Supplemental Information to

Label-Free Quantitation of Protein Modifications by Pseudo-Selected Reaction Monitoring with Internal Reference Peptides

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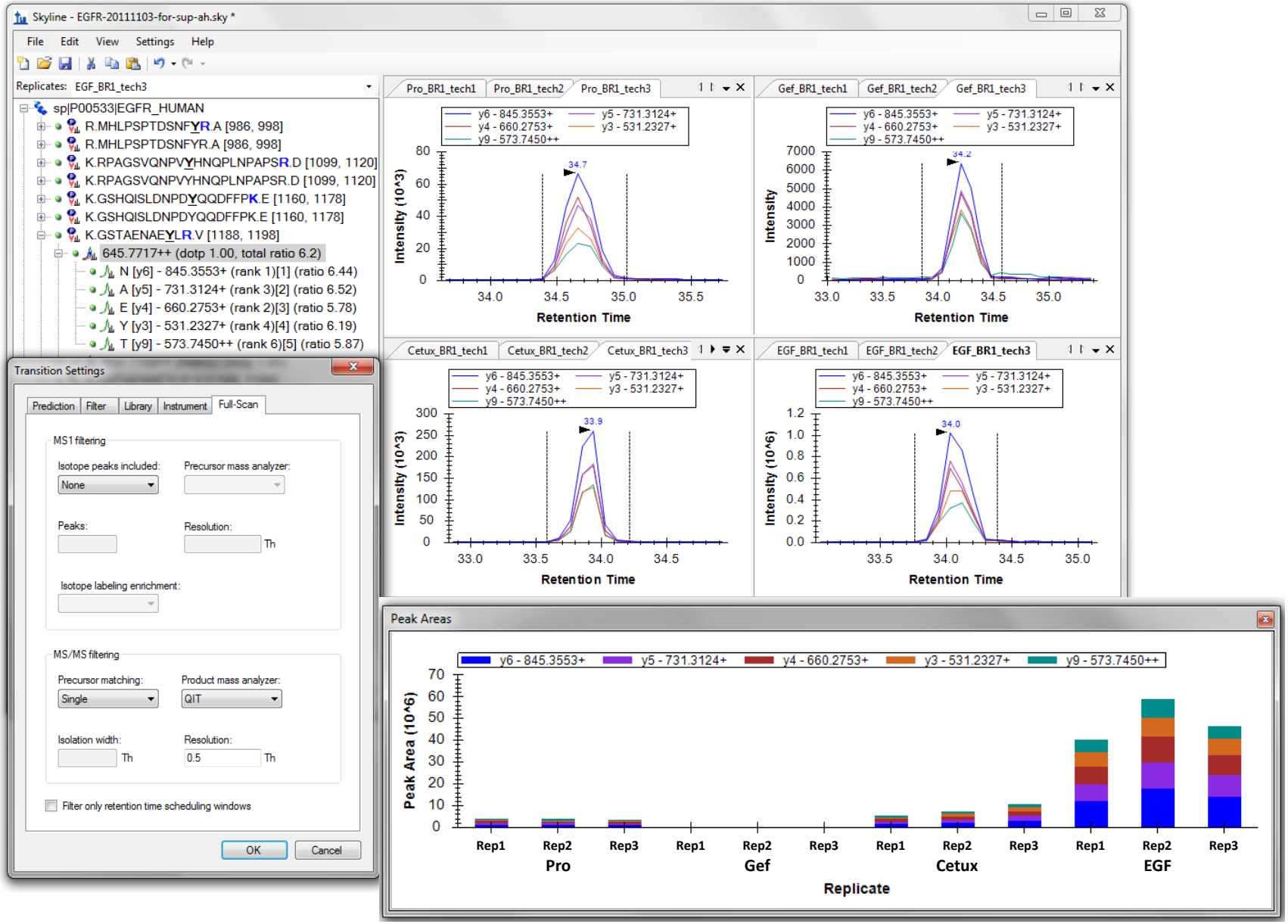
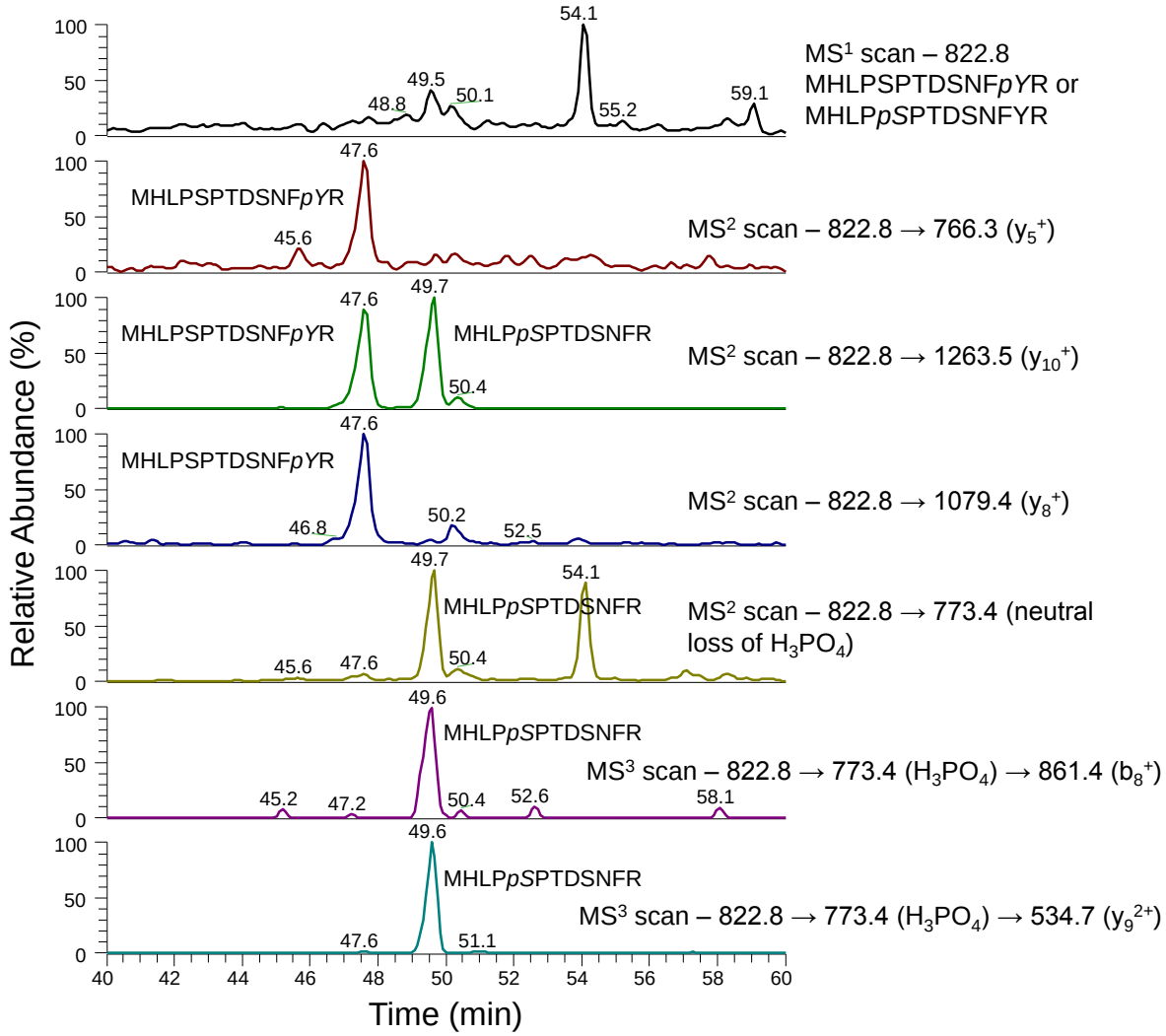
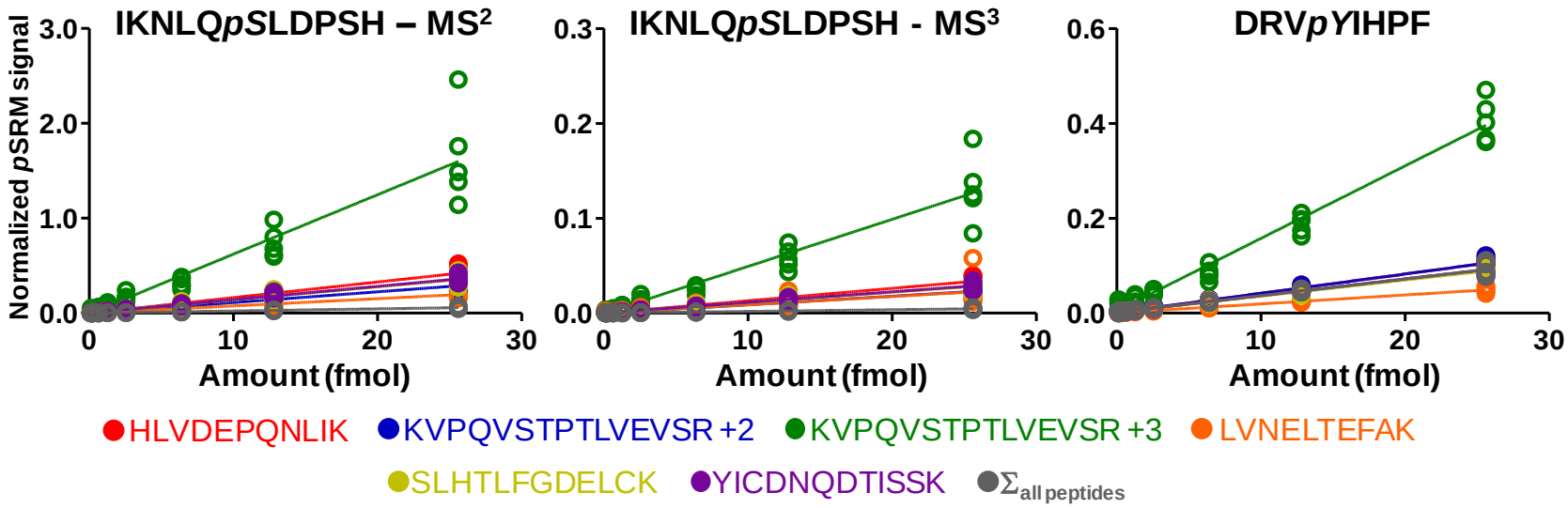


Supplemental figure S1. Skyline MS/MS settings, replicate peak areas and imported data from a targeted MS/MS from EGFR samples run on a Thermo Fisher LTQ-Velos, low resolution instrument. The differentially colored chromatographic traces are for the individual pSRM extracted transitions from the targeted MS/MS scans.



