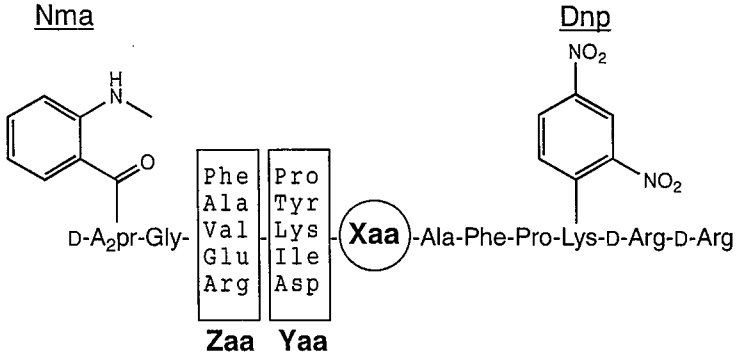
FRETS-25Xaa Series

* 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 3708-v

FRETS-25His Component-masses

FRETS-25His	Average	Monoisotopic	FRETS-25His	Average	Monoisotopic	FRETS-25His	Average	Monoisotopic	FRETS-25His	Average	Monoisotopic
A2pr(Nma)G	294.31	294.1328	K(Dnp)rr	624.65	624.3092	A2pr(Nma)GEIHA	744.80	744.3555	A2pr(Nma)GVYHAF	912.00	911.4290
A2pr(Nma)GA	365.38	365.1699	A2pr(Nma)GVPH	627.69	627.3129	A2pr(Nma)GEDHA	746.72	746.2984	A2pr(Nma)GAPHAFP	915.01	914.4399
A2pr(Nma)GV	393.44	393.2012	A2pr(Nma)GAKH	630.70	630.3238	A2pr(Nma)GFPHA	746.81	746.3500	A2pr(Nma)GRIHAF	919.04	918.4824
A2pr(Nma)GE	423.42	423.1754	A2pr(Nma)GVIH	643.73	643.3442	A2pr(Nma)GRYH	750.80	750.3562	A2pr(Nma)GRDHAF	920.97	920.4253
A2pr(Nma)GF	441.48	441.2012	A2pr(Nma)GVDH	645.66	645.2871	A2pr(Nma)GRPHA	755.82	755.3827	A2pr(Nma)GFKHAF	925.04	924.4606
A2pr(Nma)GR	450.49	450.2339	A2pr(Nma)GEPH	657.67	657.2871	A2pr(Nma)GEKHA	759.81	759.3664	A2pr(Nma)GAIHAFP	931.05	930.4712
A2pr(Nma)GAP	462.50	462.2227	A2pr(Nma)GVKH	658.75	658.3551	A2pr(Nma)GFIHA	762.86	762.3813	A2pr(Nma)GADHAFP	932.98	932.4141
A2pr(Nma)GAI	478.54	478.2540	A2pr(Nma)GAYH	665.70	665.2922	A2pr(Nma)GFDHA	764.78	764.3242	A2pr(Nma)GRKHAF	934.06	933.4933
A2pr(Nma)GAD	480.47	480.1969	Ac-K(Dnp)rr	666.69	666.3198	A2pr(Nma)GVYHA	764.83	764.3606	AFPK(Dnp)rr	940.02	939.4675
A2pr(Nma)GVP	490.55	490.2540	A2pr(Nma)GAPHA	670.72	670.3187	A2pr(Nma)GRIHA	771.87	771.4140	A2pr(Nma)GEYHAF	941.98	941.4032
A2pr(Nma)GAK	493.56	493.2649	A2pr(Nma)GEIH	673.72	673.3184	A2pr(Nma)GRDHA	773.80	773.3569	A2pr(Nma)GVPHAFP	943.06	942.4712
A2pr(Nma)GVI	506.60	506.2853	A2pr(Nma)GEDH	675.65	675.2613	A2pr(Nma)GFKHA	777.87	777.3922	A2pr(Nma)GAKHAFP	946.06	945.4821
A2pr(Nma)GVD	508.52	508.2282	A2pr(Nma)GFPH	675.73	675.3129	A2pr(Nma)GRKHA	786.88	786.4249	A2pr(Nma)GVIHAFP	959.10	958.5025
A2pr(Nma)GEP	520.54	520.2282	A2pr(Nma)GRPH	684.75	684.3456	A2pr(Nma)GEYHA	794.81	794.3348	A2pr(Nma)GFYHAF	960.04	959.4290
