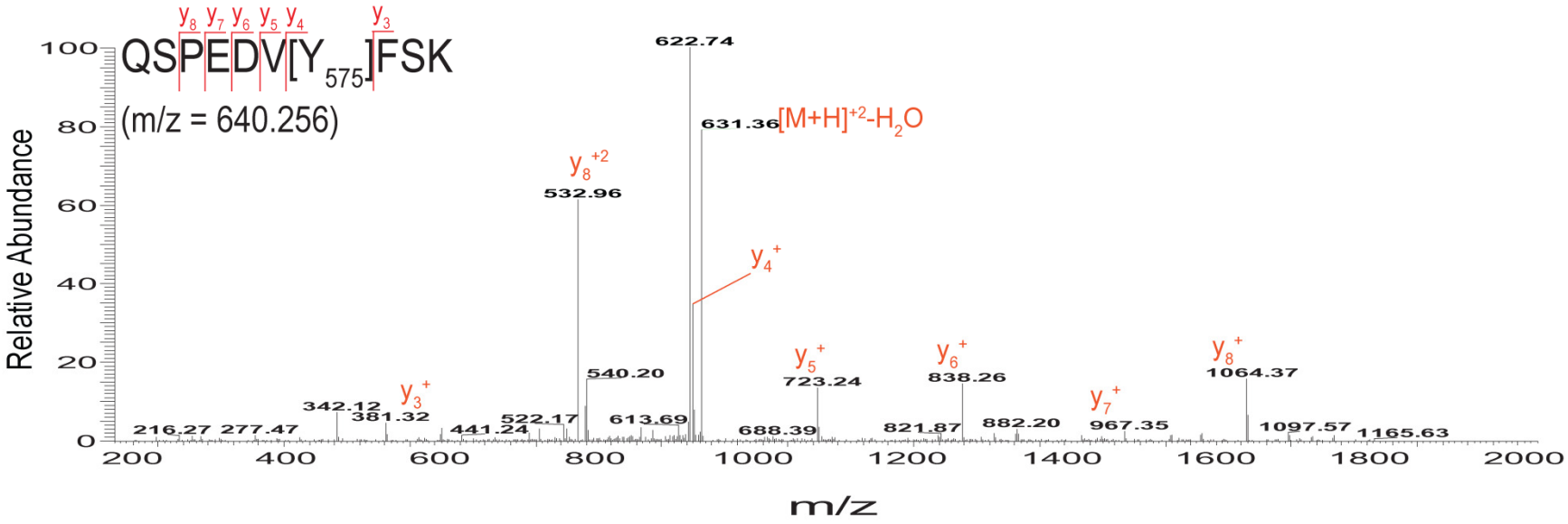
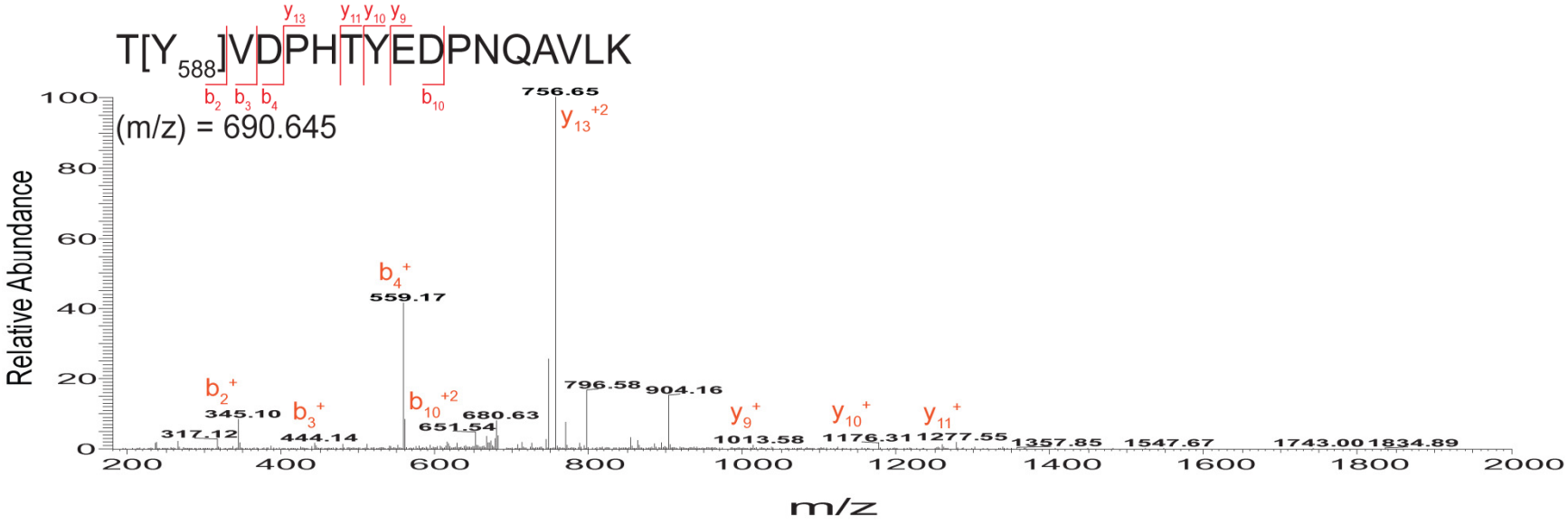
**Table S1:** SRM parameters for the peptides monitored from EphA2 C0.