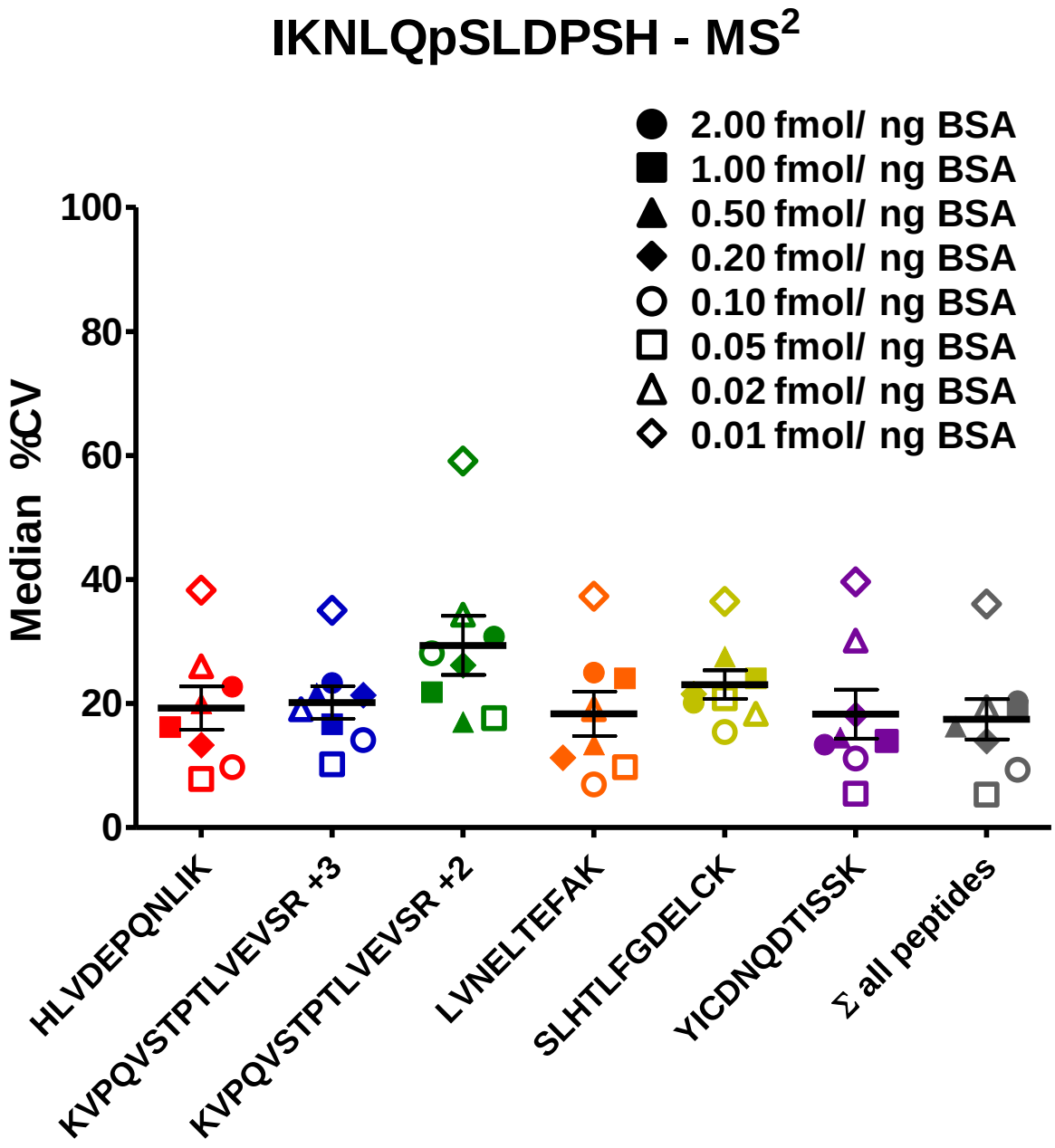
Supplemental figure S2. Extracted ion chromatograms for MHLPSPTDSNF*pY*R and MHLP*pS*PTDSNFYR using Xcalibur software (ThermoFisher, San Jose, CA). These peptides are chromatographically separated from each other and sequence specific ions used to determine which peak corresponds to pY and pS peptide.



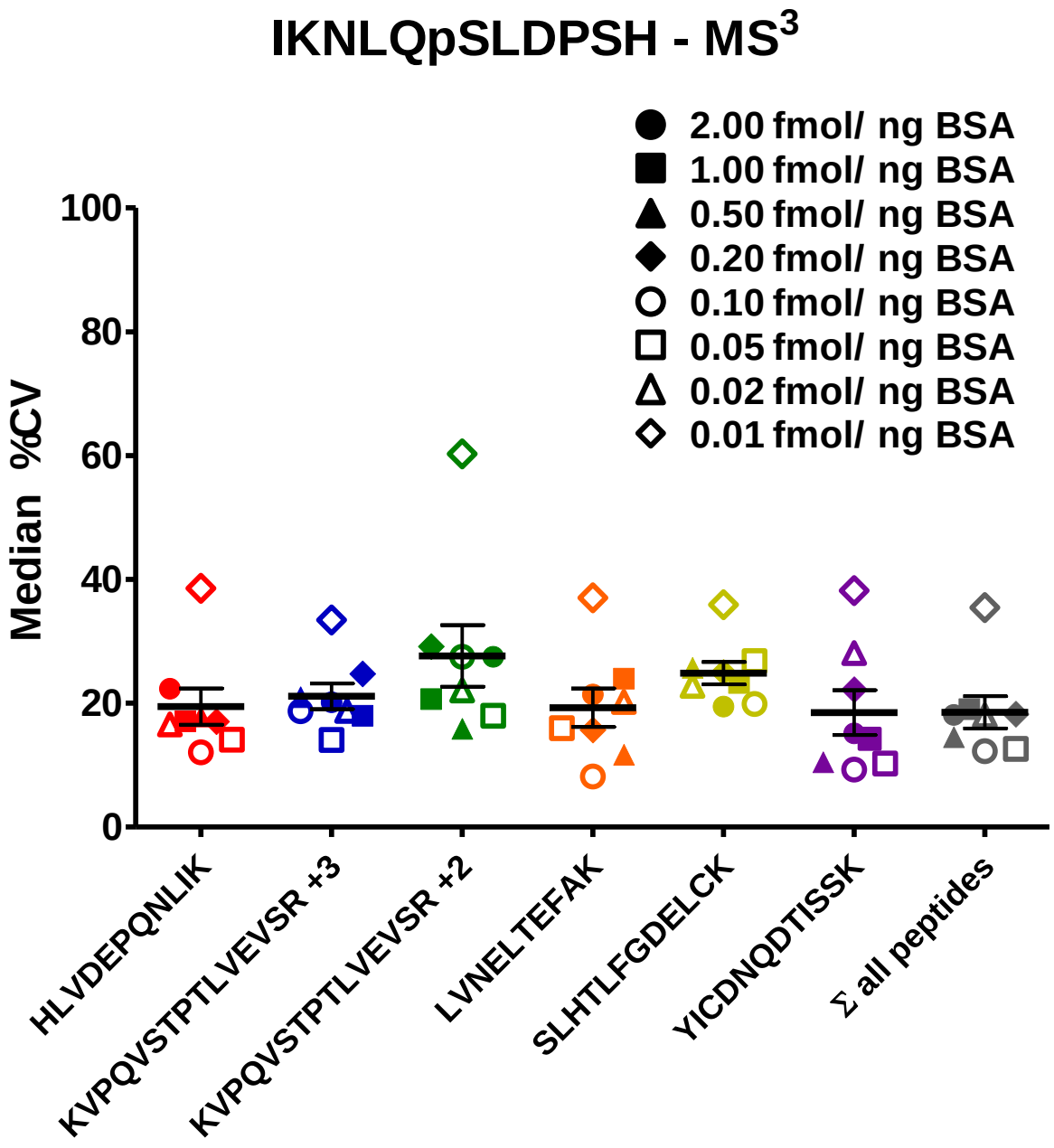
Supplemental figure S3. Standard curves for phosphopeptides spiked at increasing concentrations into BSA digest (five technical replicates). Normalized pSRM signal is calculated by dividing the phosphopeptide peak area (sum of 3-4 transitions) by the BSA reference peptide peak area (sum of 3 transitions). Color denotes different reference peptides used for normalization.

Peptide	Precursor *m/z*	Product *m/z*
DRVpYIHPF	563.8	432.7 (b_6^{2+}), 506.3 (y_7^{2+}), 864.5 (b_6)
IKNLQpSLDPSH +3	444.5	340.2 (y_3), 455.2 (y_4), 496.8 (b_8^{2+}), 411.9 $(M+3H)^{3+}$-H_3PO_4
IKNLQpSLDPSH +3 - MS^3	444.5 → 411.9	340.2 (y_3), 455.2 (y_4), 447.8 (b_8^{2+})
HLVDEPQNLIK	**653.4**	**712.4 (y_6), 956.5(y_8), 1055.6 (y_9)**
KVPQVSTPTLVEVSR +3	**547.3**	**450.8 (y_8^{2+}), 575.8 (b_{11}^{2+}), 740.4 (b_7)**
KVPQVSTPTLVEVSR +2	**820.5**	**706.9 (y_{13}^{2+}), 900.5(y_8), 1088.6(y_{10})**
LVNELTEFAK	**582.3**	**595.3 (y_5), 708.3 (y_6), 951.5 (y_8)**
SLHTLFGDELCK	**473.9**	**420.2 (y3), 699.4 (b_6), 721.3 (y_6)**
YICDNQDTISSK	**722.3**	**584.3 (y_{10}^{2+}), 1007.5 (y_9), 1167.5 (y_{10})**