A2pr(Nma)GVK	521.61	521.2962	A2pr(Nma)GAIHA	686.76	686.3500	A2pr(Nma)GFYHA	812.87	812.3606	A2pr(Nma)GVDHAFP	961.03	960.4454
A2pr(Nma)GAY	528.56	528.2332	A2pr(Nma)GADHA	688.69	688.2929	A2pr(Nma)GAPHAF	817.89	817.3871	A2pr(Nma)GRYHAF	969.06	968.4617
A2pr(Nma)GEI	536.58	536.2595	A2pr(Nma)GEKH	688.73	688.3293	A2pr(Nma)GRYHA	821.88	821.3933	A2pr(Nma)GEPHAFP	973.04	972.4454
A2pr(Nma)GED	538.51	538.2023	A2pr(Nma)GFIH	691.78	691.3442	A2pr(Nma)GAIHAF	833.93	833.4184	A2pr(Nma)GVKHAFP	974.12	973.5134
A2pr(Nma)GFP	538.60	538.2540	A2pr(Nma)GFDH	693.71	693.2871	A2pr(Nma)GADHAF	835.86	835.3613	A2pr(Nma)GAYHAFP	981.06	980.4505
A2pr(Nma)GRP	547.61	547.2867	A2pr(Nma)GVYH	693.75	693.3235	A2pr(Nma)GVPHAF	845.94	845.4184	A2pr(Nma)GEIHAFP	989.08	988.4767
A2pr(Nma)GEK	551.59	551.2704	A2pr(Nma)GVPHA	698.77	698.3500	A2pr(Nma)GAKHAF	848.95	848.4293	A2pr(Nma)GEDHAFP	991.01	990.4195
A2pr(Nma)GFI	554.64	554.2853	A2pr(Nma)GRIH	700.79	700.3769	A2pr(Nma)GVIHAF	861.99	861.4497	A2pr(Nma)GFPHAFP	991.10	990.4712
A2pr(Nma)GFD	556.57	556.2282	A2pr(Nma)GAKHA	701.77	701.3609	A2pr(Nma)GVDHAF	863.92	863.3926	A2pr(Nma)GRPHAFP	1000.11	999.5039
A2pr(Nma)GVY	556.61	556.2645	A2pr(Nma)GRDH	702.72	702.3198	FPK(Dnp)rr	868.94	868.4304	A2pr(Nma)GEKHAFP	1004.10	1003.4876
A2pr(Nma)GRI	563.65	563.3180	A2pr(Nma)GFKH	706.79	706.3551	A2pr(Nma)GEPHAF	875.93	875.3926	A2pr(Nma)GFIHAFP	1007.14	1006.5025
A2pr(Nma)GRD	565.58	565.2609	A2pr(Nma)GVIHA	714.81	714.3813	A2pr(Nma)GVKHAF	877.00	876.4606	A2pr(Nma)GFDHAFP	1009.07	1008.4454
A2pr(Nma)GFK	569.65	569.2962	A2pr(Nma)GRKH	715.80	715.3878	A2pr(Nma)GAYHAF	883.95	883.3977	A2pr(Nma)GVYHAFP	1009.12	1008.4818
A2pr(Nma)GRK	578.66	578.3289	A2pr(Nma)GVDHA	716.74	716.3242	A2pr(Nma)GEIHAF	891.97	891.4239	A2pr(Nma)GRIHAFP	1016.16	1015.5352
A2pr(Nma)GEY	586.59	586.2387	PK(Dnp)rr	721.77	721.3620	A2pr(Nma)GEDHAF	893.90	893.3668	A2pr(Nma)GRDHAFP	1018.09	1017.4781
