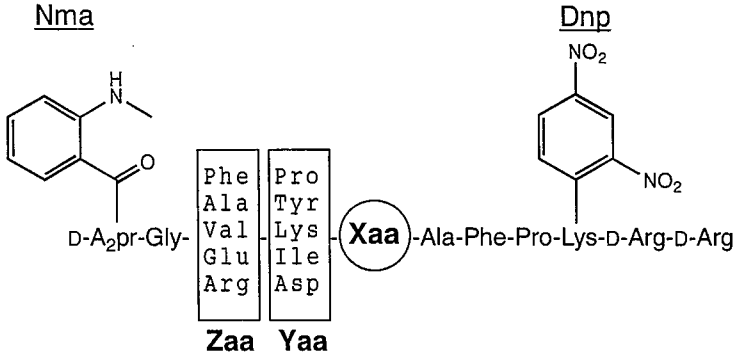
FRETS-25Xaa Series

* 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 3717-v

FRETS-25Trp Component-masses

FRETS-25Trp	Average	Monoisotopic	FRETS-25Trp	Average	Monoisotopic	FRETS-25Trp	Average	Monoisotopic	FRETS-25Trp	Average	Monoisotopic
A2pr(Nma)G	294.31	294.1328	A2pr(Nma)GADW	666.68	666.2762	A2pr(Nma)GEIWA	793.87	793.3759	A2pr(Nma)GFDWAF	961.03	960.4130
A2pr(Nma)GA	365.38	365.1699	Ac-K(Dnp)rr	666.69	666.3198	A2pr(Nma)GEDWA	795.80	795.3188	A2pr(Nma)GVYWAF	961.07	960.4494
A2pr(Nma)GV	393.44	393.2012	A2pr(Nma)GVPW	676.76	676.3333	A2pr(Nma)GFPWA	795.88	795.3704	A2pr(Nma)GAPWAFP	964.08	963.4603
A2pr(Nma)GE	423.42	423.1754	A2pr(Nma)GAKW	679.77	679.3442	A2pr(Nma)GRYW	799.88	799.3766	A2pr(Nma)GRIWAF	968.11	967.5028
A2pr(Nma)GF	441.48	441.2012	A2pr(Nma)GVIW	692.81	692.3646	A2pr(Nma)GRPWA	804.90	804.4031	A2pr(Nma)GRDWAF	970.04	969.4457
A2pr(Nma)GR	450.49	450.2339	A2pr(Nma)GVDW	694.73	694.3075	A2pr(Nma)GEKWA	808.88	808.3868	A2pr(Nma)GFKWAF	974.11	973.4810
A2pr(Nma)GAP	462.50	462.2227	A2pr(Nma)GEPW	706.75	706.3075	A2pr(Nma)GFIWA	811.93	811.4017	A2pr(Nma)GAIWAFP	980.12	979.4916
A2pr(Nma)GAI	478.54	478.2540	A2pr(Nma)GVKW	707.82	707.3755	A2pr(Nma)GFDWA	813.86	813.3446	A2pr(Nma)GADWAFP	982.05	981.4345
A2pr(Nma)GAD	480.47	480.1969	A2pr(Nma)GAYW	714.77	714.3126	A2pr(Nma)GVYWA	813.90	813.3810	A2pr(Nma)GRKWAF	983.13	982.5137
A2pr(Nma)GVP	490.55	490.2540	A2pr(Nma)GAPWA	719.79	719.3391	A2pr(Nma)GRIWA	820.94	820.4344	A2pr(Nma)GEYWAF	991.06	990.4236
A2pr(Nma)GAK	493.56	493.2649	PK(Dnp)rr	721.77	721.3620	A2pr(Nma)GRDWA	822.87	822.3773	A2pr(Nma)GVPWAFP	992.13	991.4916
A2pr(Nma)GVI	506.60	506.2853	A2pr(Nma)GEIW	722.79	722.3388	A2pr(Nma)GFKWA	826.94	826.4126	A2pr(Nma)GAKWAFP	995.13	994.5025
A2pr(Nma)GVD	508.52	508.2282	A2pr(Nma)GEDW	724.72	724.2817	A2pr(Nma)GRKWA	835.95	835.4453	A2pr(Nma)GVIWAFP	1008.17	1007.5229
A2pr(Nma)GEP	520.54	520.2282	A2pr(Nma)GFPW	724.81	724.3333	A2pr(Nma)GEYWA	843.88	843.3552	A2pr(Nma)GFYWAF	1009.12	1008.4494