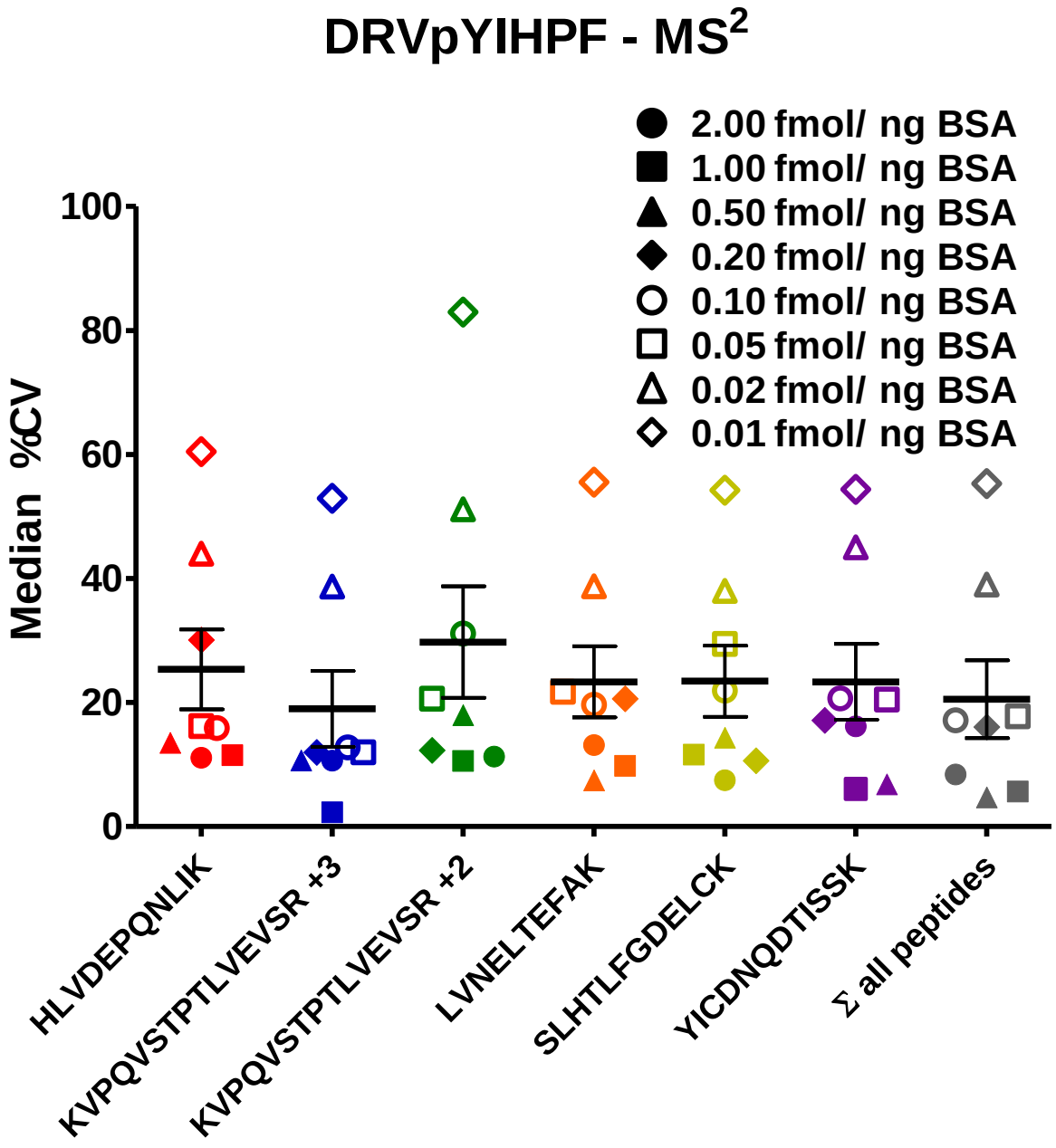
Supplemental table S4. Bovine serum albumin and spiked-in phosphorylated peptides and transitions selected for LC-pSRM-MS



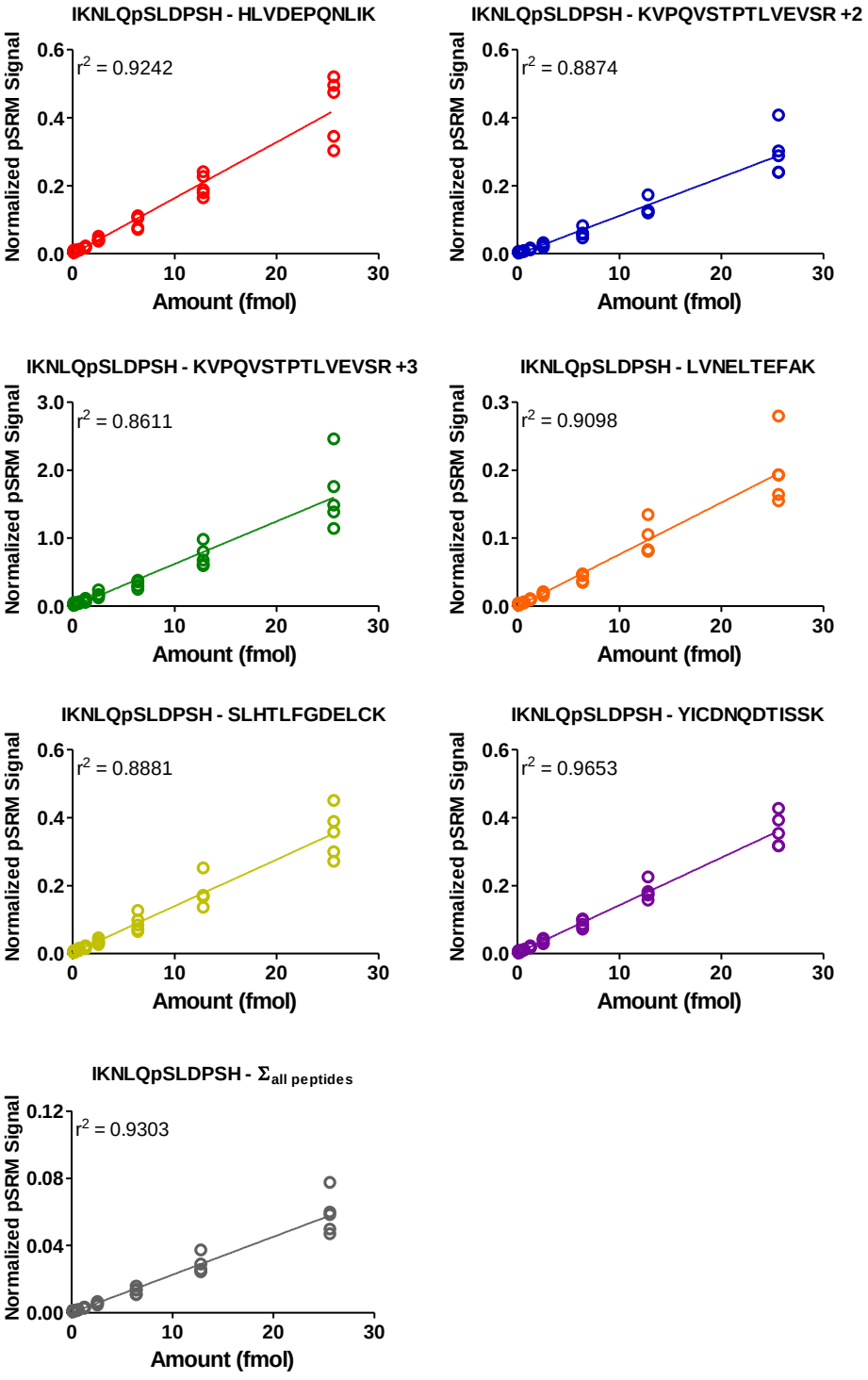
Supplemental figure S5. A plot of median CV (from five technical replicates) for phosphopeptide IKNLQ*p*SLDPSH – MS/MS shows the difference in median CV for each BSA normalizing peptide. For each of the normalizing peptides, the highest CV (open diamonds) correlates to the lowest phosphopeptide spike-in amount, 0.01 fmol ng BSA^{-1} which corresponds to 0.128 fmol on column.



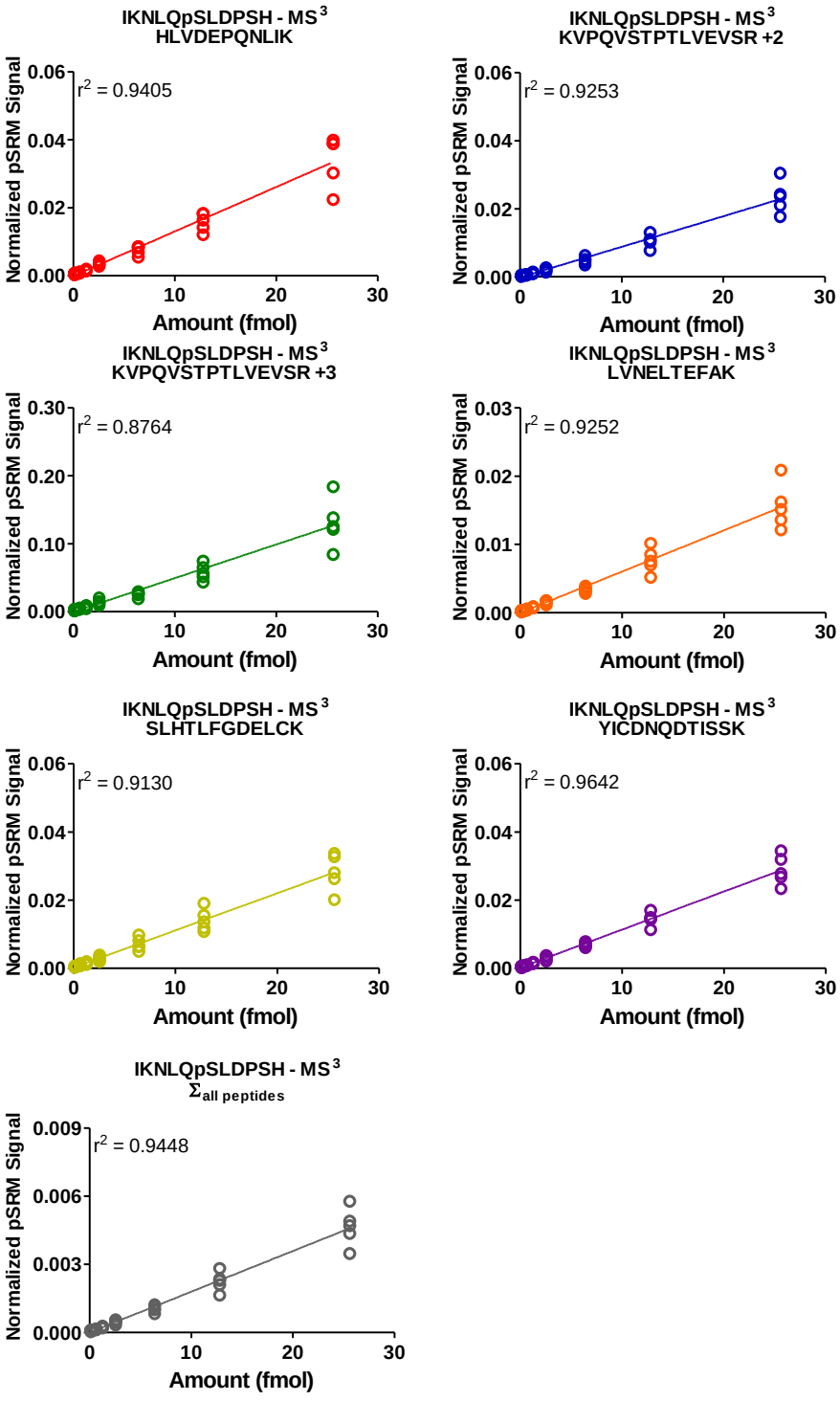
Supplemental figure S6. A plot of median CV (from five technical replicates) for phosphopeptide IKNLQ*p*SLDPSH – MS^3 shows the difference in median CV for each BSA normalizing peptide. For each of the normalizing peptides, the highest CV (open diamonds) correlates to the lowest phosphopeptide spike-in amount, 0.01 fmol ng BSA^{-1} which corresponds to 0.128 fmol on column.