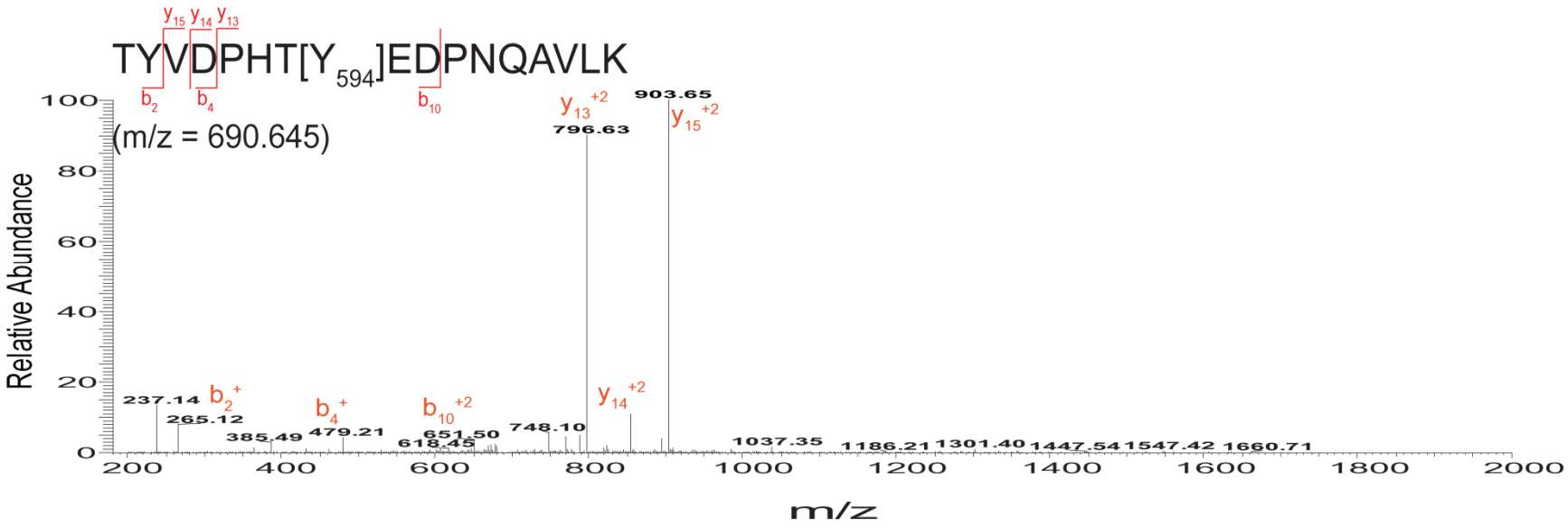
| Tyrosine monitored | [a]Sequence and charge | [c]Precursor ion (m/z) | Fragment | | Fragment (m/z) | | Collision Energy (V) | |
|---|---|---|---|---|---|---|---|---|
| | | | [d]1 | 2 | 1 | 2 | 1 | 2 |
| pY575 | QSPEDV[$Y_{575}$]FSK $^{2+}$ | 640. 3 | $y4^{1+}$ | $y8^{1+}$ | 624.2 | 1064.4 | 15 | 15 |
| Y575 | QSPEDV$Y_{575}$FSK$^{2+}$ | 600.4 | $y4^{1+}$ | $y8^{1+}$ | 544.4 | 983.8 | 12 | 15 |
| ppY588Y594 | T[$Y_{588}$]VDPHT[$Y_{594}$]EDPNQAVLK $^{3+}$ | 717.5 | $b4^{1+}$ | $y13^{2}$ | 559.4 | 796.1 | 15 | 15 |
| pY588Y594 | T[$Y_{588}$]VDPHTY$_{594}$EDPNQAVLK $^{3+}$ | 690.8 | $b4^{1+}$ | $y13^{2}$ | 559.4 | 756.4 | 15 | 15 |
| Y588pY594 | TY$_{588}$VDPHT[$Y_{594}$]EDPNQAVLK $^{3+}$ | 690.8 | $y13^{2+}$ | $y15^{2+}$ | 796.2 | 903.8 | 20 | 15 |
| Y588Y594 | TY$_{588}$VDPHTY$_{594}$EDPNQAVLK $^{3+}$ | 664.2 | $y13^{2+}$ | $y15^{2+}$ | 756.4 | 863.2 | 18 | 15 |
| pY772 | VLEDDPEAT[$Y_{772}$]TTSGGK $^{2+}$ | 882.0 | $y9^{1+}$ | $y11^{1+}$ | 965.0 | 1191.2 | 30 | 32 |
| Y772 | VLEDDPEATY$_{772}$TTSGGK $^{2+}$ | 842.0 | $y9^{1+}$ | $y11^{1+}$ | 885.2 | 1111.2 | 28 | 28 |
| pY960 | IA[$Y_{960}$]SLLGLK $^{2+}$ | 529.2 | $y7^{1+}$ | $y8^{1+}$ | 873.4 | 944.4 | 10 | 10 |
| Y960 | IAY$_{960}$SLLGLK $^{2+}$ | 489.4 | $y7^{1+}$ | $y8^{1+}$ | 793.4 | 864.4 | 10 | 10 |
| [b]STD1 | TLADFDPR$^{2+}$ | 467.8 | $y5^{1+}$ | $y6^{1+}$ | 649.3 | 720.5 | 10 | 10 |
| [b]STD2 | VSDFGLSR$^{2+}$ | 440.7 | $y5^{1+}$ | $y7^{1+}$ | 579.3 | 781.6 | 20 | 20 |
| [b]STD3 | LPSTSGSEGVPFR$^{2+}$ | 667.7 | $y9^{1+}$ | $y12^{2+}$ | 935.6 | 611.0 | 25 | 25 |