A2pr(Nma)GVK	521.61	521.2962	A2pr(Nma)GRPW	733.82	733.3660	A2pr(Nma)GFYWA	861.94	861.3810	A2pr(Nma)GVDWAFP	1010.10	1009.4658
A2pr(Nma)GAY	528.56	528.2332	A2pr(Nma)GAIWA	735.83	735.3704	A2pr(Nma)GAPWAF	866.96	866.4075	A2pr(Nma)GRYWAF	1018.13	1017.4821
A2pr(Nma)GEI	536.58	536.2595	A2pr(Nma)GADWA	737.76	737.3133	FPK(Dnp)rr	868.94	868.4304	A2pr(Nma)GEPWAFP	1022.11	1021.4658
A2pr(Nma)GED	538.51	538.2023	A2pr(Nma)GEKW	737.80	737.3497	A2pr(Nma)GRYWA	870.95	870.4137	A2pr(Nma)GVKWAFP	1023.19	1022.5338
A2pr(Nma)GFP	538.60	538.2540	A2pr(Nma)GFIW	740.85	740.3646	A2pr(Nma)GAIWAF	883.00	882.4388	A2pr(Nma)GAYWAFP	1030.13	1029.4709
A2pr(Nma)GRP	547.61	547.2867	A2pr(Nma)GFDW	742.78	742.3075	A2pr(Nma)GADWAF	884.93	884.3817	A2pr(Nma)GEIWAFP	1038.15	1037.4971
A2pr(Nma)GEK	551.59	551.2704	A2pr(Nma)GVYW	742.82	742.3439	A2pr(Nma)GVPWAF	895.01	894.4388	A2pr(Nma)GEDWAFP	1040.08	1039.4399
A2pr(Nma)GFI	554.64	554.2853	A2pr(Nma)GVPWA	747.84	747.3704	A2pr(Nma)GAKWAF	898.02	897.4497	A2pr(Nma)GFPWAFP	1040.17	1039.4916
A2pr(Nma)GFD	556.57	556.2282	A2pr(Nma)GRIW	749.86	749.3973	A2pr(Nma)GVIWAF	911.06	910.4701	A2pr(Nma)GRPWAFP	1049.18	1048.5243
A2pr(Nma)GVY	556.61	556.2645	A2pr(Nma)GAKWA	750.84	750.3813	A2pr(Nma)GVDWAF	912.99	912.4130	A2pr(Nma)GEKWAFP	1053.17	1052.5080
A2pr(Nma)GRI	563.65	563.3180	A2pr(Nma)GRDW	751.79	751.3402	A2pr(Nma)GEPWAF	925.00	924.4130	A2pr(Nma)GFIWAFP	1056.21	1055.5229
A2pr(Nma)GRD	565.58	565.2609	A2pr(Nma)GFKW	755.86	755.3755	A2pr(Nma)GVKWAF	926.07	925.4810	A2pr(Nma)GFDWAFP	1058.14	1057.4658
A2pr(Nma)GFK	569.65	569.2962	A2pr(Nma)GVIWA	763.88	763.4017	A2pr(Nma)GAYWAF	933.02	932.4181	A2pr(Nma)GVYWAFP	1058.19	1057.5022
A2pr(Nma)GRK	578.66	578.3289	A2pr(Nma)GRKW	764.87	764.4082	AFPK(Dnp)rr	940.02	939.4675	A2pr(Nma)GRIWAFP	1065.23	1064.5556
A2pr(Nma)GEY	586.59	586.2387	A2pr(Nma)GVDWA	765.81	765.3446	A2pr(Nma)GEIWAF	941.04	940.4443	A2pr(Nma)GRDWAFP	1067.16	1066.4985
