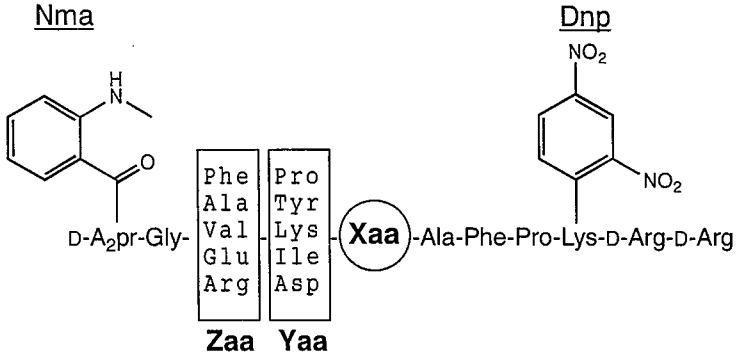
FRETS-25Xaa Series

* 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 3704-v

FRETS-25Asp Component-masses

FRETS-25Asp	Average	Monoisotopic	FRETS-25Asp	Average	Monoisotopic	FRETS-25Asp	Average	Monoisotopic	FRETS-25Asp	Average	Monoisotopic
A2pr(Nma)G	294.31	294.1328	A2pr(Nma)GAKD	608.64	608.2918	A2pr(Nma)GEIDA	722.74	722.3235	A2pr(Nma)GVYDAF	889.95	889.3970
A2pr(Nma)GA	365.38	365.1699	A2pr(Nma)GRY	613.67	613.2972	A2pr(Nma)GEDDA	724.67	724.2664	A2pr(Nma)GAPDAFP	892.95	892.4079
A2pr(Nma)GV	393.44	393.2012	A2pr(Nma)GVID	621.68	621.3122	A2pr(Nma)GFPDA	724.76	724.3180	A2pr(Nma)GRIDAF	896.99	896.4505
A2pr(Nma)GE	423.42	423.1754	A2pr(Nma)GVDD	623.61	623.2551	A2pr(Nma)GRYD	728.75	728.3242	A2pr(Nma)GRDDAF	898.92	898.3933
A2pr(Nma)GF	441.48	441.2012	K(Dnp)rr	624.65	624.3092	A2pr(Nma)GRPDA	733.77	733.3507	A2pr(Nma)GFKDAF	902.99	902.4287
A2pr(Nma)GR	450.49	450.2339	A2pr(Nma)GEPD	635.62	635.2551	A2pr(Nma)GEKDA	737.76	737.3344	A2pr(Nma)GAIDAFP	909.00	908.4392
A2pr(Nma)GAP	462.50	462.2227	A2pr(Nma)GVKD	636.70	636.3231	A2pr(Nma)GFIDA	740.80	740.3493	A2pr(Nma)GADDAFP	910.93	910.3821
A2pr(Nma)GAI	478.54	478.2540	A2pr(Nma)GAYD	643.64	643.2602	A2pr(Nma)GFDDA	742.73	742.2922	A2pr(Nma)GRKDAF	912.00	911.4613
A2pr(Nma)GAD	480.47	480.1969	A2pr(Nma)GAPDA	648.66	648.2867	A2pr(Nma)GVYDA	742.78	742.3286	A2pr(Nma)GEYDAF	919.93	919.3712
A2pr(Nma)GVP	490.55	490.2540	A2pr(Nma)GEID	651.67	651.2864	A2pr(Nma)GRIDA	749.82	749.3820	A2pr(Nma)GVPDAFP	921.01	920.4392
A2pr(Nma)GAK	493.56	493.2649	A2pr(Nma)GEDD	653.60	653.2293	A2pr(Nma)GRDDA	751.74	751.3249	A2pr(Nma)GAKDAFP	924.01	923.4501
A2pr(Nma)GVI	506.60	506.2853	A2pr(Nma)GFPD	653.68	653.2809	A2pr(Nma)GFKDA	755.82	755.3602	A2pr(Nma)GVIDAFP	937.05	936.4705
A2pr(Nma)GVD	508.52	508.2282	A2pr(Nma)GRPD	662.69	662.3136	A2pr(Nma)GRKDA	764.83	764.3929	A2pr(Nma)GFYDAF	937.99	937.3970
A2pr(Nma)GEP	520.54	520.2282	A2pr(Nma)GAIDA	664.71	664.3180	A2pr(Nma)GEYDA	772.76	772.3028	A2pr(Nma)GVDDAFP	938.98	938.4134