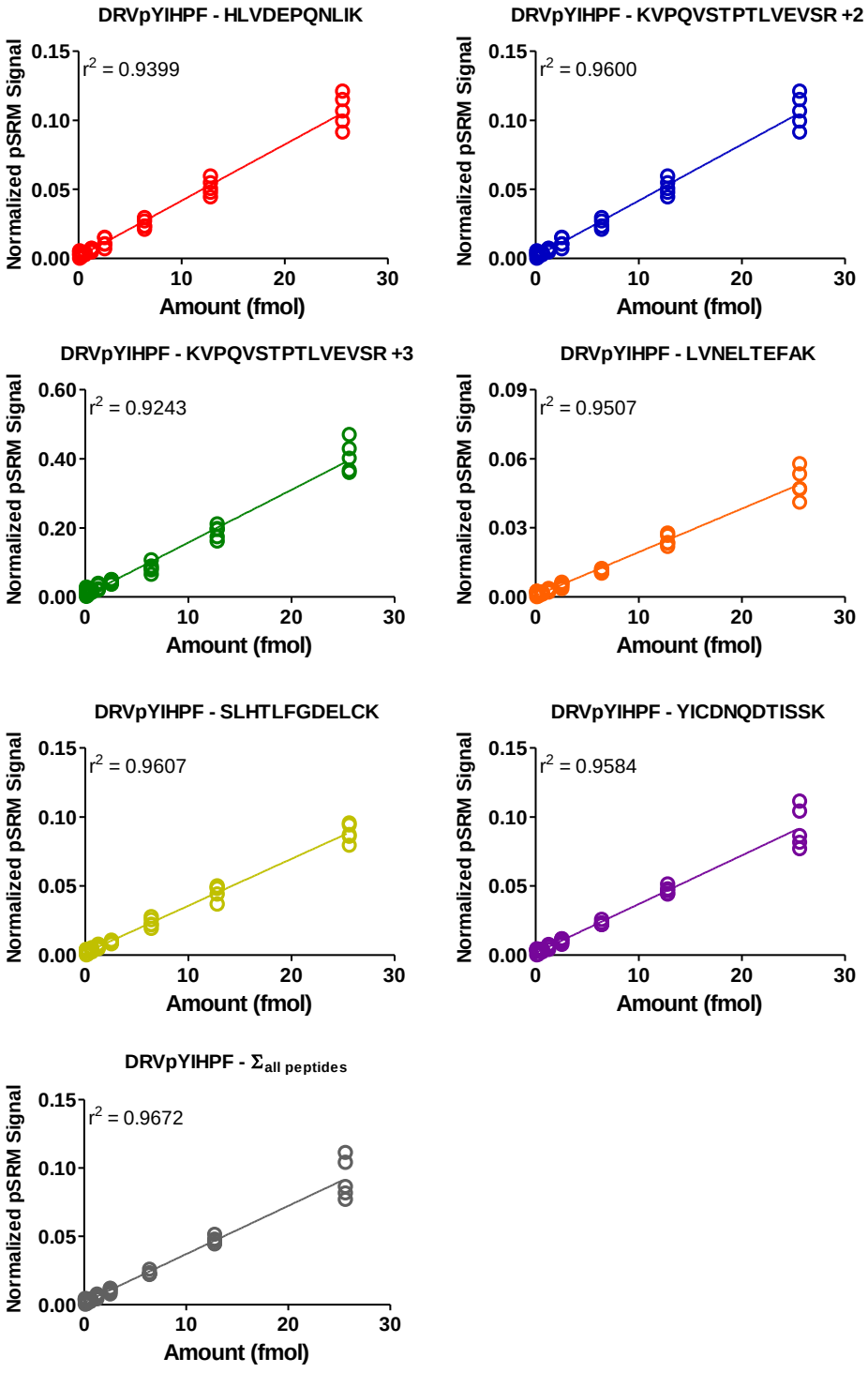
Supplemental figure S7. A plot of median CV (from five technical replicates) for phosphopeptide DRV*p*YIHPF shows the difference in median CV for each BSA normalizing peptide. For each of the normalizing peptides, the highest CV (open diamonds) correlates to the lowest phosphopeptide spike-in amount, 0.01 fmol ng BSA^{-1} which corresponds to 0.128 fmol on column.



Supplemental figure S8. Individual standard curves for phosphopeptide IKNLQ*p*SLDPSH spiked into BSA digest. Normalized pSRM signals of IKNLQ*p*SLDPSH – MS^2 were plotted for each phosphopeptide spike-in amount (0.128-25.6 fmol on column). Individual open circles at each concentration indicate the five technical replicates for each sample.



Supplemental figure S9. Individual standard curves for phosphopeptide IKNLQ*p*SLDPSH spiked into BSA digest. Normalized pSRM signals of IKNLQ*p*SLDPSH – MS^3 were plotted for each phosphopeptide spike-in amount (0.128-25.6 fmol on column). Individual open circles at each concentration indicate the five technical replicates for each sample.



Supplemental figure S10. Individual standard curves for phosphopeptide DRV*pY*IHPF spiked into BSA digest. Normalized pSRM signals of DRV*pY*IHPF were plotted for each phosphopeptide spike-in amount (0.128-25.6 fmol on column). Individual open circles at each concentration indicate the five technical replicates for each sample.

hEGFR

1210 amino acids

MRPSGTAGAALLALLAALCPASRALEEKKVCQGTSNKLTQLGTFEDHFLS
LQRMFNNCEVVLGNLEITYVQRNYDLSFLKTIQEVAGYVLIALNTVERIP
LENLQIIRGNMYYENSYALAVLSNYDANKTGLKELPMRNLQEILHGAVRF
SNNPALCNVESIQWRDIVSSDFLSNMSMDFQNHLGSCQKCDPSCPNGSCW
GAGEENCQKLTKIICAQQCSGRCRGKSPSDCCHNQCAAGCTGPRESDCLV
CRKFRDEATCKDTCPPLMLYNPTTYQMDVNPEGKYSFGATCVKKCPRNYV
VTDHGSCVRACGADSYEMEEDGVRKCKKCEGPCRKVCNGIGIGEFKDSLS
INATNIKHFKNCTSISGDLHILPVAFRGDSFTHTPPLDPQELDILKTVKE
ITGFLLIQAWPENRTDLHAFENLEIIRGRTKQHGQFSLAVVSLNITSLGL
RSLKEISDGDVIISGNKNLCYANTINWKKLFGTSGQKTKIISNRGENSCK
ATGQVCHALCSPEGCWGPEPRDCVSCRNVSRGRECVDKCNLLEGEPREFV
ENSECIQCHPECLPQAMNITCTGRGPDNCIQCAHYIDGPHCVKTCPAGVM
GENNTLVWKYADAGHVCHLCHPNCTYGCTGPGLEGCPTNGPKIPSIATGM
VGALLLLLVVALGIGLFMRRRHIVRKRTLRRLLQERELVEPLTPSGEAPN
QALLRILKETEFKKIKVLGSGAFGTVYKGLWIPEGEKVKIPVAIKELREA
TSPKANKEILDEAYVMASVDNPHVCRLLGICLTSTVQLITQLMPFGCLLD
YVREHKDNIGSQYLLNWCVQIAKGMNYLEDRRLVHRDLAARNVLVKTPQH
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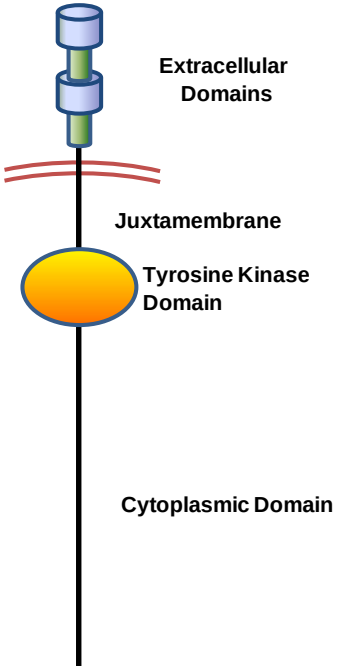
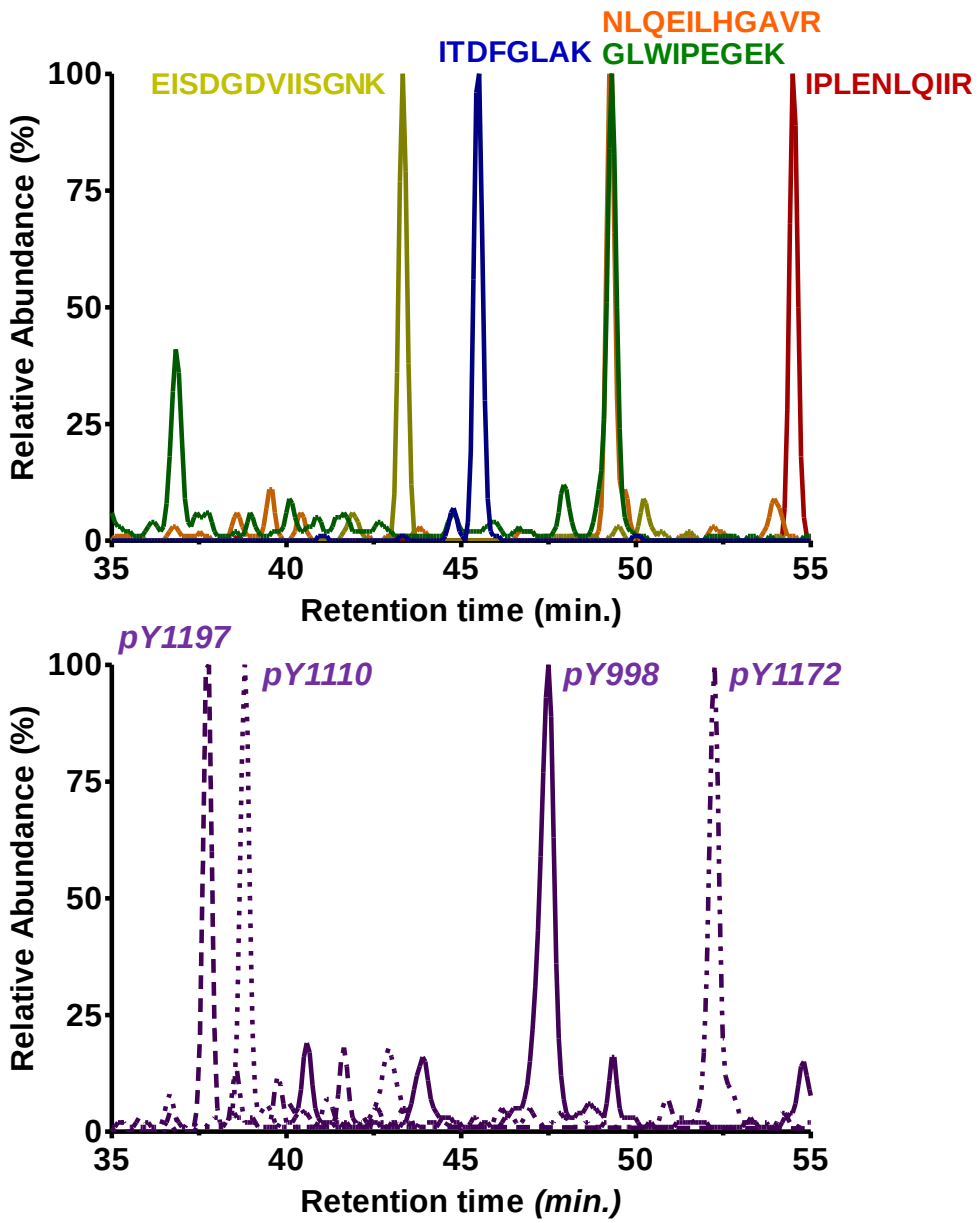