A2pr(Nma)GFY	604.65	604.2645	A2pr(Nma)GEYW	772.80	772.3180	A2pr(Nma)GEDWAF	942.97	942.3872	A2pr(Nma)GFKWAFP	1071.23	1070.5338
A2pr(Nma)GRY	613.67	613.2972	A2pr(Nma)GEPWA	777.82	777.3446	A2pr(Nma)GFPWAF	943.06	942.4388	A2pr(Nma)GRKWAFP	1080.24	1079.5665
K(Dnp)rr	624.65	624.3092	A2pr(Nma)GVKWA	778.90	778.4126	A2pr(Nma)GRPWAF	952.07	951.4715	A2pr(Nma)GEYWAFP	1088.17	1087.4763
A2pr(Nma)GAPW	648.71	648.3020	A2pr(Nma)GAYWA	785.85	785.3497	A2pr(Nma)GEKWAF	956.05	955.4552	A2pr(Nma)GFYWAFP	1106.23	1105.5022
A2pr(Nma)GAIW	664.75	664.3333	A2pr(Nma)GFYW	790.86	790.3439	A2pr(Nma)GFIWAF	959.10	958.4701	A2pr(Nma)GRYWAFP	1115.24	1114.5348

FRETS-25Trp Component-masses

FRETS-25Trp	Average	Monoisotopic	FRETS-25Trp	Average	Monoisotopic	FRETS-25Trp	Average	Monoisotopic	FRETS-25Trp	Average	Monoisotopic
WAFPK(Dnp)rr	1126.23	1125.5468	A2pr(Nma)GRIWAFPK(Dnp)	1359.49	1358.6520	A2pr(Nma)GAIWAFPK(Dnp)r	1430.57	1429.6891	A2pr(Nma)GRKWAFPK(Dnp)r	1530.69	1529.7640
PWAFPK(Dnp)rr	1223.34	1222.5996	AYWAFPK(Dnp)rr	1360.48	1359.6473	A2pr(Nma)GADWAFPK(Dnp)r	1432.50	1431.6320	A2pr(Nma)GEYWAFPK(Dnp)r	1538.62	1537.6739
IWAFPK(Dnp)rr	1239.38	1238.6309	A2pr(Nma)GRDWAFPK(Dnp)	1361.42	1360.5949	FYWAFPK(Dnp)rr	1436.57	1435.6786	A2pr(Nma)GFYWAFPK(Dnp)r	1556.68	1555.6997
DWAFPK(Dnp)rr	1241.31	1240.5738	A2pr(Nma)GFKWAFPK(Dnp)	1365.49	1364.6302	GRPWAFPK(Dnp)rr	1436.58	1435.7221	A2pr(Nma)GRYWAFPK(Dnp)r	1565.69	1564.7324
KWAFPK(Dnp)rr	1254.40	1253.6418	GAIWAFPK(Dnp)rr	1367.51	1366.6895	GEKWAFPK(Dnp)rr	1440.56	1439.7058	A2pr(Nma)GAPWAFPK(Dnp)rr	1570.71	1569.7589
A2pr(Nma)GAPWAFPK(Dnp)	1258.34	1257.5567	EIWAFPK(Dnp)rr	1368.50	1367.6735	A2pr(Nma)GVPWAFPK(Dnp)r	1442.58	1441.6891	A2pr(Nma)GAIWAFPK(Dnp)rr	1586.75	1585.7902
A2pr(Nma)GAIWAFPK(Dnp)	1274.38	1273.5880	GADWAFPK(Dnp)rr	1369.44	1368.6323	GFIWAFPK(Dnp)rr	1443.61	1442.7208	A2pr(Nma)GADWAFPK(Dnp)rr	1588.68	1587.7331
A2pr(Nma)GADWAFPK(Dnp)	1276.31	1275.5309	EDWAFPK(Dnp)rr	1370.43	1369.6163	GFDWAFPK(Dnp)rr	1445.54	1444.6636	A2pr(Nma)GVPWAFPK(Dnp)rr	1598.76	1597.7902