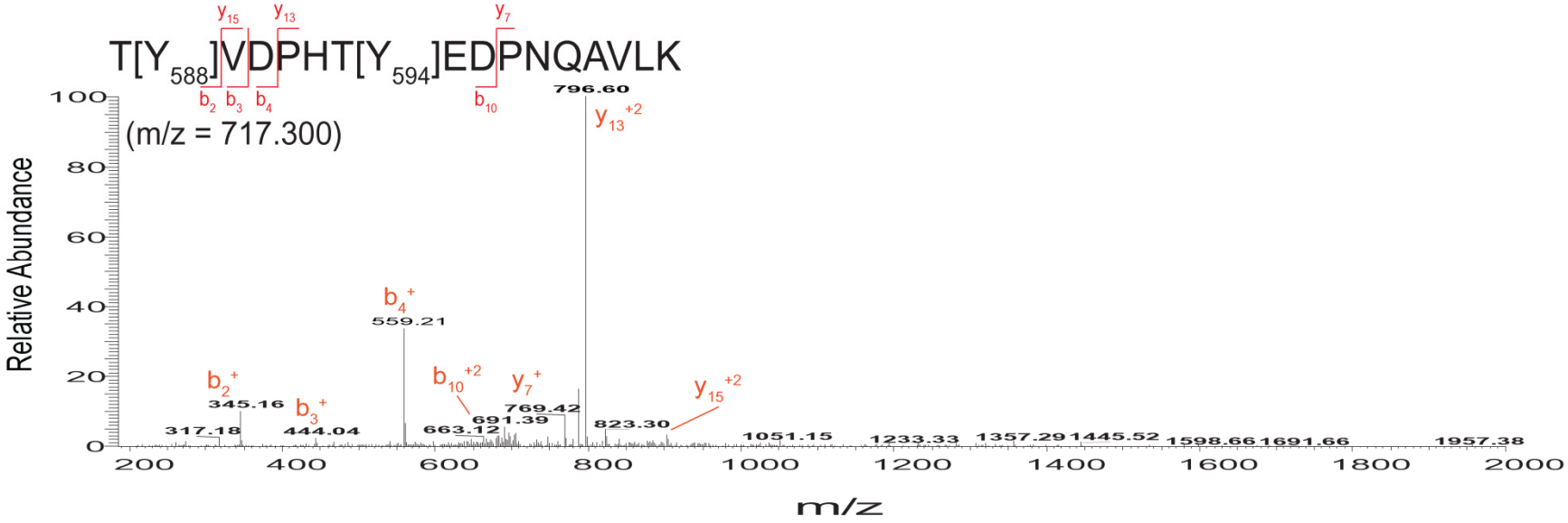
a) Phosphotyrosines monitored are within square brackets and numbered according to their position on EphA2 amino acid sequence.
b) STD represents standard peptides used for normalization.
c) Dwell time for each transition was 20 ms for all peptides.
d) Two transitions were monitored for all peptides to ensure specificity and are indicated by the column headings “1” and “2.

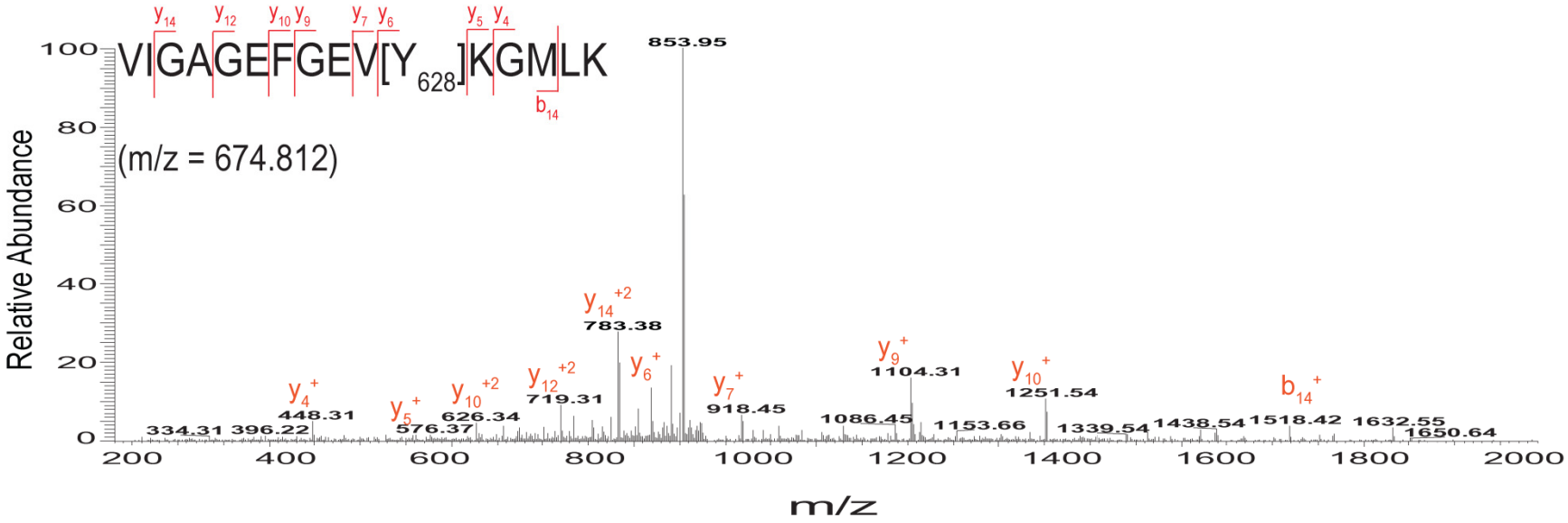
**Figure S1.** Product ion (PI) scans of autophosphorylated tyrosines identified on EphA2 C0. PI scans were acquired on a Thermo Scientific LTQ-Orbitrap coupled to an Aglilent 1100 HPLC. The peptide sequence is indicated above the spectrum and phosphorylated residues are shown in parentheses. Only y and b series ions are labeled. Phosphorylated tyrosines with residue numbers are indicated by brackets.

