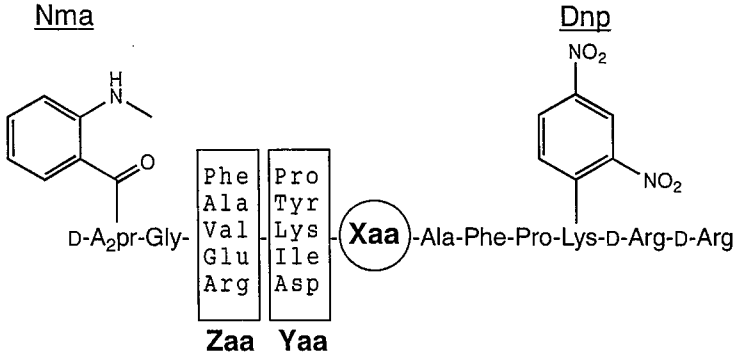
FRETS-25Xaa Series

* 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 3702-v

FRETS-25Arg Component-masses

FRETS-25Arg	Average	Monoisotopic	FRETS-25Arg	Average	Monoisotopic	FRETS-25Arg	Average	Monoisotopic	FRETS-25Arg	Average	Monoisotopic
A2pr(Nma)G	294.31	294.1328	A2pr(Nma)GADR	636.66	636.2980	A2pr(Nma)GEIRA	763.84	763.3977	A2pr(Nma)GVYRAF	931.05	930.4712
A2pr(Nma)GA	365.38	365.1699	A2pr(Nma)GVPR	646.74	646.3551	A2pr(Nma)GEDRA	765.77	765.3406	A2pr(Nma)GAPRAFP	934.05	933.4821
A2pr(Nma)GV	393.44	393.2012	A2pr(Nma)GAKR	649.74	649.3660	A2pr(Nma)GFPRA	765.86	765.3922	A2pr(Nma)GRIRAF	938.09	937.5246
A2pr(Nma)GE	423.42	423.1754	A2pr(Nma)GVIR	662.78	662.3864	A2pr(Nma)GRYR	769.85	769.3984	A2pr(Nma)GRDRAF	940.02	939.4675
A2pr(Nma)GF	441.48	441.2012	A2pr(Nma)GVDR	664.71	664.3293	A2pr(Nma)GRPRA	774.87	774.4249	AFPK(Dnp)rr	940.02	939.4675
A2pr(Nma)GR	450.49	450.2339	Ac-K(Dnp)rr	666.69	666.3198	A2pr(Nma)GEKRA	778.86	778.4086	A2pr(Nma)GFKRAF	944.09	943.5028
A2pr(Nma)GAP	462.50	462.2227	A2pr(Nma)GEPR	676.72	676.3293	A2pr(Nma)GFIRA	781.90	781.4235	A2pr(Nma)GAIRAFP	950.09	949.5134
A2pr(Nma)GAI	478.54	478.2540	A2pr(Nma)GVKR	677.80	677.3973	A2pr(Nma)GFDRA	783.83	783.3664	A2pr(Nma)GADRAFP	952.02	951.4563
A2pr(Nma)GAD	480.47	480.1969	A2pr(Nma)GAYR	684.74	684.3344	A2pr(Nma)GVYRA	783.87	783.4028	A2pr(Nma)GRKRAF	953.10	952.5355
A2pr(Nma)GVP	490.55	490.2540	A2pr(Nma)GAPRA	689.76	689.3609	A2pr(Nma)GRIRA	790.91	790.4562	A2pr(Nma)GEYRAF	961.03	960.4454
A2pr(Nma)GAK	493.56	493.2649	A2pr(Nma)GEIR	692.76	692.3606	A2pr(Nma)GRDRA	792.84	792.3991	A2pr(Nma)GVPRAFP	962.11	961.5134
A2pr(Nma)GVI	506.60	506.2853	A2pr(Nma)GEDR	694.69	694.3035	A2pr(Nma)GFKRA	796.92	796.4344	A2pr(Nma)GAKRAFP	965.11	964.5243
A2pr(Nma)GVD	508.52	508.2282	A2pr(Nma)GFPR	694.78	694.3551	A2pr(Nma)GRKRA	805.93	805.4671	A2pr(Nma)GVIRAFP	978.15	977.5447
A2pr(Nma)GEP	520.54	520.2282	A2pr(Nma)GRPR	703.79	703.3878	A2pr(Nma)GEYRA	813.86	813.3770	A2pr(Nma)GFYRAF	979.09	978.4712