A2pr(Nma)GAPH	599.64	599.2816	A2pr(Nma)GEYH	723.73	723.2976	A2pr(Nma)GFPHAF	893.99	893.4184	A2pr(Nma)GFKHAFP	1022.16	1021.5134
A2pr(Nma)GFY	604.65	604.2645	A2pr(Nma)GEPHA	728.75	728.3242	A2pr(Nma)GRPHAF	903.00	902.4511	A2pr(Nma)GRKHAFP	1031.17	1030.5461
A2pr(Nma)GRY	613.67	613.2972	A2pr(Nma)GVKHA	729.83	729.3922	A2pr(Nma)GEKHAF	906.98	906.4348	A2pr(Nma)GEYHAFP	1039.10	1038.4559
A2pr(Nma)GAIH	615.68	615.3129	A2pr(Nma)GAYHA	736.77	736.3293	A2pr(Nma)GFIHAF	910.03	909.4497	A2pr(Nma)GFYHAFP	1057.16	1056.4818
A2pr(Nma)GADH	617.61	617.2558	A2pr(Nma)GFYH	741.79	741.3235	A2pr(Nma)GFDHAF	911.96	911.3926	A2pr(Nma)GRYHAFP	1066.17	1065.5144

Code 3708-v

FRETS-25His Component-masses

FRETS-25His	Average	Monoisotopic	FRETS-25His	Average	Monoisotopic	FRETS-25His	Average	Monoisotopic	FRETS-25His	Average	Monoisotopic
HAFPK(Dnp)rr	1077.16	1076.5264	A2pr(Nma)GRIHAFPK(Dnp)	1310.42	1309.6316	A2pr(Nma)GAIHAFPK(Dnp)r	1381.50	1380.6687	A2pr(Nma)GRKHAFPK(Dnp)r	1481.62	1480.7436
PHAFPK(Dnp)rr	1174.27	1173.5792	AYHAFPK(Dnp)rr	1311.41	1310.6269	A2pr(Nma)GADHAFPK(Dnp)r	1383.43	1382.6116	A2pr(Nma)GEYHAFPK(Dnp)r	1489.55	1488.6535
IHAFPK(Dnp)rr	1190.31	1189.6105	A2pr(Nma)GRDHAFPK(Dnp)	1312.35	1311.5745	FYHAFPK(Dnp)rr	1387.50	1386.6582	A2pr(Nma)GFYHAFPK(Dnp)r	1507.61	1506.6793
DHAFPK(Dnp)rr	1192.24	1191.5534	A2pr(Nma)GFKHAFPK(Dnp)	1316.42	1315.6098	GRPHAFPK(Dnp)rr	1387.51	1386.7017	A2pr(Nma)GRYHAFPK(Dnp)r	1516.62	1515.7120
KHAFPK(Dnp)rr	1205.33	1204.6214	GAIHAFPK(Dnp)rr	1318.44	1317.6691	GEKHAFPK(Dnp)rr	1391.49	1390.6854	A2pr(Nma)GAPHAFPK(Dnp)rr	1521.64	1520.7385
A2pr(Nma)GAPHAFPK(Dnp)	1209.27	1208.5363	EIHAFPK(Dnp)rr	1319.43	1318.6531	A2pr(Nma)GVPHAFPK(Dnp)r	1393.51	1392.6687	A2pr(Nma)GAIHAFPK(Dnp)rr	1537.68	1536.7698
A2pr(Nma)GAIHAFPK(Dnp)	1225.31	1224.5676	GADHAFPK(Dnp)rr	1320.37	1319.6119	GFIHAFPK(Dnp)rr	1394.54	1393.7004	A2pr(Nma)GADHAFPK(Dnp)rr	1539.61	1538.7127
A2pr(Nma)GADHAFPK(Dnp)	1227.24	1226.5105	EDHAFPK(Dnp)rr	1321.36	1320.5959	GFDHAFPK(Dnp)rr	1396.47	1395.6432	A2pr(Nma)GVPHAFPK(Dnp)rr	1549.69	1548.7698