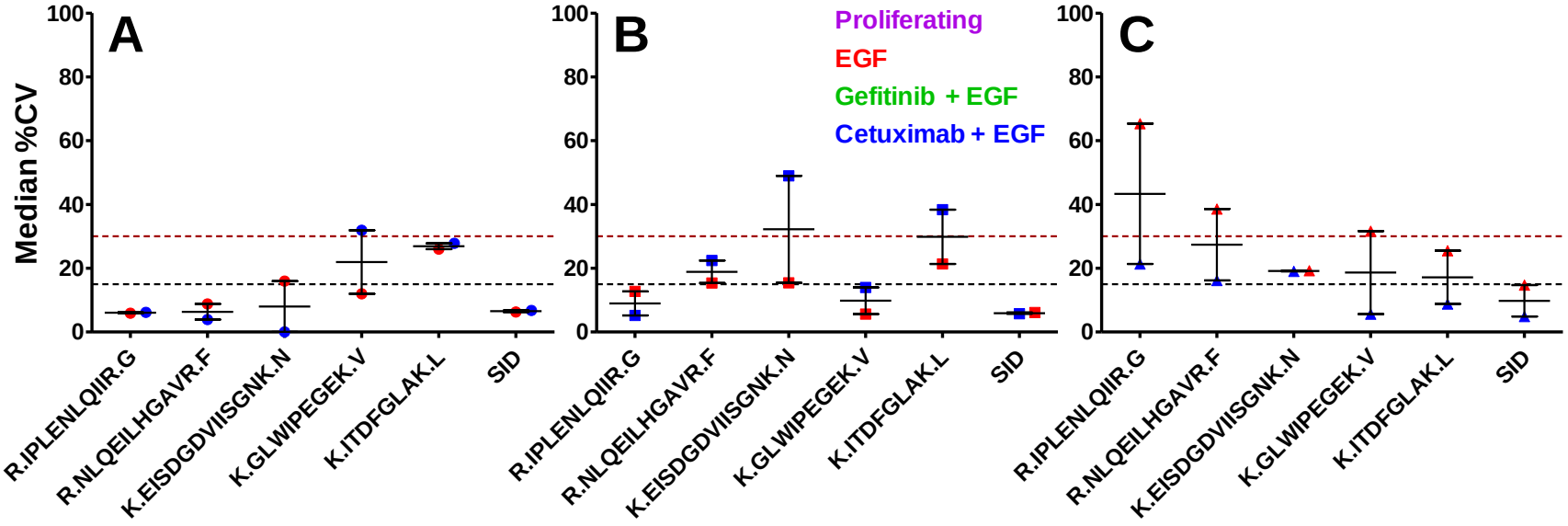
Supplemental Figure S11. Human epidermal growth factor receptor (hEGFR) amino acid sequence. Normalizing and phosphorylated peptides are highlighted in different colors. The nonphosphorylated peptides that were chosen to be used as normalizing peptides are located in the extracellular domain (IPLENLQIIR, NLQEILHGAVR and EISDGDVIISGNK), juxtamembrane (GLWIPEGEK) and tyrosine kinase domain (ITDFGLAK). Two phosphopeptides that we monitored are located in the tyrosine kinase domain (MHLP*pS*PTDSNFYR and MHLPSPTDSNF*pY*R) The other four phosphopeptides (*pY* and *pS*) that were monitored are located in the cytoplasmic domain (RPAGSVQNPV*pY*HNQPLNPAPSR, GSHQI*pS*LDNPDYQQDFFPK, GSHQISLDNPD*pY*QQDFFPK and GSTAENAE*pY*LR).



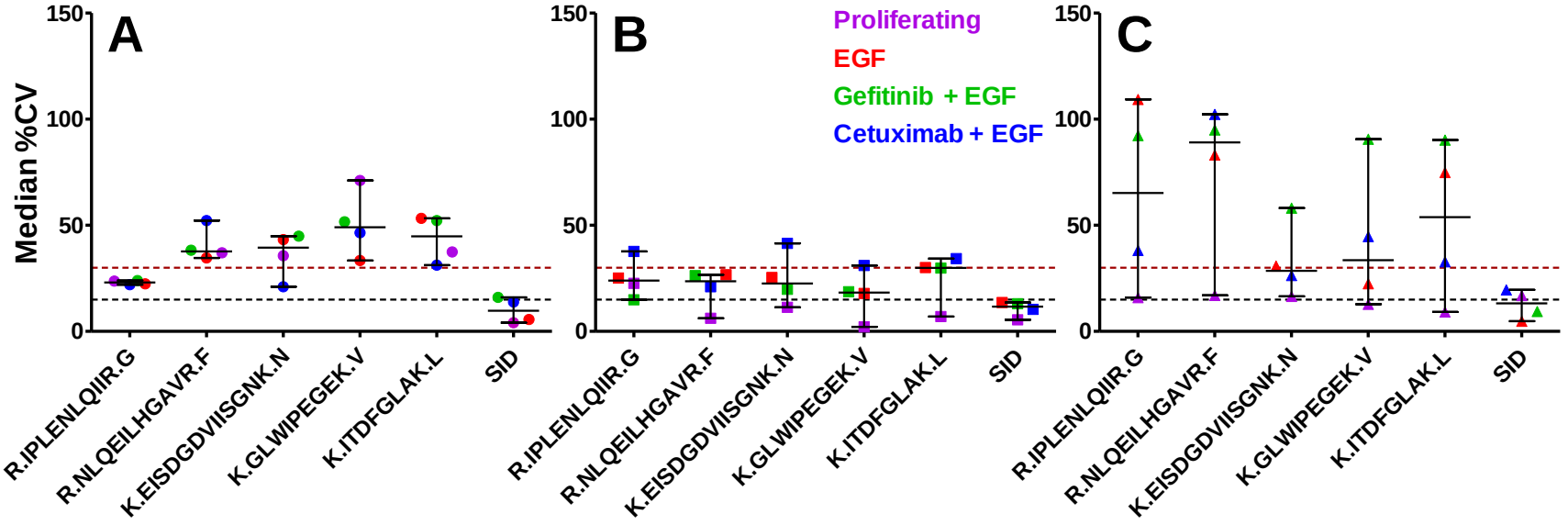
Supplemental figure S12. The retention time for normalizing peptides ranged across the peptide elution retention time. Retention time plots for all internal nonphosphorylated reference peptides (top) and stable-isotope labeled *pY* peptides, Y998 – MHLPSPTDSNF*pY*R, Y1110 – RPAGSVQNPV*pY*HNQPLNPAPSR, Y1172 – GSHQISLDNPD*pY*QQDFFPK, and Y1197 – GSTAENAE*pY*LR) (bottom). Retention time plots were generated from biological replicate 3, technical replicate 3. The underlined amino acid indicates which amino acid was stable isotope labeled.

Peptide[1]	Precursor *m/z*	Product *m/z*
MHLPSPTDSNF*p*YR	822.8	1263.5 (y_{10}), 1079.4 (y_8), 766.3 (y_5), 688.8 (y_{11}^{2+}), 382.2 (b_3)
MHLPSPTDSNFYR	782.9	1296.6 (y_{11}), 1183.5 (y_{10}), 648.8 (y_{11}^{2+}), 592.3 (y_{10}^{2+}), 1390.6 (b_{12})
MHLP*p*SPTDSNFYR – MS^3	822.84 → 773.85	534.7 (y_9^{2+}), 639.8 (y_{11}^{2+}), 861.4 (b_8), 1165.5 (y_{10}), 1278.6 (y_{11})
RPAGSVQNPV*p*YHNQPLNPAPSR	827.1	1473.7 (y_{12}), 1113.5 (y_{20}^{2+}), 1078.0 (y_{19}^{2+}), 892.4 (y_{15}^{2+}), 977.0 (b_{17}^{2+})
RPAGSVQNPVYHNQPLNPAPSR	800.4	851.5 (y_8), 1009.5 (y_{18}^{2+}), 710.9 (b_{13}^{2+}), 774.9 (b_{14}^{2+}), 937.0 (b_{17}^{2+})
GSHQISLDNPD*p*YQQDFFPK	772.7	1267.5 (y_9), 1152.5 (y_8), 797.3 (y_{12}^{2+}), 739.8 (y_{11}^{2+}), 682.8 (y_{10}^{2+})
GSHQISLDNPDYQQDFFPK	746.0	1072.5 (y_8), 642.8 (y_{10}^{2+}), 952.5 (b_9), 923.4 (b_{16}^{2+}), 996.9(b_{17}^{2+})
GSHQI*p*SLDNPDYQQDFFPK – MS^3	772.7 → 740.01	914.4 (b_{16}^{2+}), 934.4 (b_9), 987.9 (b_{17}^{2+})
GSTAENAE*p*YLR	645.8	845.4 (y_6), 731.3 (y_5), 660.3 (y_4), 531.2 (y_3), 573.8 (y_9^{2+})
GSTAENAEYLR	605.78	894.4 (y_7), 765.4 (y_6), 580.3 (y_4), 451.3 (y_3), 533.8 (y_9^{2+})
IPLENLQIIR	**604.9**	**998.6 (y_8), 885.5 (y_7), 756.5 (y_6), 529.4 (y_4), 548.3 (y_9^{2+})**
NLQEILHGAVR	**625.4**	**894.5 (y_8), 765.5 (y7), 652.4 (y_6), 539.3 (y_5), 402.3 (y_4)**
EISDGDVIISGNK	**673.8**	**1104.6 (y_{11}), 631.4 (y_6), 518.3 (y_5), 405.2 (y_4), 502.2 (b_5^{2+})**
GLWIPEGEK	**514.8**	**858.4 (y_7), 672.4 (y_6), 559.3 (y_5), 429.7 (y_7^{2+}), 470.3 (b_4)**
ITDFGLAK	**432.7**	**751.4 (y_7), 650.4 (y_6), 535.3 (y_5), 388.3 (y_4), 215.1 (b_2)**
MHLPSPTDSNF*p*YR^	**827.9**	**1273.5 (y_{10}), 1089.4 (y_8), 776.3 (y_5), 693.8 (y_{11}^{2+}), 382.2 (b_3)**
RPAGSVQNPV*p*YHNQPLNPAPSR^	**830.4**	**1483.7 (y_{12}), 1118.5 (y_{20}^{2+}), 1083.0 (y_{19}^{2+}), 897.4 (y_{15}^{2+}), 976.9 (b_{17}^{2+})**
GSHQISLDNPD*p*YQQDFFPK^	**775.3**	**1275.5 (y_9), 1160.5 (y_8), 807.3 (y_{12}^{2+}), 743.8 (y_{11}^{2+}), 686.8 (y_{10}^{2+})**
GSTAENAE*p*YLR^	**650.8**	**855.4 (y_6), 741.3 (y_5), 670.3 (y_4), 541.2 (y_3), 578.8 (y_9^{2+})**