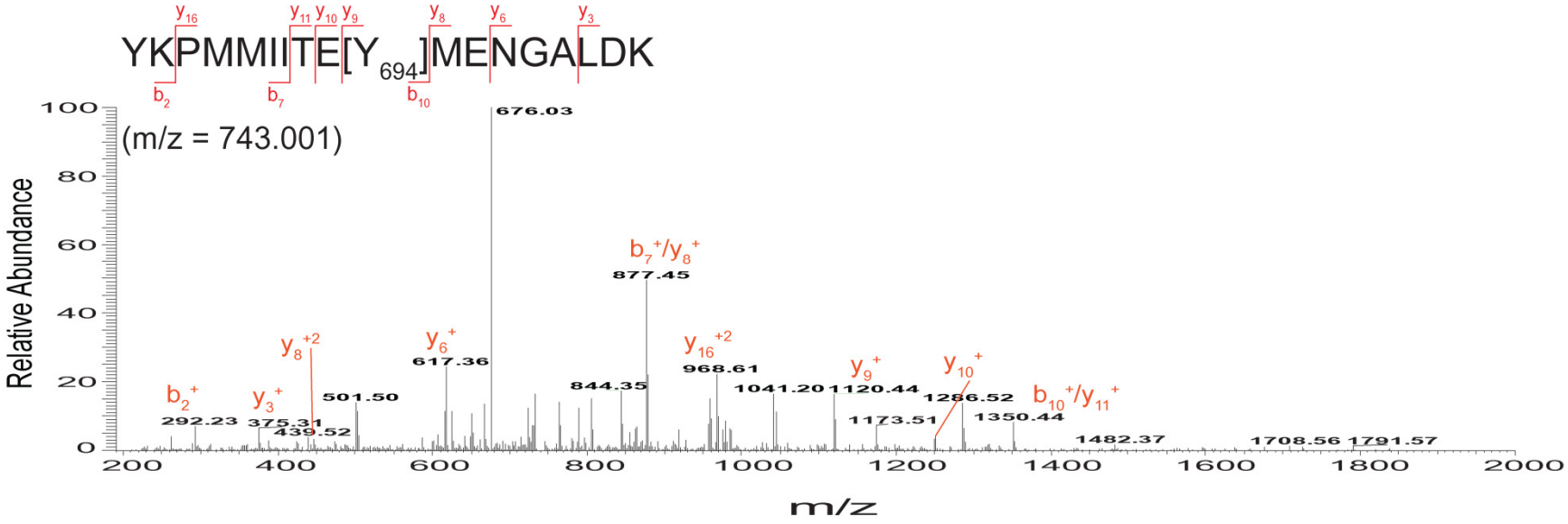

QSPEDV[Y575]FSK
(m/z = 640.256)
Relative Abundance
m/z
622.74
631.36
[M+H]+2-H2O
532.96
540.20
723.24
838.26
1064.37
342.12
381.32
216.27
277.47
441.24
522.17
613.69
688.39
821.87
882.20
967.35
1097.57
1165.63

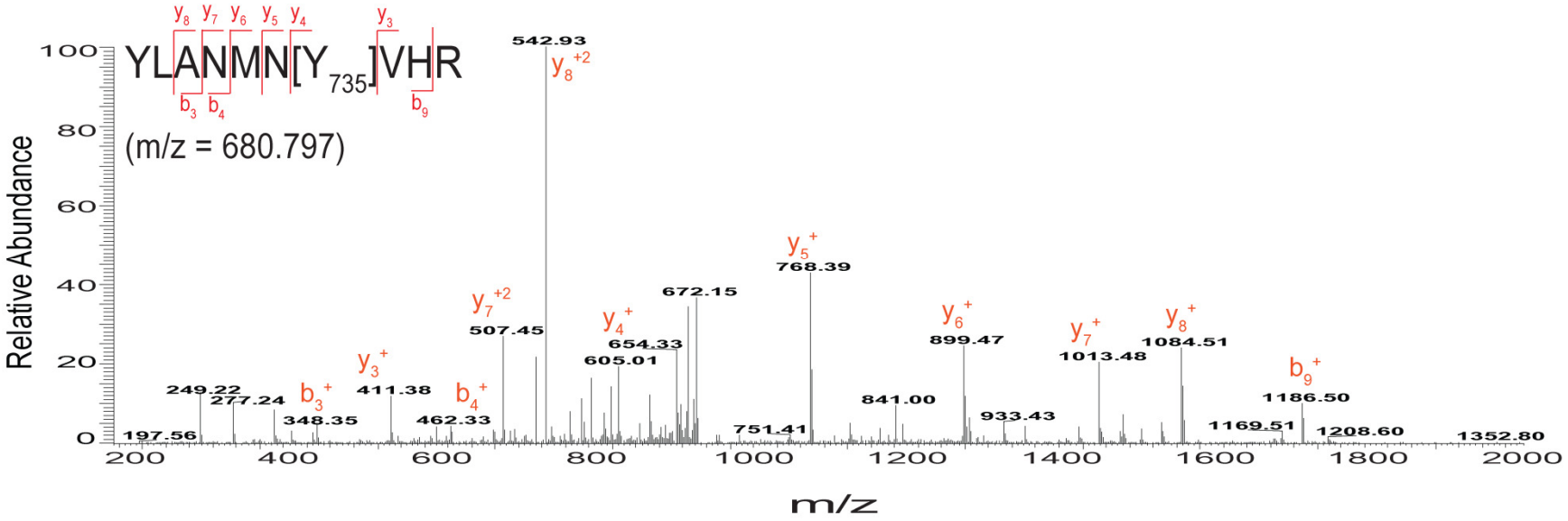

T[Y588]VDPHTYEDPNQAVLK
(m/z) = 690.645
Relative Abundance
m/z
756.65
559.17
345.10
317.12
444.14
651.54
680.63
796.58
904.16
1013.58
1176.31
1277.55
1357.85
1547.67
1743.00
1834.89