A2pr(Nma)GVPWAFPK(Dnp)	1286.39	1285.5880	FPWAFPK(Dnp)rr	1370.52	1369.6680	A2pr(Nma)GAKWAFPK(Dnp)r	1445.58	1444.7000	A2pr(Nma)GAKWAFPK(Dnp)rr	1601.77	1600.8011
A2pr(Nma)GAKWAFPK(Dnp)	1289.40	1288.5989	A2pr(Nma)GRKWAFPK(Dnp)	1374.50	1373.6629	GVYWAFPK(Dnp)rr	1445.58	1444.7000	A2pr(Nma)GVIWAFPK(Dnp)rr	1614.81	1613.8215
YWAFPK(Dnp)rr	1289.40	1288.6101	GVPWAFPK(Dnp)rr	1379.52	1378.6895	RYWAFPK(Dnp)rr	1445.59	1444.7113	A2pr(Nma)GVDWAFPK(Dnp)rr	1616.74	1615.7644
APWAFPK(Dnp)rr	1294.42	1293.6367	RPWAFPK(Dnp)rr	1379.53	1378.7007	GRIWAFPK(Dnp)rr	1452.62	1451.7534	A2pr(Nma)GEPWAFPK(Dnp)rr	1628.75	1627.7644
A2pr(Nma)GVIWAFPK(Dnp)	1302.44	1301.6193	A2pr(Nma)GEYWAFPK(Dnp)	1382.43	1381.5728	GRDWAFPK(Dnp)rr	1454.55	1453.6963	A2pr(Nma)GVKWAFPK(Dnp)rr	1629.82	1628.8324
A2pr(Nma)GVDWAFPK(Dnp)	1304.37	1303.5622	GAKWAFPK(Dnp)rr	1382.53	1381.7004	A2pr(Nma)GVIWAFPK(Dnp)r	1458.62	1457.7204	A2pr(Nma)GAYWAFPK(Dnp)rr	1636.77	1635.7695
AIWAFPK(Dnp)rr	1310.46	1309.6680	EKWAFPK(Dnp)rr	1383.51	1382.6844	GFKWAFPK(Dnp)rr	1458.62	1457.7317	A2pr(Nma)GEIWAFPK(Dnp)rr	1644.79	1643.7957
ADWAFPK(Dnp)rr	1312.39	1311.6109	FIWAFPK(Dnp)rr	1386.56	1385.6993	A2pr(Nma)GVDWAFPK(Dnp)r	1460.55	1459.6633	A2pr(Nma)GEDWAFPK(Dnp)rr	1646.72	1645.7386
A2pr(Nma)GEPWAFPK(Dnp)	1316.38	1315.5622	FDWAFPK(Dnp)rr	1388.49	1387.6422	GRKWAFPK(Dnp)rr	1467.64	1466.7643	A2pr(Nma)GFPWAFPK(Dnp)rr	1646.81	1645.7902
A2pr(Nma)GVKWAFPK(Dnp)	1317.45	1316.6302	VYWAFPK(Dnp)rr	1388.53	1387.6786	A2pr(Nma)GEPWAFPK(Dnp)r	1472.56	1471.6633	A2pr(Nma)GRPWAFPK(Dnp)rr	1655.82	1654.8229
VPWAFPK(Dnp)rr	1322.47	1321.6680	GVIWAFPK(Dnp)rr	1395.57	1394.7208	A2pr(Nma)GVKWAFPK(Dnp)r	1473.64	1472.7313	A2pr(Nma)GEKWAFPK(Dnp)rr	1659.80	1658.8066
A2pr(Nma)GAYWAFPK(Dnp)	1324.40	1323.5673	RIWAFPK(Dnp)rr	1395.57	1394.7320	GEYWAFPK(Dnp)rr	1475.57	1474.6742	A2pr(Nma)GFIWAFPK(Dnp)rr	1662.85	1661.8215