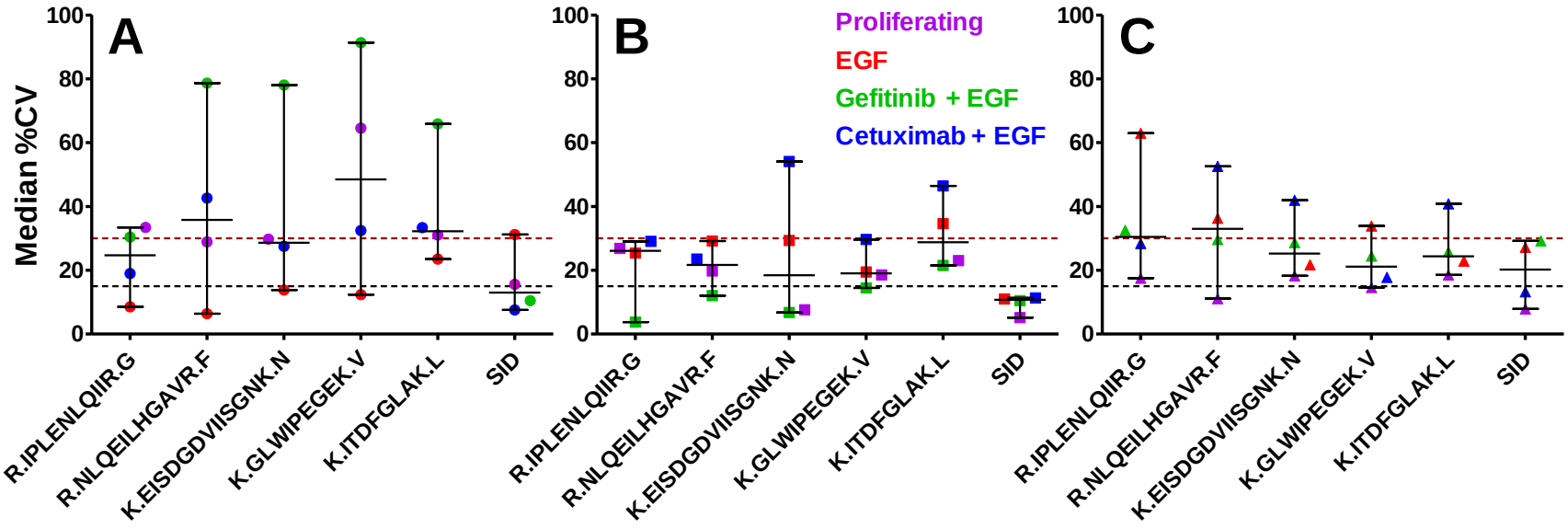
Supplemental table S13. EGFR phosphorylated and nonphosphorylated peptides and transitions selected for LC-pSRM-MS.
[1]Peptides marked with ^ are isotopically labeled, the ^ indicates which amino acid was stable isotope labeled.



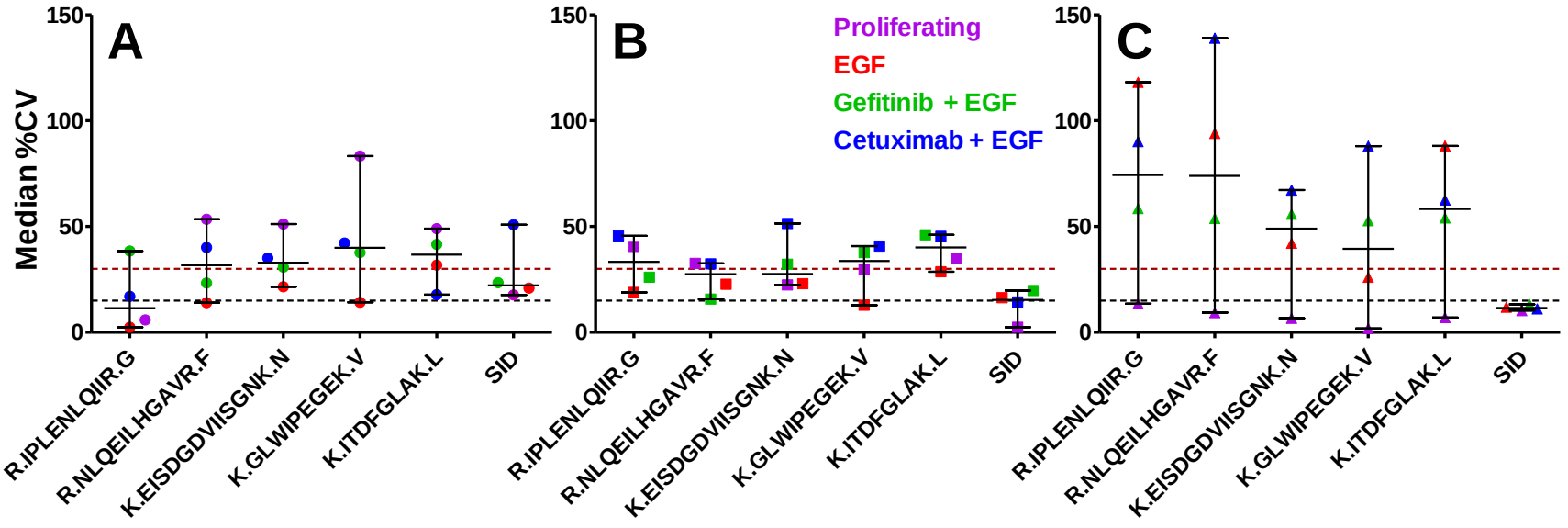
Supplemental figure S14. Median CV plots for peptide MHLPSPTDSNF*pY*R. The CV's were calculated by the technical replicate analysis on a per treatment and internal reference/stable isotope labeled peptide basis. (A-C) All data (three technical replicates for each treatment) was used to calculate median CV's for biological replicate 1 (A), 2 (B) and 3 (C). The black dashed line represents 15% CV, and the red dashed line represents 30% CV. Data was not detected in proliferating, co-treated gefitinib followed by EGF samples.



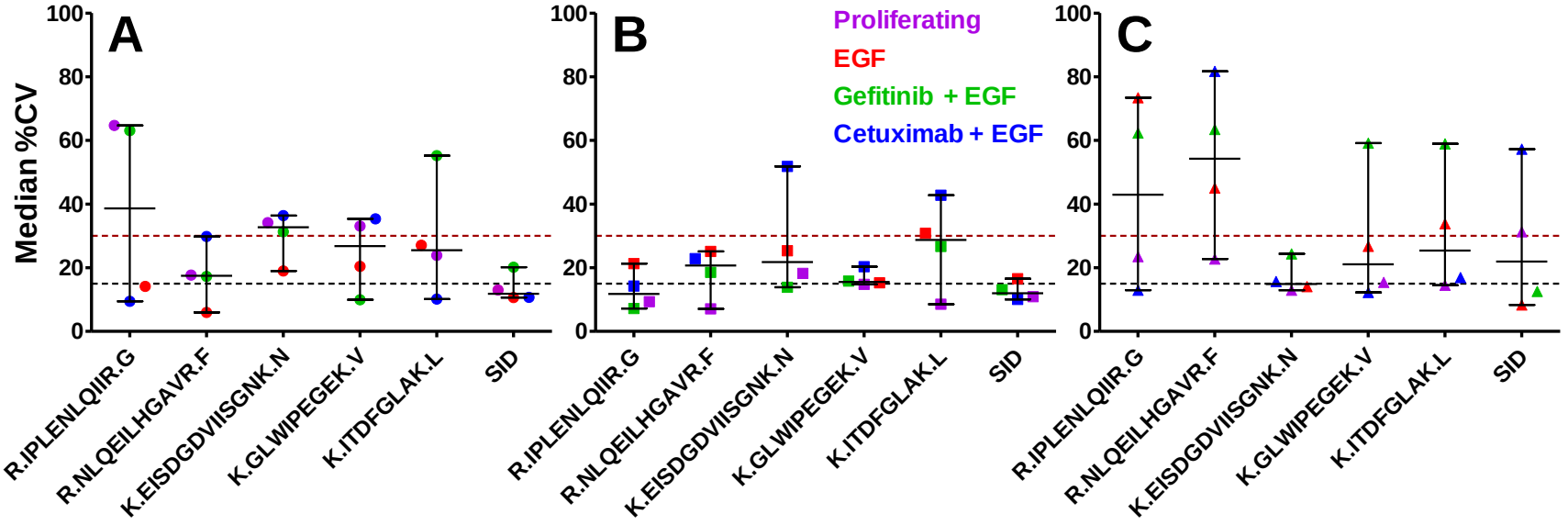
Supplemental figure S15. Median CV plots for peptide RPAGSVQNPV*pY*HNQPLNPAPSR. The CV's were calculated by the technical replicate analysis on a per treatment and internal reference/stable isotope labeled peptide basis. (A-C) All data (three technical replicates for each treatment) was used to calculate median CV's for biological replicate 1 (A), 2 (B) and 3 (C). The black dashed line represents 15% CV, and the red dashed line represents 30% CV.



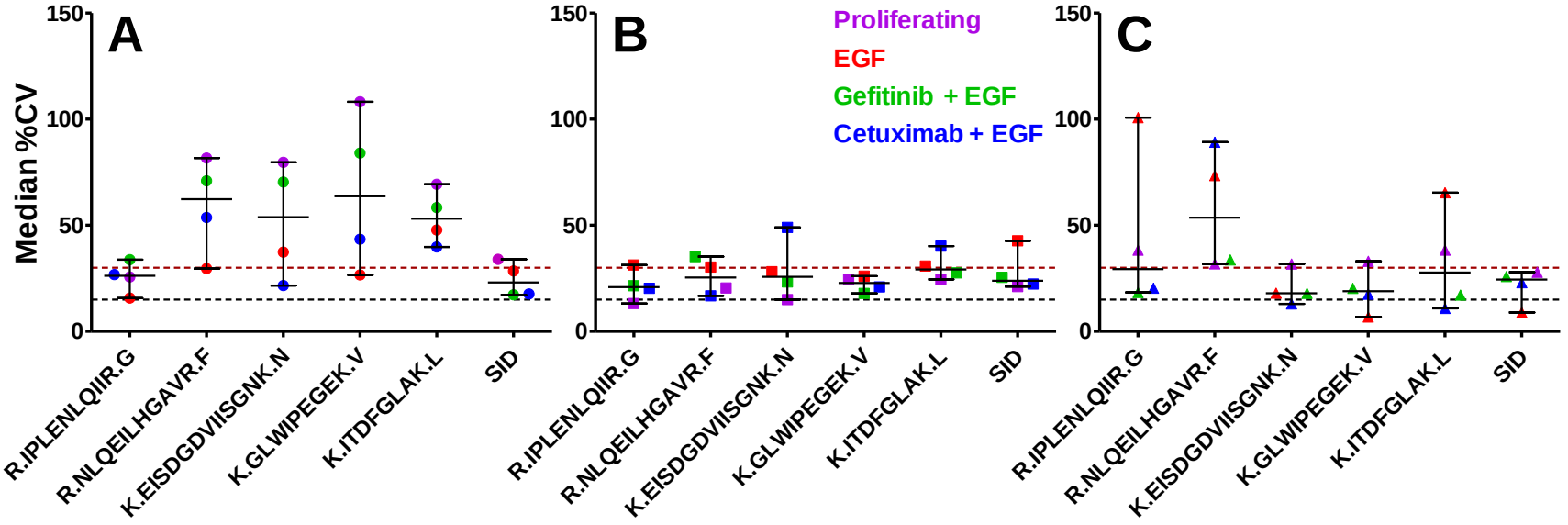
Supplemental figure S16. Median CV plots for peptide GSHQISLDNPD*pY*QQDFFPK. The CV's were calculated by the technical replicate analysis on a per treatment and internal reference/stable isotope labeled peptide basis. (A-C) All data (three technical replicates for each treatment) was used to calculate median CV's for biological replicate 1 (A), 2 (B) and 3 (C). The black dashed line represents 15% CV, and the red dashed line represents 30% CV.