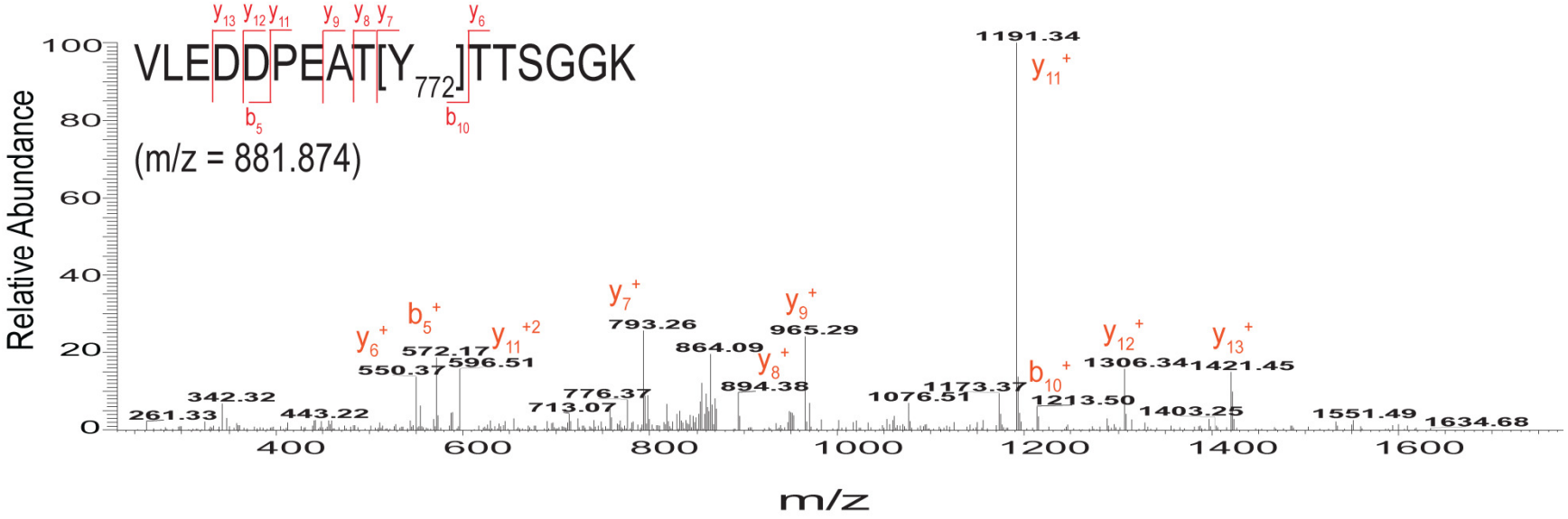

TYVDPHT[Y594]EDPNQAVLK
(m/z = 690.645)
Relative Abundance
m/z
796.63
903.65
237.14
265.12
385.49
479.21
618.45
651.50
748.10
1037.35
1186.21
1301.40
1447.54
1547.42
1660.71

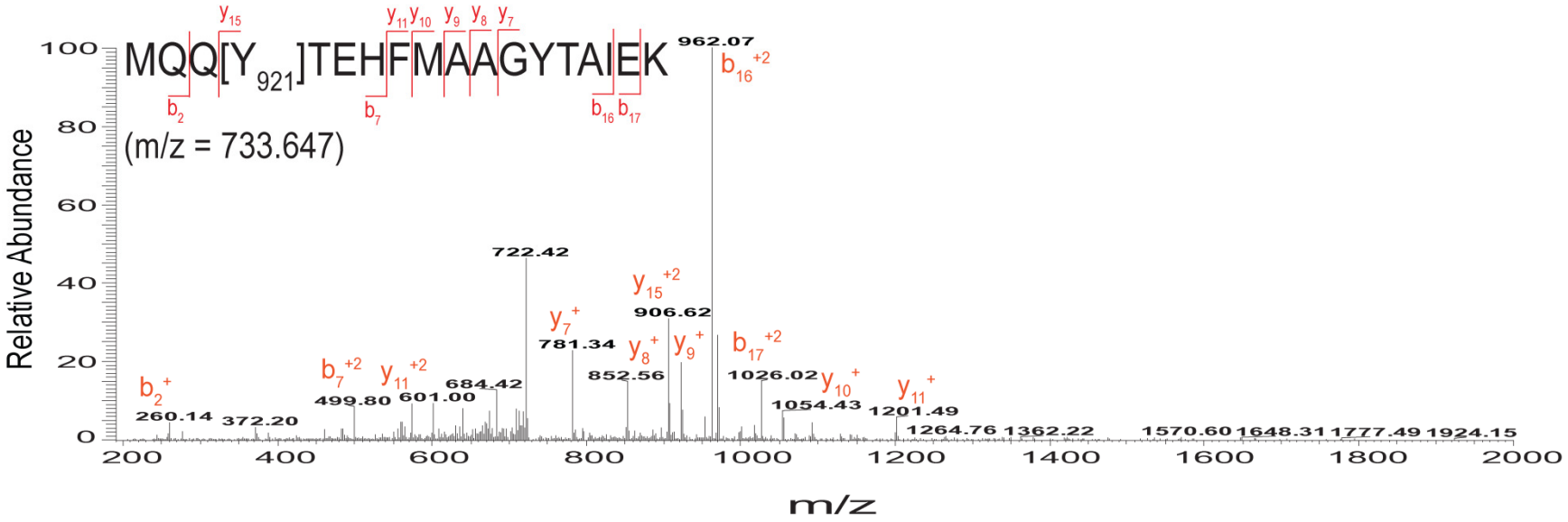

T[Y588]VDPHT[Y594]EDPNQAVLK
(m/z = 717.300)
Relative Abundance
m/z
796.60
559.21
345.16
317.18
444.04
663.12
691.39
769.42
823.30
1051.15
1233.33
1357.29
1445.52
1598.66
1691.66
1957.38


VIGAGEFGEV[Y628]KGMLK
(m/z = 674.812)
Relative Abundance
m/z
853.95
783.38
448.31
626.34
576.37
719.31
918.45
1104.31
1251.54
1086.45
1153.66
1339.54
1438.54
1518.42
1632.55
1650.64
334.31
396.22


YKPMMIITE[Y694]MENGALDK
(m/z = 743.001)
Relative Abundance
m/z
676.03
877.45
617.36
968.61
844.35
1041.20
1120.44
1286.52
1173.51
1350.44
1482.37
1708.56
1791.57
501.50
439.52
375.31
292.23


YLANMN[Y735]VHR
(m/z = 680.797)
Relative Abundance
m/z
542.93
768.39
672.15
507.45
654.33
605.01
899.47
1084.51
1013.48
1186.50
411.38
249.22
277.24
348.35
462.33
841.00
933.43
751.41
1169.51
1208.60
1352.80
197.56


VLEDDPEAT[Y772]TTSGGK
(m/z = 881.874)
Relative Abundance
m/z
1191.34
793.26
965.29
572.17
596.51
550.37
864.09
894.38
1306.34
1421.45
1213.50
1173.37
1076.51
776.37
713.07
342.32
261.33
443.22
1403.25
1551.49
1634.68


MQQ[Y921]TEHFMAAGYTAIEK
(m/z = 733.647)
Relative Abundance
m/z
962.07
722.42
906.62
781.34
852.56
1026.02
1054.43
1201.49
499.80
601.00
684.42
260.14
372.20
1264.76
1362.22
1570.60
1648.31
1777.49
1924.15