A2pr(Nma)GVK	521.61	521.2962	A2pr(Nma)GAIRA	705.81	705.3922	A2pr(Nma)GFYRA	831.92	831.4028	A2pr(Nma)GVDRAFP	980.08	979.4876
A2pr(Nma)GAY	528.56	528.2332	A2pr(Nma)GADRA	707.74	707.3351	A2pr(Nma)GAPRAF	836.94	836.4293	A2pr(Nma)GRYRAF	988.10	987.5039
A2pr(Nma)GEI	536.58	536.2595	A2pr(Nma)GEKR	707.78	707.3715	A2pr(Nma)GRYRA	840.93	840.4355	A2pr(Nma)GEPRAFP	992.09	991.4876
A2pr(Nma)GED	538.51	538.2023	A2pr(Nma)GFIR	710.82	710.3864	A2pr(Nma)GAIRAF	852.98	852.4606	A2pr(Nma)GVKRAFP	993.16	992.5556
A2pr(Nma)GFP	538.60	538.2540	A2pr(Nma)GFDR	712.75	712.3293	A2pr(Nma)GADRAF	854.91	854.4035	A2pr(Nma)GAYRAFP	1000.11	999.4926
A2pr(Nma)GRP	547.61	547.2867	A2pr(Nma)GVYR	712.80	712.3657	A2pr(Nma)GVPRAF	864.99	864.4606	A2pr(Nma)GEIRAFP	1008.13	1007.5189
A2pr(Nma)GEK	551.59	551.2704	A2pr(Nma)GVPRA	717.82	717.3922	A2pr(Nma)GAKRAF	867.99	867.4715	A2pr(Nma)GEDRAFP	1010.06	1009.4617
A2pr(Nma)GFI	554.64	554.2853	A2pr(Nma)GRIR	719.84	719.4191	FPK(Dnp)rr	868.94	868.4304	A2pr(Nma)GFPRAFP	1010.15	1009.5134
A2pr(Nma)GFD	556.57	556.2282	A2pr(Nma)GAKRA	720.82	720.4031	A2pr(Nma)GVIRAF	881.03	880.4919	A2pr(Nma)GRPRAFP	1019.16	1018.5461
A2pr(Nma)GVY	556.61	556.2645	A2pr(Nma)GRDR	721.77	721.3620	A2pr(Nma)GVDRAF	882.96	882.4348	A2pr(Nma)GEKRAFP	1023.15	1022.5298
A2pr(Nma)GRI	563.65	563.3180	PK(Dnp)rr	721.77	721.3620	A2pr(Nma)GEPRAF	894.97	894.4348	A2pr(Nma)GFIRAFP	1026.19	1025.5447
A2pr(Nma)GRD	565.58	565.2609	A2pr(Nma)GFKR	725.84	725.3973	A2pr(Nma)GVKRAF	896.05	895.5028	A2pr(Nma)GFDRAFP	1028.12	1027.4876
A2pr(Nma)GFK	569.65	569.2962	A2pr(Nma)GVIRA	733.86	733.4235	A2pr(Nma)GAYRAF	903.00	902.4399	A2pr(Nma)GVYRAFP	1028.16	1027.5240
A2pr(Nma)GRK	578.66	578.3289	A2pr(Nma)GRKR	734.85	734.4300	A2pr(Nma)GEIRAF	911.02	910.4661	A2pr(Nma)GRIRAFP	1035.20	1034.5774
A2pr(Nma)GEY	586.59	586.2387	A2pr(Nma)GVDRA	735.79	735.3664	A2pr(Nma)GEDRAF	912.95	912.4090	A2pr(Nma)GRDRAFP	1037.13	1036.5203