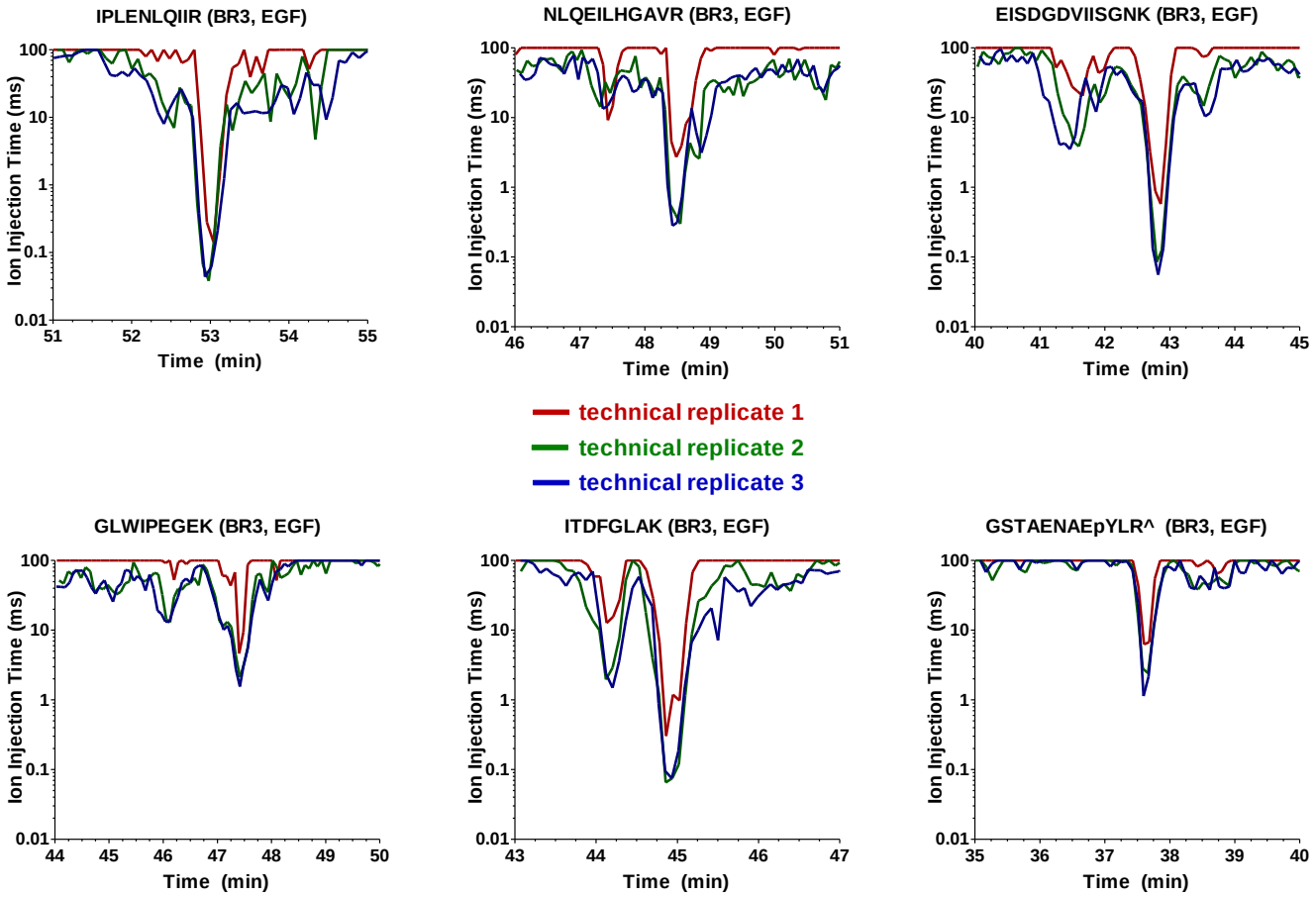
Supplemental figure S17. Median CV plots for peptide GSTAENAE*pY*LR for each biological replicate. The CV's were calculated by the technical replicate analysis on a per treatment and internal reference/stable isotope labeled peptide basis. (A-C) All data (three technical replicates for each treatment) was used to calculate median CV's for biological replicate 1 (A), 2 (B) and 3 (C). The black dashed line represents 15% CV, and the red dashed line represents 30% CV.



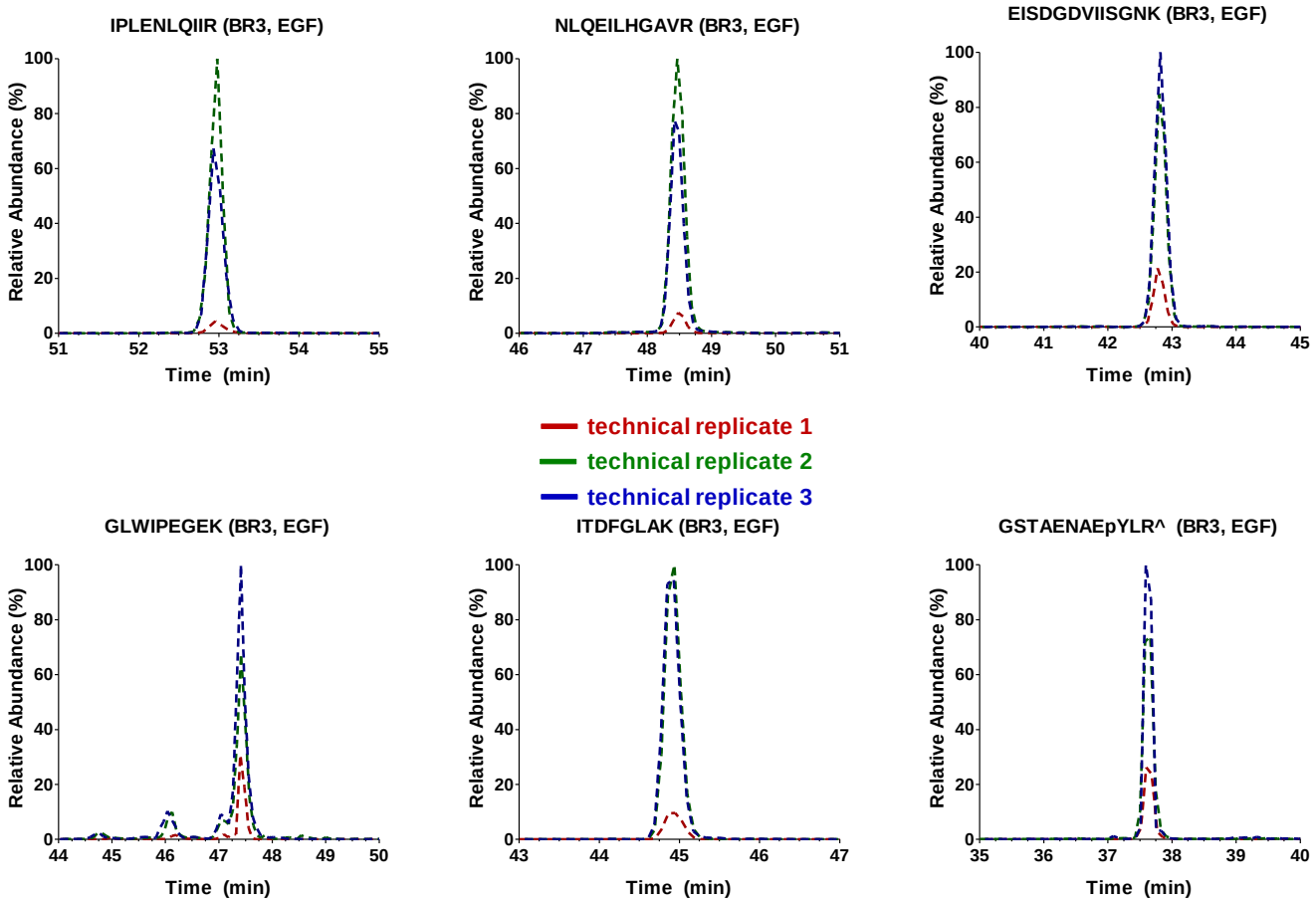
Supplemental figure S18. Median CV plots for MS^3 data for peptide MHLP*p*SPTDSNFYR. The CV's were calculated by the technical replicate analysis on a per treatment and internal reference/stable isotope labeled peptide basis. (A-C) All data (three technical replicates for each treatment) was used to calculate median CV's for biological replicate 1 (A), 2 (B) and 3 (C). The black dashed line represents 15% CV, and the red dashed line represents 30% CV.