A2pr(Nma)GVK	521.61	521.2962	A2pr(Nma)GADDA	666.64	666.2609	A2pr(Nma)GFYDA	790.82	790.3286	AFPK(Dnp)rr	940.02	939.4675
A2pr(Nma)GAY	528.56	528.2332	A2pr(Nma)GEKD	666.68	666.2973	A2pr(Nma)GAPDAF	795.84	795.3552	A2pr(Nma)GRYDAF	947.00	946.4297
A2pr(Nma)GEI	536.58	536.2595	Ac-K(Dnp)rr	666.69	666.3198	A2pr(Nma)GRYDA	799.83	799.3613	A2pr(Nma)GEPDAFP	950.99	950.4134
A2pr(Nma)GED	538.51	538.2023	A2pr(Nma)GFID	669.73	669.3122	A2pr(Nma)GAIDAF	811.88	811.3865	A2pr(Nma)GVKDAFP	952.06	951.4814
A2pr(Nma)GFP	538.60	538.2540	A2pr(Nma)GFDD	671.66	671.2551	A2pr(Nma)GADDAF	813.81	813.3293	A2pr(Nma)GAYDAFP	959.01	958.4185
A2pr(Nma)GRP	547.61	547.2867	A2pr(Nma)GVYD	671.70	671.2915	A2pr(Nma)GVPDAF	823.89	823.3865	A2pr(Nma)GEIDAFP	967.03	966.4447
A2pr(Nma)GEK	551.59	551.2704	A2pr(Nma)GVPDA	676.72	676.3180	A2pr(Nma)GAKDAF	826.90	826.3974	A2pr(Nma)GEDDAFP	968.96	968.3876
A2pr(Nma)GFI	554.64	554.2853	A2pr(Nma)GRID	678.74	678.3449	A2pr(Nma)GVIDAF	839.93	839.4178	A2pr(Nma)GFPDAFP	969.05	968.4392
A2pr(Nma)GFD	556.57	556.2282	A2pr(Nma)GAKDA	679.72	679.3289	A2pr(Nma)GVDDAF	841.86	841.3606	A2pr(Nma)GRPDAFP	978.06	977.4719
A2pr(Nma)GVY	556.61	556.2645	A2pr(Nma)GRDD	680.67	680.2878	A2pr(Nma)GEPDAF	853.87	853.3606	A2pr(Nma)GEKDAFP	982.05	981.4556
A2pr(Nma)GRI	563.65	563.3180	A2pr(Nma)GFKD	684.74	684.3231	A2pr(Nma)GVKDAF	854.95	854.4287	A2pr(Nma)GFIDAFP	985.09	984.4705
A2pr(Nma)GRD	565.58	565.2609	A2pr(Nma)GVIDA	692.76	692.3493	A2pr(Nma)GAYDAF	861.90	861.3657	A2pr(Nma)GFDDAFP	987.02	986.4134
A2pr(Nma)GFK	569.65	569.2962	A2pr(Nma)GRKD	693.75	693.3558	FPK(Dnp)rr	868.94	868.4304	A2pr(Nma)GVYDAFP	987.07	986.4498
A2pr(Nma)GAPD	577.59	577.2496	A2pr(Nma)GVDDA	694.69	694.2922	A2pr(Nma)GEIDAF	869.92	869.3919	A2pr(Nma)GRIDAFP	994.10	993.5032
A2pr(Nma)GRK	578.66	578.3289	A2pr(Nma)GEYD	701.68	701.2657	A2pr(Nma)GEDDAF	871.85	871.3348	A2pr(Nma)GRDDAFP	996.03	995.4461