AKWAFPK(Dnp)rr	1325.48	1324.6789	GVDWAFPK(Dnp)rr	1397.50	1396.6636	A2pr(Nma)GAYWAFPK(Dnp)r	1480.58	1479.6684	A2pr(Nma)GFDWAFPK(Dnp)rr	1664.78	1663.7644
A2pr(Nma)GEIWAFPK(Dnp)	1332.42	1331.5935	RDWAFPK(Dnp)rr	1397.50	1396.6749	A2pr(Nma)GEIWAFPK(Dnp)r	1488.60	1487.6946	A2pr(Nma)GVYWAFPK(Dnp)rr	1664.82	1663.8008
A2pr(Nma)GEDWAFPK(Dnp)	1334.35	1333.5364	A2pr(Nma)GFYWAFPK(Dnp)	1400.49	1399.5986	A2pr(Nma)GEDWAFPK(Dnp)r	1490.53	1489.6375	A2pr(Nma)GRIWAFPK(Dnp)rr	1671.86	1670.8542
A2pr(Nma)GFPWAFPK(Dnp)	1334.44	1333.5880	FKWAFPK(Dnp)rr	1401.57	1400.7102	A2pr(Nma)GFPWAFPK(Dnp)r	1490.62	1489.6891	A2pr(Nma)GRDWAFPK(Dnp)rr	1673.79	1672.7971
VIWAFPK(Dnp)rr	1338.52	1337.6993	A2pr(Nma)GRYWAFPK(Dnp)	1409.51	1408.6313	GFYWAFPK(Dnp)rr	1493.63	1492.7000	A2pr(Nma)GFKWAFPK(Dnp)rr	1677.86	1676.8324
VDWAFPK(Dnp)rr	1340.45	1339.6422	GEPWAFPK(Dnp)rr	1409.51	1408.6636	A2pr(Nma)GRPWAFPK(Dnp)r	1499.63	1498.7218	A2pr(Nma)GRKWAFPK(Dnp)rr	1686.88	1685.8651
A2pr(Nma)GRPWAFPK(Dnp)	1343.45	1342.6207	GVKWAFPK(Dnp)rr	1410.58	1409.7317	GRYWAFPK(Dnp)rr	1502.64	1501.7327	A2pr(Nma)GEYWAFPK(Dnp)rr	1694.81	1693.7750
A2pr(Nma)GEKWAFPK(Dnp)	1347.43	1346.6044	RKWAFPK(Dnp)rr	1410.58	1409.7429	A2pr(Nma)GEKWAFPK(Dnp)r	1503.62	1502.7055	A2pr(Nma)GFYWAFPK(Dnp)rr	1712.87	1711.8008
A2pr(Nma)GFIWAFPK(Dnp)	1350.48	1349.6193	A2pr(Nma)GAPWAFPK(Dnp)r	1414.53	1413.6578	A2pr(Nma)GFIWAFPK(Dnp)r	1506.66	1505.7204	A2pr(Nma)GRYWAFPK(Dnp)rr	1721.88	1720.8335
GAPWAFPK(Dnp)rr	1351.47	1350.6582	GAYWAFPK(Dnp)rr	1417.53	1416.6687	A2pr(Nma)GFDWAFPK(Dnp)r	1508.59	1507.6633			
A2pr(Nma)GFDWAFPK(Dnp)	1352.41	1351.5622	EYWAFPK(Dnp)rr	1418.51	1417.6527	A2pr(Nma)GVYWAFPK(Dnp)r	1508.64	1507.6997			
A2pr(Nma)GVYWAFPK(Dnp)	1352.45	1351.5986	GEIWAFPK(Dnp)rr	1425.55	1424.6949	A2pr(Nma)GRIWAFPK(Dnp)r	1515.68	1514.7531			
EPWAFPK(Dnp)rr	1352.46	1351.6422	GEDWAFPK(Dnp)rr	1427.48	1426.6378	A2pr(Nma)GRDWAFPK(Dnp)r	1517.61	1516.6960			
VKWAFPK(Dnp)rr	1353.53	1352.7102	GFPWAFPK(Dnp)rr	1427.57	1426.6895	A2pr(Nma)GFKWAFPK(Dnp)r	1521.68	1520.7313			