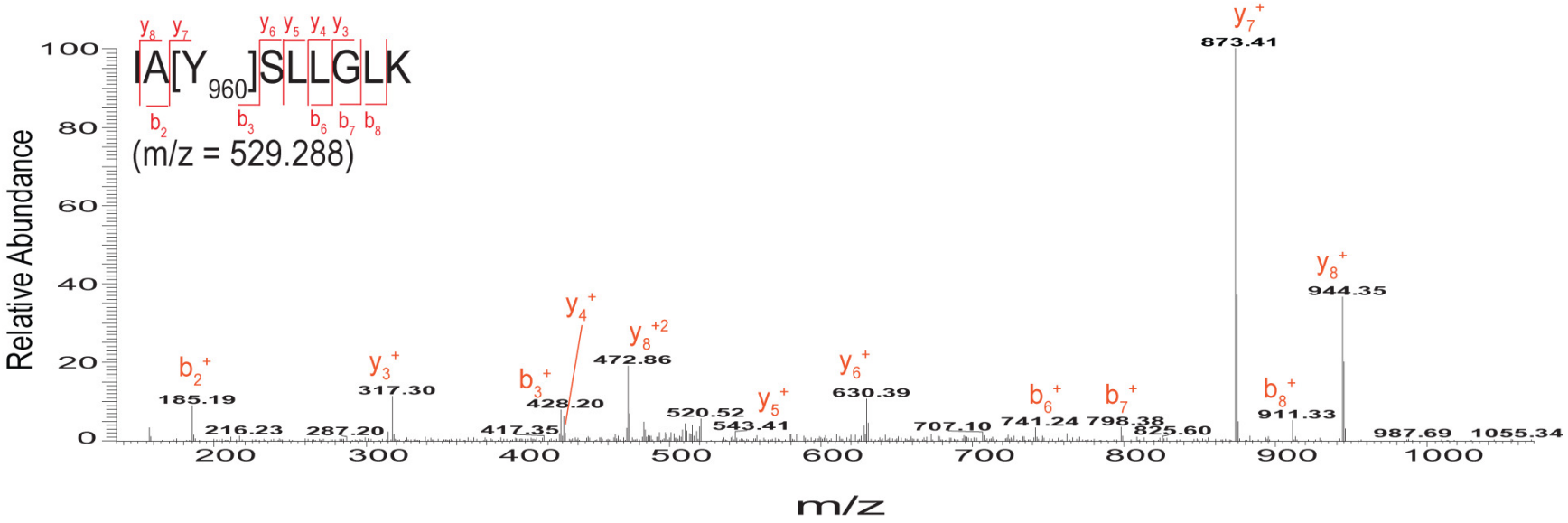
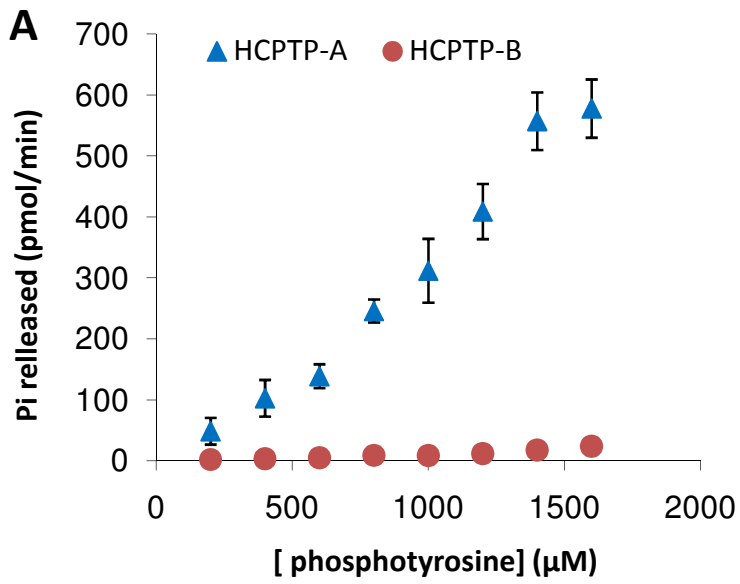

IA[Y960]SLLGLK
(m/z = 529.288)
Relative Abundance
m/z
873.41
944.35
472.86
317.30
428.20
630.39
185.19
216.23
287.20
417.35
520.52
543.41
707.10
741.24
798.38
825.60
911.33
987.69
1055.34

**Figure S2:** Dephosphorylation of phosphotyrosine and EphA2 phosphopeptides VLEDDPEAT(pY$_{772}$)TTSGGK and VRLPGHQKRIA(pY$_{960}$)SLLGLKDQ by HCPTP variants. A shows the rate of dephosphorylation of phosphotyrosine and B shows the rate of dephosphorylation of the pY772 and pY960 peptides. Phosphate release was monitored at $A_{630}$ using the malachite green reagent.



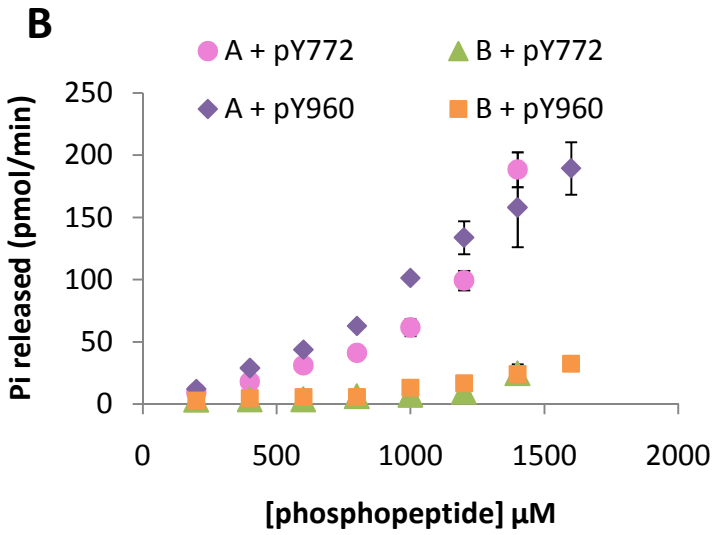



**Figure S3:** Comparison of selected ion monitoring (SIM) vs. selected reaction monitoring (SRM) to follow dephosphorylation on specific tyrosines. % phosphorylation remaining at various time points for 3 phosphotyrosines in EphA2 C0 treated with HCPTP-B by SIM and SRM are plotted. pY772 and pY575 can be monitored by either method. However monitoring pY960 by SIM does not reflect the true dephosphorylation rate as another peptide with similar m/z coelutes and masks the pY960 signal.