A2pr(Nma)GEY	586.59	586.2387	A2pr(Nma)GEPDA	706.70	706.2922	A2pr(Nma)GFPDAF	871.93	871.3865	A2pr(Nma)GFKDAFP	1000.11	999.4814
A2pr(Nma)GAID	593.63	593.2809	A2pr(Nma)GVKDA	707.78	707.3602	A2pr(Nma)GRPDAF	880.95	880.4192	A2pr(Nma)GRKDAFP	1009.12	1008.5141
A2pr(Nma)GADD	595.56	595.2238	A2pr(Nma)GAYDA	714.72	714.2973	A2pr(Nma)GEKDAF	884.93	884.4028	A2pr(Nma)GEYDAFP	1017.05	1016.4240
A2pr(Nma)GFY	604.65	604.2645	A2pr(Nma)GFYD	719.74	719.2915	A2pr(Nma)GFIDAF	887.98	887.4178	A2pr(Nma)GFYDAFP	1035.11	1034.4498
A2pr(Nma)GVPD	605.64	605.2809	PK(Dnp)rr	721.77	721.3620	A2pr(Nma)GFDDAF	889.91	889.3606	A2pr(Nma)GRYDAFP	1044.12	1043.4825

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FRETS-25Asp Component-masses

FRETS-25Asp	Average	Monoisotopic	FRETS-25Asp	Average	Monoisotopic	FRETS-25Asp	Average	Monoisotopic	FRETS-25Asp	Average	Monoisotopic
DAFPK(Dnp)rr	1055.10	1054.4944	A2pr(Nma)GRIDAFPK(Dnp)	1288.37	1287.5996	A2pr(Nma)GAIDAFPK(Dnp)r	1359.45	1358.6367	A2pr(Nma)GRKDAFPK(Dnp)r	1459.57	1458.7116
PDAFPK(Dnp)rr	1152.22	1151.5472	AYDAFPK(Dnp)rr	1289.36	1288.5949	A2pr(Nma)GADDAFPK(Dnp)r	1361.38	1360.5796	A2pr(Nma)GEYDAFPK(Dnp)r	1467.50	1466.6215
IDAFPK(Dnp)rr	1168.26	1167.5785	A2pr(Nma)GRDDAFPK(Dnp)	1290.30	1289.5425	FYDAFPK(Dnp)rr	1365.45	1364.6262	A2pr(Nma)GFYDAFPK(Dnp)r	1485.56	1484.6473
DDAFPK(Dnp)rr	1170.19	1169.5214	A2pr(Nma)GFKDAFPK(Dnp)	1294.37	1293.5778	GRPDAFPK(Dnp)rr	1365.46	1364.6698	A2pr(Nma)GRYDAFPK(Dnp)r	1494.57	1493.6800
KDAFPK(Dnp)rr	1183.28	1182.5894	GAIDAFPK(Dnp)rr	1296.39	1295.6371	GEKDAFPK(Dnp)rr	1369.44	1368.6535	A2pr(Nma)GAPDAFPK(Dnp)rr	1499.59	1498.7066
A2pr(Nma)GAPDAFPK(Dnp)	1187.22	1186.5043	EIDAFPK(Dnp)rr	1297.38	1296.6211	A2pr(Nma)GVPDAFPK(Dnp)r	1371.46	1370.6367	A2pr(Nma)GAIDAFPK(Dnp)rr	1515.63	1514.7379
A2pr(Nma)GAIDAFPK(Dnp)	1203.26	1202.5356	GADDAFPK(Dnp)rr	1298.32	1297.5800	GFIDAFPK(Dnp)rr	1372.49	1371.6684	A2pr(Nma)GADDAFPK(Dnp)rr	1517.56	1516.6807
A2pr(Nma)GADDAFPK(Dnp)	1205.19	1204.4785	EDDAFPK(Dnp)rr	1299.31	1298.5640	GFDDAFPK(Dnp)rr	1374.42	1373.6113	A2pr(Nma)GVPDAFPK(Dnp)rr	1527.64	1526.7379