A2pr(Nma)GFY	604.65	604.2645	A2pr(Nma)GEYR	742.78	742.3398	A2pr(Nma)GFPRAF	913.03	912.4606	A2pr(Nma)GFKRAFP	1041.21	1040.5556
A2pr(Nma)GRY	613.67	613.2972	A2pr(Nma)GEPRA	747.80	747.3664	A2pr(Nma)GRPRAF	922.04	921.4933	A2pr(Nma)GRKRAFP	1050.22	1049.5883
A2pr(Nma)GAPR	618.69	618.3238	A2pr(Nma)GVKRA	748.87	748.4344	A2pr(Nma)GEKRAF	926.03	925.4770	A2pr(Nma)GEYRAFP	1058.15	1057.4981
K(Dnp)rr	624.65	624.3092	A2pr(Nma)GAYRA	755.82	755.3715	A2pr(Nma)GFIRAF	929.08	928.4919	A2pr(Nma)GFYRAFP	1076.21	1075.5240
A2pr(Nma)GAIR	634.73	634.3551	A2pr(Nma)GFYR	760.84	760.3657	A2pr(Nma)GFDRAF	931.01	930.4348	A2pr(Nma)GRYRAFP	1085.22	1084.5566

FRETS-25Arg Component-masses

FRETS-25Arg	Average	Monoisotopic	FRETS-25Arg	Average	Monoisotopic	FRETS-25Arg	Average	Monoisotopic	FRETS-25Arg	Average	Monoisotopic
RAFPK(Dnp)rr	1096.20	1095.5686	A2pr(Nma)GRIRAFPK(Dnp)	1329.47	1328.6738	A2pr(Nma)GAIRAFPK(Dnp)r	1400.54	1399.7109	A2pr(Nma)GRKRAFPK(Dnp)r	1500.67	1499.7858
PRAFPK(Dnp)rr	1193.32	1192.6214	AYRAFPK(Dnp)rr	1330.45	1329.6691	A2pr(Nma)GADRAFPK(Dnp)r	1402.47	1401.6538	A2pr(Nma)GEYRAFPK(Dnp)r	1508.60	1507.6957
IRAFPK(Dnp)rr	1209.36	1208.6527	A2pr(Nma)GRDRAFPK(Dnp)	1331.40	1330.6167	FYRAFPK(Dnp)rr	1406.55	1405.7004	A2pr(Nma)GFYRAFPK(Dnp)r	1526.66	1525.7215
DRAFPK(Dnp)rr	1211.29	1210.5956	A2pr(Nma)GFKRAFPK(Dnp)	1335.47	1334.6520	GRPRAFPK(Dnp)rr	1406.55	1405.7439	A2pr(Nma)GRYRAFPK(Dnp)r	1535.67	1534.7542
KRAFPK(Dnp)rr	1224.37	1223.6636	GAIRAFPK(Dnp)rr	1337.49	1336.7113	GEKRAFPK(Dnp)rr	1410.54	1409.7276	A2pr(Nma)GAPRAFPK(Dnp)rr	1540.69	1539.7807
A2pr(Nma)GAPRAFPK(Dnp)	1228.32	1227.5785	EIRAFPK(Dnp)rr	1338.47	1337.6953	A2pr(Nma)GVPRAFPK(Dnp)r	1412.55	1411.7109	A2pr(Nma)GAIRAFPK(Dnp)rr	1556.73	1555.8120
A2pr(Nma)GAIRAFPK(Dnp)	1244.36	1243.6098	GADRAFPK(Dnp)rr	1339.42	1338.6541	GFIRAFPK(Dnp)rr	1413.59	1412.7426	A2pr(Nma)GADRAFPK(Dnp)rr	1558.66	1557.7549
A2pr(Nma)GADRAFPK(Dnp)	1246.29	1245.5527	EDRAFPK(Dnp)rr	1340.40	1339.6381	GFDRAFPK(Dnp)rr	1415.52	1414.6854	A2pr(Nma)GVPRAFPK(Dnp)rr	1568.74	1567.8120