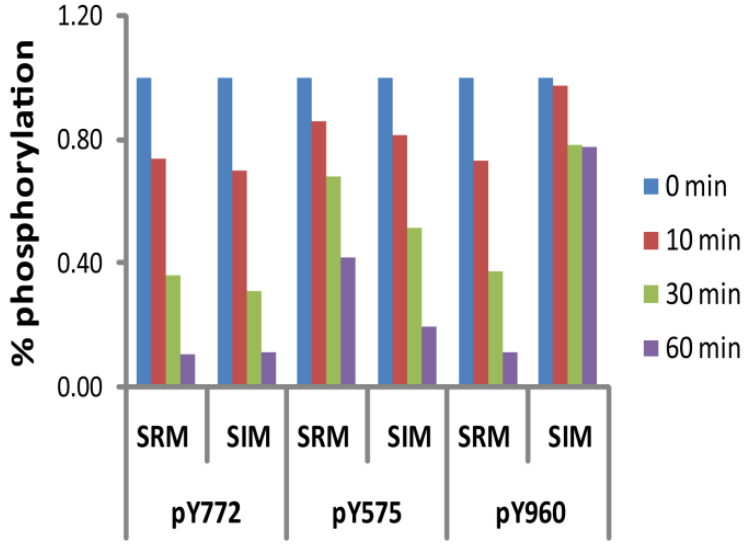
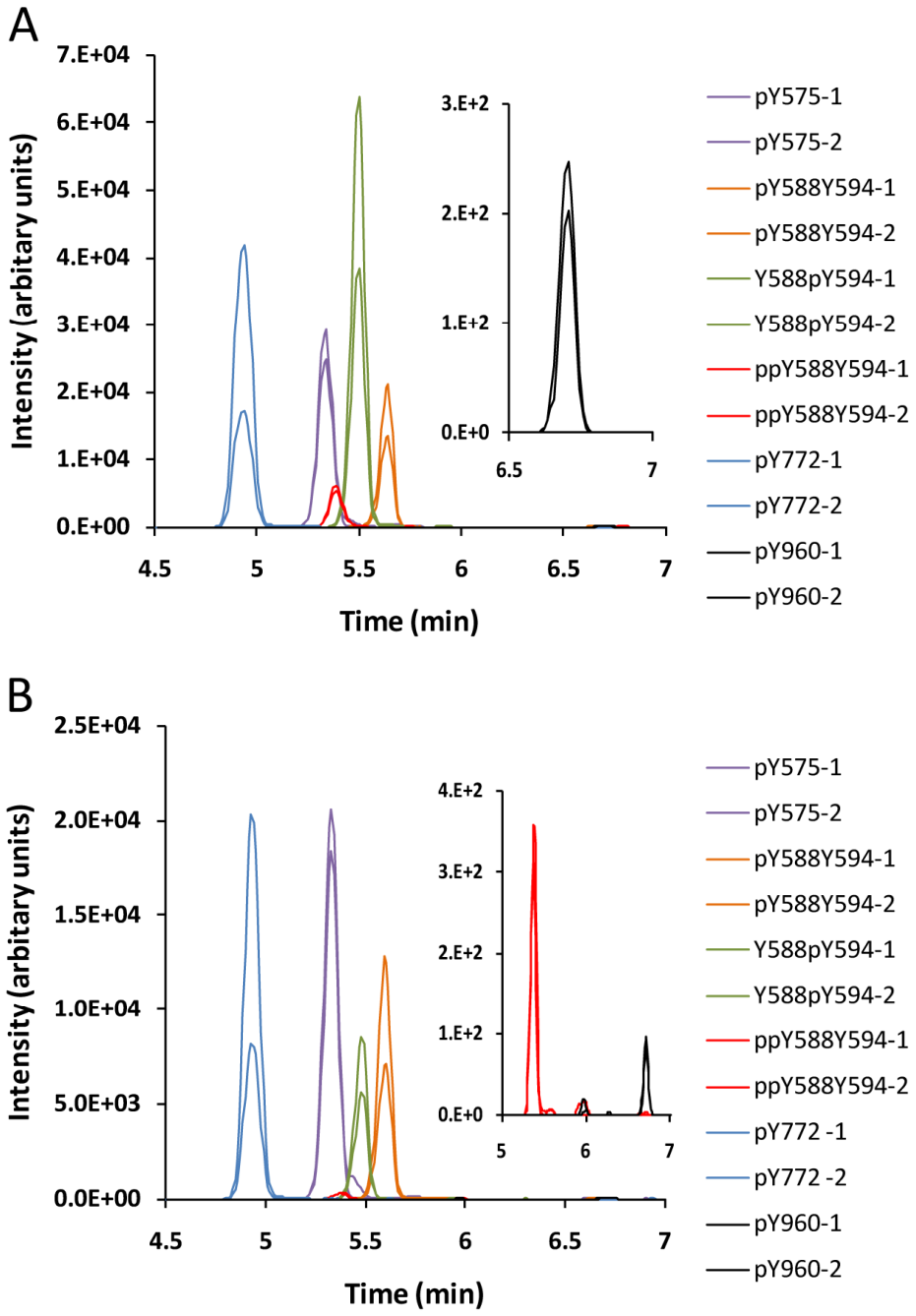
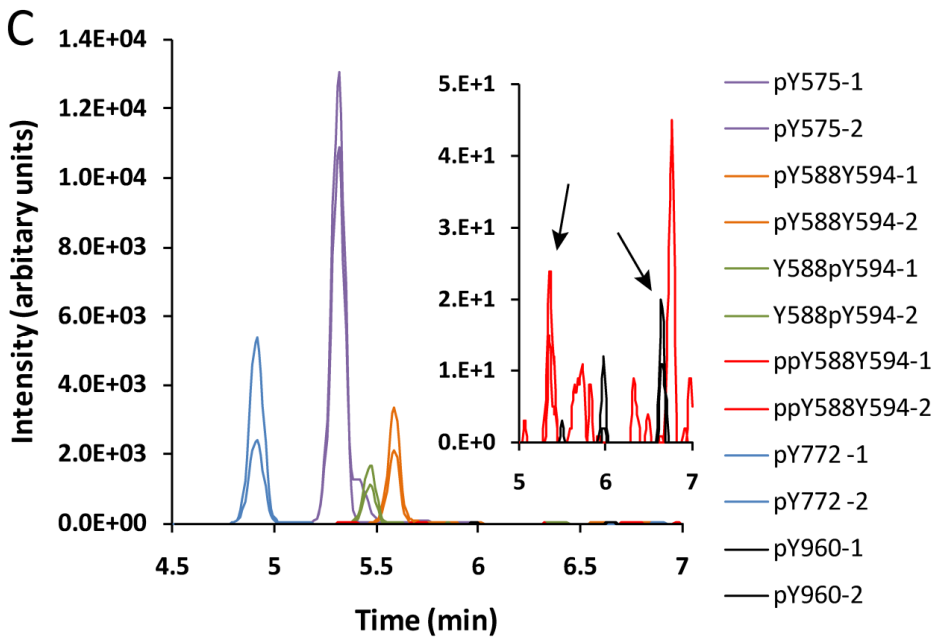