A2pr(Nma)GVPDAFPK(Dnp)	1215.27	1214.5356	FPDAFPK(Dnp)rr	1299.39	1298.6156	A2pr(Nma)GAKDAFPK(Dnp)r	1374.46	1373.6476	A2pr(Nma)GAKDAFPK(Dnp)rr	1530.65	1529.7488
A2pr(Nma)GAKDAFPK(Dnp)	1218.27	1217.5465	A2pr(Nma)GRKDAFPK(Dnp)	1303.38	1302.6105	GVYDAFPK(Dnp)rr	1374.46	1373.6476	A2pr(Nma)GVIDAFPK(Dnp)rr	1543.68	1542.7692
YDAFPK(Dnp)rr	1218.28	1217.5578	GVPDAFPK(Dnp)rr	1308.40	1307.6371	RYDAFPK(Dnp)rr	1374.46	1373.6589	A2pr(Nma)GVDDAFPK(Dnp)rr	1545.61	1544.7120
APDAFPK(Dnp)rr	1223.30	1222.5843	RPDAFPK(Dnp)rr	1308.41	1307.6483	GRIDAFPK(Dnp)rr	1381.50	1380.7011	A2pr(Nma)GEPDAFPK(Dnp)rr	1557.62	1556.7120
A2pr(Nma)GVIDAFPK(Dnp)	1231.31	1230.5669	A2pr(Nma)GEYDAFPK(Dnp)	1311.31	1310.5204	GRDDAFPK(Dnp)rr	1383.43	1382.6440	A2pr(Nma)GVKDAFPK(Dnp)rr	1558.70	1557.7801
A2pr(Nma)GVDDAFPK(Dnp)	1233.24	1232.5098	GAKDAFPK(Dnp)rr	1311.41	1310.6480	A2pr(Nma)GVIDAFPK(Dnp)r	1387.50	1386.6680	A2pr(Nma)GAYDAFPK(Dnp)rr	1565.65	1564.7171
AIDAFPK(Dnp)rr	1239.34	1238.6156	EKDAFPK(Dnp)rr	1312.39	1311.6320	GFKDAFPK(Dnp)rr	1387.50	1386.6793	A2pr(Nma)GEIDAFPK(Dnp)rr	1573.67	1572.7433
ADDAFPK(Dnp)rr	1241.27	1240.5585	FIDAFPK(Dnp)rr	1315.44	1314.6469	A2pr(Nma)GVDDAFPK(Dnp)r	1389.43	1388.6109	A2pr(Nma)GEDDAFPK(Dnp)rr	1575.60	1574.6862
A2pr(Nma)GEPDAFPK(Dnp)	1245.25	1244.5098	FDDAFPK(Dnp)rr	1317.37	1316.5898	GRKDAFPK(Dnp)rr	1396.51	1395.7120	A2pr(Nma)GFPDAFPK(Dnp)rr	1575.68	1574.7379
A2pr(Nma)GVKDAFPK(Dnp)	1246.33	1245.5778	VYDAFPK(Dnp)rr	1317.41	1316.6262	A2pr(Nma)GEPDAFPK(Dnp)r	1401.44	1400.6109	A2pr(Nma)GRPDAFPK(Dnp)rr	1584.70	1583.7706
VPDAFPK(Dnp)rr	1251.35	1250.6156	GVIDAFPK(Dnp)rr	1324.44	1323.6684	A2pr(Nma)GVKDAFPK(Dnp)r	1402.51	1401.6789	A2pr(Nma)GEKDAFPK(Dnp)rr	1588.68	1587.7542
A2pr(Nma)GAYDAFPK(Dnp)	1253.28	1252.5149	RIDAFPK(Dnp)rr	1324.45	1323.6796	GEYDAFPK(Dnp)rr	1404.44	1403.6218	A2pr(Nma)GFIDAFPK(Dnp)rr	1591.73	1590.7692