A2pr(Nma)GVPRAFPK(Dnp)	1256.37	1255.6098	FPRAFPK(Dnp)rr	1340.49	1339.6898	A2pr(Nma)GAKRAFPK(Dnp)r	1415.56	1414.7218	A2pr(Nma)GAKRAFPK(Dnp)rr	1571.74	1570.8229
A2pr(Nma)GAKRAFPK(Dnp)	1259.37	1258.6207	A2pr(Nma)GRKRAFPK(Dnp)	1344.48	1343.6847	GVYRAFPK(Dnp)rr	1415.56	1414.7218	A2pr(Nma)GVIRAFPK(Dnp)rr	1584.78	1583.8433
YRAFPK(Dnp)rr	1259.38	1258.6319	GVPRAFPK(Dnp)rr	1349.50	1348.7113	RYRAFPK(Dnp)rr	1415.56	1414.7330	A2pr(Nma)GVDRAFPK(Dnp)rr	1586.71	1585.7862
APRAFPK(Dnp)rr	1264.40	1263.6585	RPRAFPK(Dnp)rr	1349.50	1348.7225	GRIRAFPK(Dnp)rr	1422.60	1421.7752	A2pr(Nma)GEPRAFPK(Dnp)rr	1598.72	1597.7862
A2pr(Nma)GVIRAFPK(Dnp)	1272.41	1271.6411	A2pr(Nma)GEYRAFPK(Dnp)	1352.41	1351.5945	GRDRAFPK(Dnp)rr	1424.53	1423.7181	A2pr(Nma)GVKRAFPK(Dnp)rr	1599.80	1598.8542
A2pr(Nma)GVDRAFPK(Dnp)	1274.34	1273.5840	GAKRAFPK(Dnp)rr	1352.50	1351.7221	A2pr(Nma)GVIRAFPK(Dnp)r	1428.60	1427.7422	A2pr(Nma)GAYRAFPK(Dnp)rr	1606.74	1605.7913
AIRAFPK(Dnp)rr	1280.44	1279.6898	EKRAFPK(Dnp)rr	1353.49	1352.7062	GFKRAFPK(Dnp)rr	1428.60	1427.7534	A2pr(Nma)GEIRAFPK(Dnp)rr	1614.77	1613.8175
ADRAFPK(Dnp)rr	1282.37	1281.6327	FIRAFPK(Dnp)rr	1356.53	1355.7211	A2pr(Nma)GVDRAFPK(Dnp)r	1430.53	1429.6851	A2pr(Nma)GEDRAFPK(Dnp)rr	1616.70	1615.7604
A2pr(Nma)GEPRAFPK(Dnp)	1286.35	1285.5840	FDRAFPK(Dnp)rr	1358.46	1357.6640	GRKRAFPK(Dnp)rr	1437.61	1436.7861	A2pr(Nma)GFPRAFPK(Dnp)rr	1616.78	1615.8120
A2pr(Nma)GVKRAFPK(Dnp)	1287.43	1286.6520	VYRAFPK(Dnp)rr	1358.51	1357.7004	A2pr(Nma)GEPRAFPK(Dnp)r	1442.54	1441.6851	A2pr(Nma)GRPRAFPK(Dnp)rr	1625.79	1624.8447
VPRAFPK(Dnp)rr	1292.45	1291.6898	GVIRAFPK(Dnp)rr	1365.54	1364.7426	A2pr(Nma)GVKRAFPK(Dnp)r	1443.61	1442.7531	A2pr(Nma)GEKRAFPK(Dnp)rr	1629.78	1628.8284
A2pr(Nma)GAYRAFPK(Dnp)	1294.37	1293.5891	RIRAFPK(Dnp)rr	1365.55	1364.7538	GEYRAFPK(Dnp)rr	1445.54	1444.6960	A2pr(Nma)GFIRAFPK(Dnp)rr	1632.83	1631.8433