**Figure S4.** SRM traces for the phosphorylated and standard peptides from one dephosphorylation time point study. A, B and C show respectively the SRM chromatographic trace for transition1 (T1) and transition2 (T2) for the 0 min, 30 min and 60 min time points for all phosphorylated tyrosines monitored. The inset shows peaks that are too small for the scale of the figure. Arrows in part C point to the coeluting peaks that correspond to the peptide of interest. The peaks can be clearly detected at the 60 min time point even when there is a small degree of noise in the data for low abundance peaks. D. Shows the SRM traces for the standard peptides monitored for the above three time points. The variation in the area of the peak measured among the standard peptides for all the time points monitored for the run is 7.2 %.




C
Intensity (arbitary units)
1.4E+04
1.2E+04
1.0E+04
8.0E+03
6.0E+03
4.0E+03
2.0E+03
0.0E+00
4.5
5
5.5
6
6.5
7
Time (min)
5.E+1
4.E+1
3.E+1
2.E+1
1.E+1
0.E+0
5
6
7
pY575-1
pY575-2
pY588Y594-1
pY588Y594-2
Y588pY594-1
Y588pY594-2
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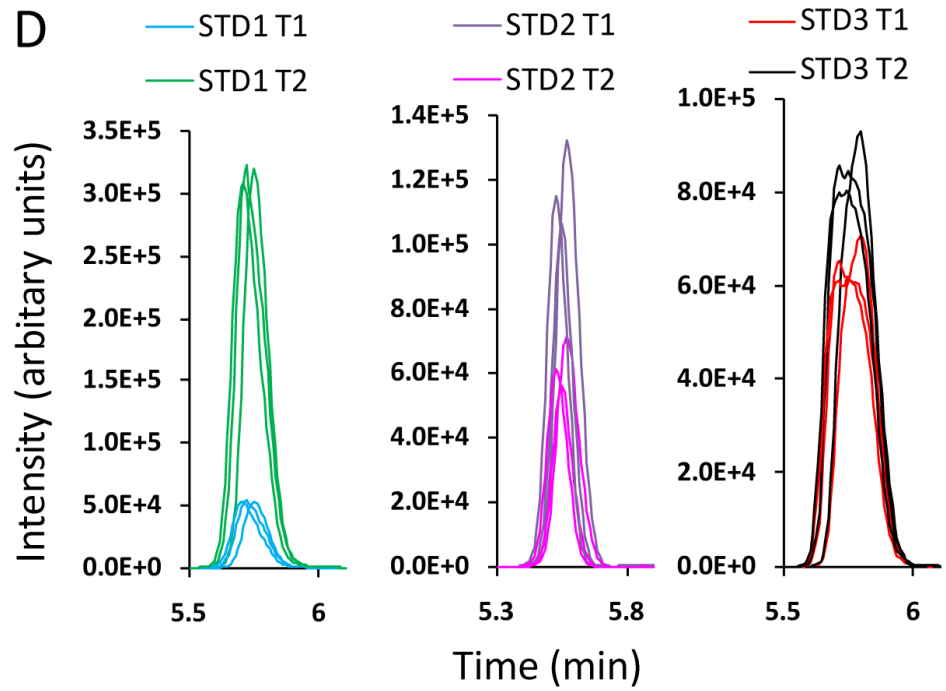
pY772 -1
pY772 -2
pY960-1
pY960-2


D
STD1 T1
STD1 T2
STD2 T1
STD2 T2
STD3 T1
STD3 T2
Intensity (arbitary units)
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3.0E+5
2.5E+5
2.0E+5
1.5E+5
1.0E+5
5.0E+4
0.0E+0
5.5
6
1.4E+5
1.2E+5
1.0E+5
8.0E+4
6.0E+4
4.0E+4
2.0E+4
0.0E+0
5.3
5.8
1.0E+5
8.0E+4
6.0E+4
4.0E+4
2.0E+4
0.0E+0
5.5
6
Time (min)

**Figure S5.** Rates of dephosphorylation and $R^2$ (correlation coefficient) values obtained by fitting of an exponential rate equation to the SRM dephosphorylation plots for all tyrosines monitored are shown. The same data in Figures 2 and 3 are plotted here as amount of phosphotyrosine (nmol) vs. time for each individual tyrosine monitored. S3A shows the decrease in phosphotyrosine content for the 5 tyrosines monitored on EphA2 when treated with HCPTP-A. S3B shows the decrease in phosphotyrosine content for all 5 tyrosines monitored on EphA2 when treated with HCPTP-B. The concentration of the substrate (nmol) was determined from the stoichiometry calculations. All data are the average of 3 independent experiments and the error bars represent the standard deviation.