AKDAFPK(Dnp)rr	1254.35	1253.6265	GVDDAFPK(Dnp)rr	1326.37	1325.6113	A2pr(Nma)GAYDAFPK(Dnp)r	1409.46	1408.6160	A2pr(Nma)GFDDAFPK(Dnp)rr	1593.66	1592.7120
A2pr(Nma)GEIDAFPK(Dnp)	1261.30	1260.5411	RDDAFPK(Dnp)rr	1326.38	1325.6225	A2pr(Nma)GEIDAFPK(Dnp)r	1417.48	1416.6422	A2pr(Nma)GVYDAFPK(Dnp)rr	1593.70	1592.7484
A2pr(Nma)GEDDAFPK(Dnp)	1263.23	1262.4840	A2pr(Nma)GFYDAFPK(Dnp)	1329.37	1328.5462	A2pr(Nma)GEDDAFPK(Dnp)r	1419.41	1418.5851	A2pr(Nma)GRIDAFPK(Dnp)rr	1600.74	1599.8019
A2pr(Nma)GFPDAFPK(Dnp)	1263.31	1262.5356	FKDAFPK(Dnp)rr	1330.45	1329.6578	A2pr(Nma)GFPDAFPK(Dnp)r	1419.50	1418.6367	A2pr(Nma)GRDDAFPK(Dnp)rr	1602.67	1601.7447
VIDAFPK(Dnp)rr	1267.39	1266.6469	A2pr(Nma)GRYDAFPK(Dnp)	1338.38	1337.5789	GFYDAFPK(Dnp)rr	1422.50	1421.6476	A2pr(Nma)GFKDAFPK(Dnp)rr	1606.74	1605.7801
VDDAFPK(Dnp)rr	1269.32	1268.5898	GEPDAFPK(Dnp)rr	1338.38	1337.6113	A2pr(Nma)GRPDAFPK(Dnp)r	1428.51	1427.6694	A2pr(Nma)GRKDAFPK(Dnp)rr	1615.75	1614.8128
A2pr(Nma)GRPDAFPK(Dnp)	1272.32	1271.5683	GVKDAFPK(Dnp)rr	1339.46	1338.6793	GRYDAFPK(Dnp)rr	1431.51	1430.6803	A2pr(Nma)GEYDAFPK(Dnp)rr	1623.68	1622.7226
A2pr(Nma)GEKDAFPK(Dnp)	1276.31	1275.5520	RKDAFPK(Dnp)rr	1339.46	1338.6905	A2pr(Nma)GEKDAFPK(Dnp)r	1432.50	1431.6531	A2pr(Nma)GFYDAFPK(Dnp)rr	1641.74	1640.7484
A2pr(Nma)GFIDAFPK(Dnp)	1279.36	1278.5669	A2pr(Nma)GAPDAFPK(Dnp)r	1343.40	1342.6054	A2pr(Nma)GFIDAFPK(Dnp)r	1435.54	1434.6680	A2pr(Nma)GRYDAFPK(Dnp)rr	1650.75	1649.7811
GAPDAFPK(Dnp)rr	1280.35	1279.6058	GAYDAFPK(Dnp)rr	1346.41	1345.6163	A2pr(Nma)GFDDAFPK(Dnp)r	1437.47	1436.6109			
A2pr(Nma)GFDDAFPK(Dnp)	1281.29	1280.5098	EYDAFPK(Dnp)rr	1347.39	1346.6004	A2pr(Nma)GVYDAFPK(Dnp)r	1437.51	1436.6473			
A2pr(Nma)GVYDAFPK(Dnp)	1281.33	1280.5462	GEIDAFPK(Dnp)rr	1354.43	1353.6426	A2pr(Nma)GRIDAFPK(Dnp)r	1444.55	1443.7007			
EPDAFPK(Dnp)rr	1281.33	1280.5898	GEDDAFPK(Dnp)rr	1356.36	1355.5854	A2pr(Nma)GRDDAFPK(Dnp)r	1446.48	1445.6436			
VKDAFPK(Dnp)rr	1282.41	1281.6578	GFPDAFPK(Dnp)rr	1356.44	1355.6371	A2pr(Nma)GFKDAFPK(Dnp)r	1450.56	1449.6789			