AKRAFPK(Dnp)rr	1295.45	1294.7007	GVDRAFPK(Dnp)rr	1367.47	1366.6854	A2pr(Nma)GAYRAFPK(Dnp)r	1450.56	1449.6902	A2pr(Nma)GFDRAFPK(Dnp)rr	1634.76	1633.7862
A2pr(Nma)GEIRAFPK(Dnp)	1302.39	1301.6153	RDRAFPK(Dnp)rr	1367.48	1366.6967	A2pr(Nma)GEIRAFPK(Dnp)r	1458.58	1457.7164	A2pr(Nma)GVYRAFPK(Dnp)rr	1634.80	1633.8226
A2pr(Nma)GEDRAFPK(Dnp)	1304.32	1303.5582	A2pr(Nma)GFYRAFPK(Dnp)	1370.47	1369.6204	A2pr(Nma)GEDRAFPK(Dnp)r	1460.51	1459.6593	A2pr(Nma)GRIRAFPK(Dnp)rr	1641.84	1640.8760
A2pr(Nma)GFPRAFPK(Dnp)	1304.41	1303.6098	FKRAFPK(Dnp)rr	1371.55	1370.7320	A2pr(Nma)GFPRAFPK(Dnp)r	1460.60	1459.7109	A2pr(Nma)GRDRAFPK(Dnp)rr	1643.77	1642.8189
VIRAFPK(Dnp)rr	1308.49	1307.7211	A2pr(Nma)GRYRAFPK(Dnp)	1379.48	1378.6531	GFYRAFPK(Dnp)rr	1463.60	1462.7218	A2pr(Nma)GFKRAFPK(Dnp)rr	1647.84	1646.8542
VDRAFPK(Dnp)rr	1310.42	1309.6640	GEPRAFPK(Dnp)rr	1379.48	1378.6854	A2pr(Nma)GRPRAFPK(Dnp)r	1469.61	1468.7436	A2pr(Nma)GRKRAFPK(Dnp)rr	1656.85	1655.8869
A2pr(Nma)GRPRAFPK(Dnp)	1313.42	1312.6425	GVKRAFPK(Dnp)rr	1380.56	1379.7534	GRYRAFPK(Dnp)rr	1472.61	1471.7545	A2pr(Nma)GEYRAFPK(Dnp)rr	1664.78	1663.7968
A2pr(Nma)GEKRAFPK(Dnp)	1317.41	1316.6262	RKRAFPK(Dnp)rr	1380.56	1379.7647	A2pr(Nma)GEKRAFPK(Dnp)r	1473.59	1472.7273	A2pr(Nma)GFYRAFPK(Dnp)rr	1682.84	1681.8226
A2pr(Nma)GFIRAFPK(Dnp)	1320.45	1319.6411	A2pr(Nma)GAPRAFPK(Dnp)r	1384.50	1383.6796	A2pr(Nma)GFIRAFPK(Dnp)r	1476.64	1475.7422	A2pr(Nma)GRYRAFPK(Dnp)rr	1691.85	1690.8553
GAPRAFPK(Dnp)rr	1321.45	1320.6800	GAYRAFPK(Dnp)rr	1387.51	1386.6905	A2pr(Nma)GFDRAFPK(Dnp)r	1478.57	1477.6851			
A2pr(Nma)GFDRAFPK(Dnp)	1322.38	1321.5840	EYRAFPK(Dnp)rr	1388.49	1387.6745	A2pr(Nma)GVYRAFPK(Dnp)r	1478.61	1477.7215			
A2pr(Nma)GVYRAFPK(Dnp)	1322.43	1321.6204	GEIRAFPK(Dnp)rr	1395.53	1394.7167	A2pr(Nma)GRIRAFPK(Dnp)r	1485.65	1484.7749			
EPRAFPK(Dnp)rr	1322.43	1321.6640	GEDRAFPK(Dnp)rr	1397.46	1396.6596	A2pr(Nma)GRDRAFPK(Dnp)r	1487.58	1486.7178			
VKRAFPK(Dnp)rr	1323.51	1322.7320	GFPRAFPK(Dnp)rr	1397.54	1396.7113	A2pr(Nma)GFKRAFPK(Dnp)r	1491.65	1490.7531			