A2pr(Nma)GVPHAFPK(Dnp)	1237.32	1236.5676	FPHAFPK(Dnp)rr	1321.45	1320.6476	A2pr(Nma)GAKHAFPK(Dnp)r	1396.51	1395.6796	A2pr(Nma)GAKHAFPK(Dnp)rr	1552.70	1551.7807
A2pr(Nma)GAKHAFPK(Dnp)	1240.33	1239.5785	A2pr(Nma)GRKHAFPK(Dnp)	1325.43	1324.6425	GVYHAFPK(Dnp)rr	1396.51	1395.6796	A2pr(Nma)GVIHAFPK(Dnp)rr	1565.74	1564.8011
YHAFPK(Dnp)rr	1240.33	1239.5897	GVPHAFPK(Dnp)rr	1330.45	1329.6691	RYHAFPK(Dnp)rr	1396.52	1395.6908	A2pr(Nma)GVDHAFPK(Dnp)rr	1567.67	1566.7440
APHAFPK(Dnp)rr	1245.35	1244.6163	RPHAFPK(Dnp)rr	1330.46	1329.6803	GRIHAFPK(Dnp)rr	1403.55	1402.7330	A2pr(Nma)GEPHAFPK(Dnp)rr	1579.68	1578.7440
A2pr(Nma)GVIHAFPK(Dnp)	1253.36	1252.5989	A2pr(Nma)GEYHAFPK(Dnp)	1333.36	1332.5523	GRDHAFPK(Dnp)rr	1405.48	1404.6759	A2pr(Nma)GVKHAFPK(Dnp)rr	1580.75	1579.8120
A2pr(Nma)GVDHAFPK(Dnp)	1255.29	1254.5418	GAKHAFPK(Dnp)rr	1333.46	1332.6800	A2pr(Nma)GVIHAFPK(Dnp)r	1409.55	1408.7000	A2pr(Nma)GAYHAFPK(Dnp)rr	1587.70	1586.7491
AIHAFPK(Dnp)rr	1261.39	1260.6476	EKHAFPK(Dnp)rr	1334.44	1333.6640	GFKHAFPK(Dnp)rr	1409.55	1408.7113	A2pr(Nma)GEIHAFPK(Dnp)rr	1595.72	1594.7753
ADHAFPK(Dnp)rr	1263.32	1262.5905	FIHAFPK(Dnp)rr	1337.49	1336.6789	A2pr(Nma)GVDHAFPK(Dnp)r	1411.48	1410.6429	A2pr(Nma)GEDHAFPK(Dnp)rr	1597.65	1596.7182
A2pr(Nma)GEPHAFPK(Dnp)	1267.31	1266.5418	FDHAFPK(Dnp)rr	1339.42	1338.6218	GRKHAFPK(Dnp)rr	1418.57	1417.7439	A2pr(Nma)GFPHAFPK(Dnp)rr	1597.74	1596.7698
A2pr(Nma)GVKHAFPK(Dnp)	1268.38	1267.6098	VYHAFPK(Dnp)rr	1339.46	1338.6582	A2pr(Nma)GEPHAFPK(Dnp)r	1423.49	1422.6429	A2pr(Nma)GRPHAFPK(Dnp)rr	1606.75	1605.8025
VPHAFPK(Dnp)rr	1273.40	1272.6476	GVIHAFPK(Dnp)rr	1346.50	1345.7004	A2pr(Nma)GVKHAFPK(Dnp)r	1424.57	1423.7109	A2pr(Nma)GEKHAFPK(Dnp)rr	1610.73	1609.7862
A2pr(Nma)GAYHAFPK(Dnp)	1275.33	1274.5469	RIHAFPK(Dnp)rr	1346.50	1345.7116	GEYHAFPK(Dnp)rr	1426.49	1425.6538	A2pr(Nma)GFIHAFPK(Dnp)rr	1613.78	1612.8011
AKHAFPK(Dnp)rr	1276.41	1275.6585	GVDHAFPK(Dnp)rr	1348.43	1347.6432	A2pr(Nma)GAYHAFPK(Dnp)r	1431.51	1430.6480	A2pr(Nma)GFDHAFPK(Dnp)rr	1615.71	1614.7440
A2pr(Nma)GEIHAFPK(Dnp)	1283.35	1282.5731	RDHAFPK(Dnp)rr	1348.43	1347.6545	A2pr(Nma)GEIHAFPK(Dnp)r	1439.53	1438.6742	A2pr(Nma)GVYHAFPK(Dnp)rr	1615.75	1614.7804
A2pr(Nma)GEDHAFPK(Dnp)	1285.28	1284.5160	A2pr(Nma)GFYHAFPK(Dnp)	1351.42	1350.5782	A2pr(Nma)GEDHAFPK(Dnp)r	1441.46	1440.6171	A2pr(Nma)GRIHAFPK(Dnp)rr	1622.79	1621.8338
A2pr(Nma)GFPHAFPK(Dnp)	1285.37	1284.5676	FKHAFPK(Dnp)rr	1352.50	1351.6898	A2pr(Nma)GFPHAFPK(Dnp)r	1441.55	1440.6687	A2pr(Nma)GRDHAFPK(Dnp)rr	1624.72	1623.7767
VIHAFPK(Dnp)rr	1289.44	1288.6789	A2pr(Nma)GRYHAFPK(Dnp)	1360.43	1359.6109	GFYHAFPK(Dnp)rr	1444.55	1443.6796	A2pr(Nma)GFKHAFPK(Dnp)rr	1628.79	1627.8120
VDHAFPK(Dnp)rr	1291.37	1290.6218	GEPHAFPK(Dnp)rr	1360.44	1359.6432	A2pr(Nma)GRPHAFPK(Dnp)r	1450.56	1449.7014	A2pr(Nma)GRKHAFPK(Dnp)rr	1637.81	1636.8447
A2pr(Nma)GRPHAFPK(Dnp)	1294.38	1293.6003	GVKHAFPK(Dnp)rr	1361.51	1360.7113	GRYHAFPK(Dnp)rr	1453.57	1452.7123	A2pr(Nma)GEYHAFPK(Dnp)rr	1645.73	1644.7546
A2pr(Nma)GEKHAFPK(Dnp)	1298.36	1297.5840	RKHAFPK(Dnp)rr	1361.51	1360.7225	A2pr(Nma)GEKHAFPK(Dnp)r	1454.55	1453.6851	A2pr(Nma)GFYHAFPK(Dnp)rr	1663.79	1662.7804
A2pr(Nma)GFIHAFPK(Dnp)	1301.41	1300.5989	A2pr(Nma)GAPHAFPK(Dnp)r	1365.45	1364.6374	A2pr(Nma)GFIHAFPK(Dnp)r	1457.59	1456.7000	A2pr(Nma)GRYHAFPK(Dnp)rr	1672.81	1671.8131
GAPHAFPK(Dnp)rr	1302.40	1301.6378	GAYHAFPK(Dnp)rr	1368.46	1367.6483	A2pr(Nma)GFDHAFPK(Dnp)r	1459.52	1458.6429			
A2pr(Nma)GFDHAFPK(Dnp)	1303.34	1302.5418	EYHAFPK(Dnp)rr	1369.44	1368.6323	A2pr(Nma)GVYHAFPK(Dnp)r	1459.57	1458.6793			
A2pr(Nma)GVYHAFPK(Dnp)	1303.38	1302.5782	GEIHAFPK(Dnp)rr	1376.48	1375.6745	A2pr(Nma)GRIHAFPK(Dnp)r	1466.61	1465.7327			
EPHAFPK(Dnp)rr	1303.39	1302.6218	GEDHAFPK(Dnp)rr	1378.41	1377.6174	A2pr(Nma)GRDHAFPK(Dnp)r	1468.53	1467.6756			
VKHAFPK(Dnp)rr	1304.46	1303.6898	GFPHAFPK(Dnp)rr	1378.50	1377.6691	A2pr(Nma)GFKHAFPK(Dnp)r	1472.61	1471.7109			