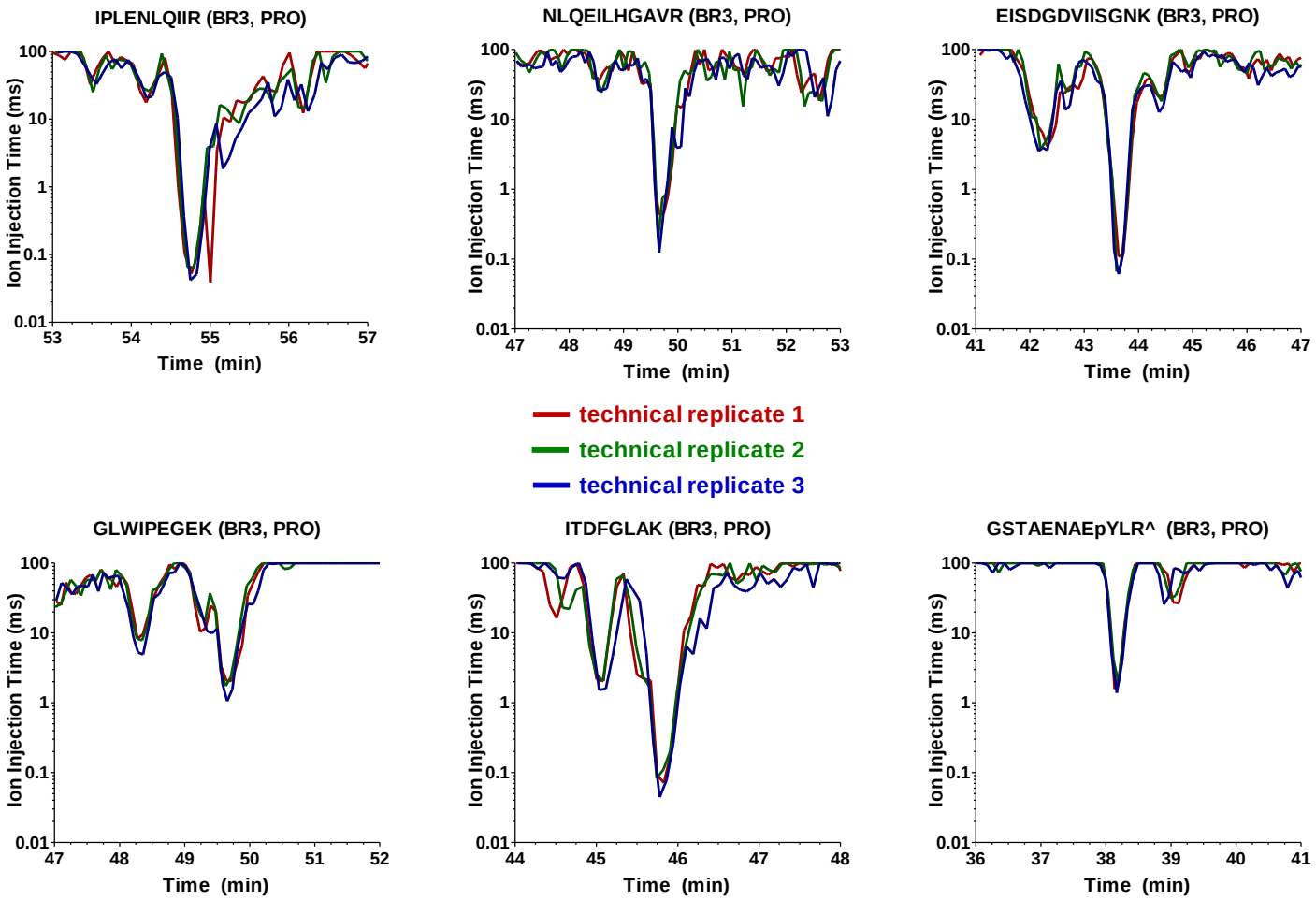
Supplemental figure S19. Median CV plots for MS^3 data for peptide GSHQI*p*SLDNPDYQQDFFPK. The CV's were calculated by the technical replicate analysis on a per treatment and internal reference/stable isotope labeled peptide basis. (A-C) All data (three technical replicates for each treatment) was used to calculate median CV's for biological replicate 1 (A), 2 (B) and 3 (C). The black dashed line represents 15% CV, and the red dashed line represents 30% CV.



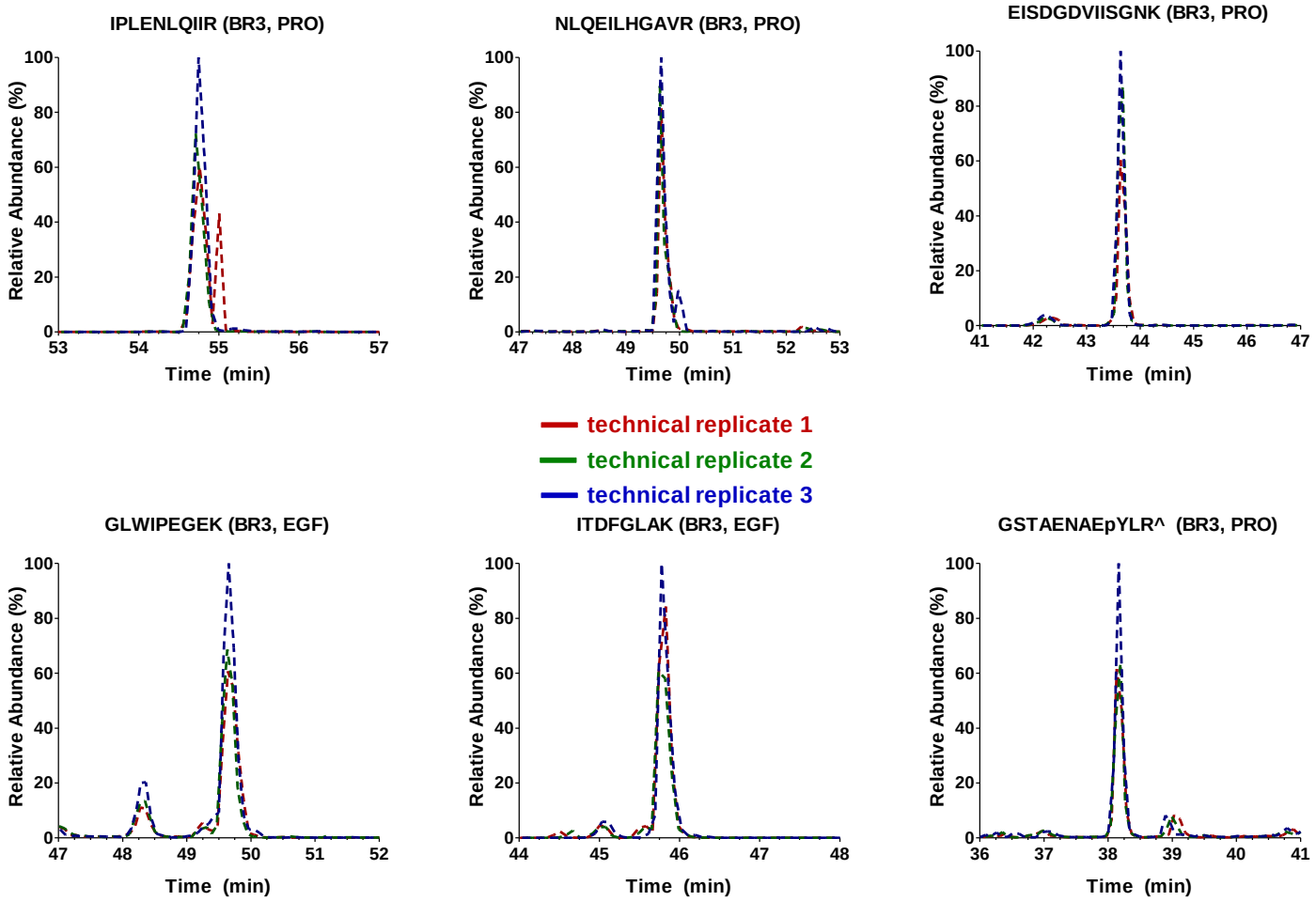
Supplemental figure S20. Time vs. ion injection time for five internal reference peptides and a stable isotope labeled peptide (GSTAENAE*p*YLR) for EGF treated samples (biological replicate 3). The maximum allowable ion injection time in these experiments is 100ms. These data show the ion injection time for technical replicate 1 was drastically different than both technical replicates 2 and 3, *i.e.*, technical replicate 1 always required a longer ion injection time for the same sample, suggesting the instrument was behaving differently during the EGF treated technical replicate 1 run. These data indicate the reason why large CV values for EGF treated biological replicate 3 was observed.



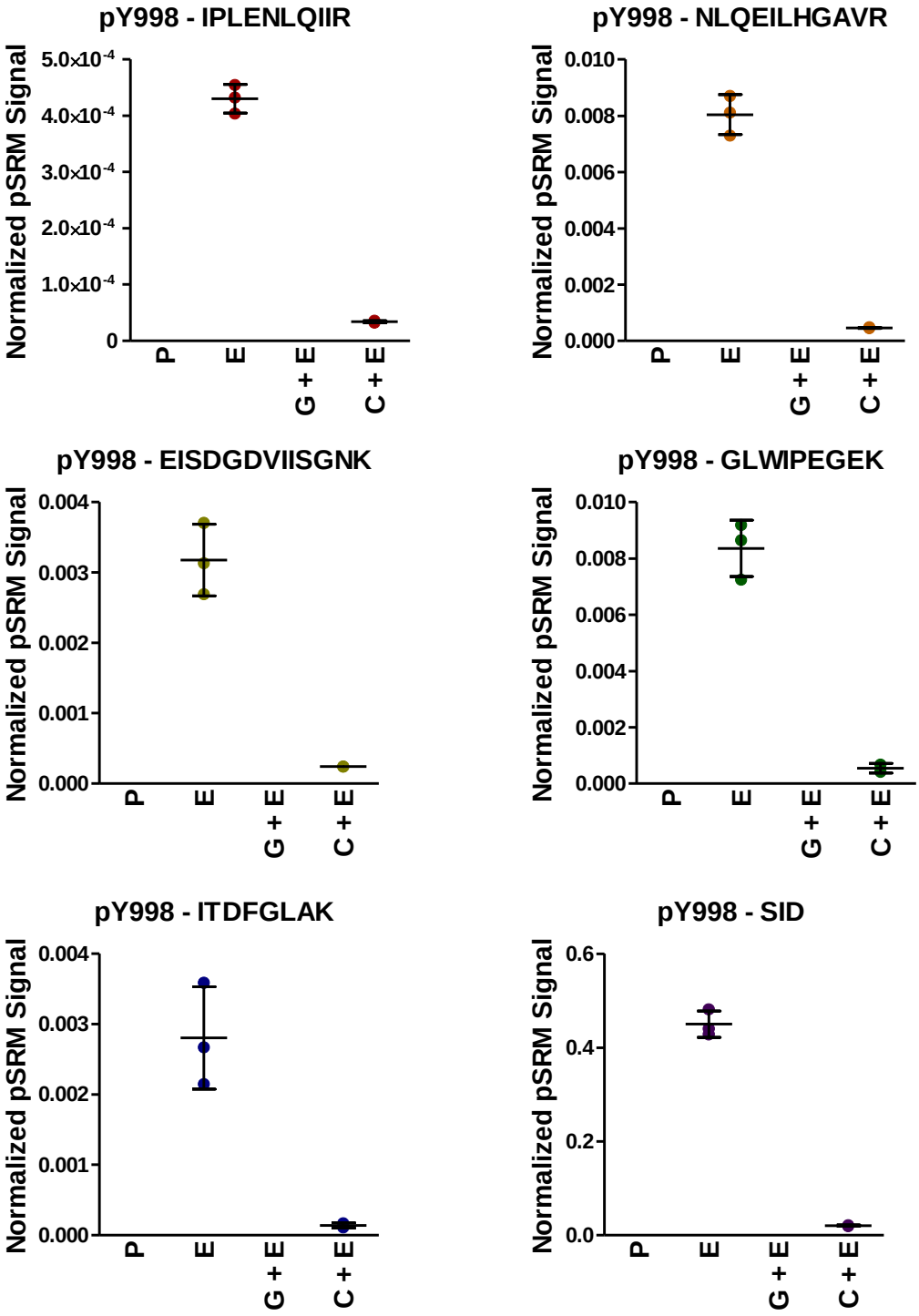
Supplemental figure S21. Extraction ion chromatograms (time vs. relative abundance (%)) for five internal reference peptides and a stable isotope labeled peptide (GSTAENAE*pY*LR) for EGF treated samples (biological replicate 3). These data show that the relative abundance (%) for technical replicate 1 is drastically different than both technical replicates 2 and 3, *i.e.*, technical replicate 1 has a low intensity for the same sample, suggesting the instrument was behaving significantly different during the EGF treated biological replicate 3 (technical replicate 1 run). These data indicate the reason why large CV values for EGF treated biological replicate 3 was observed.



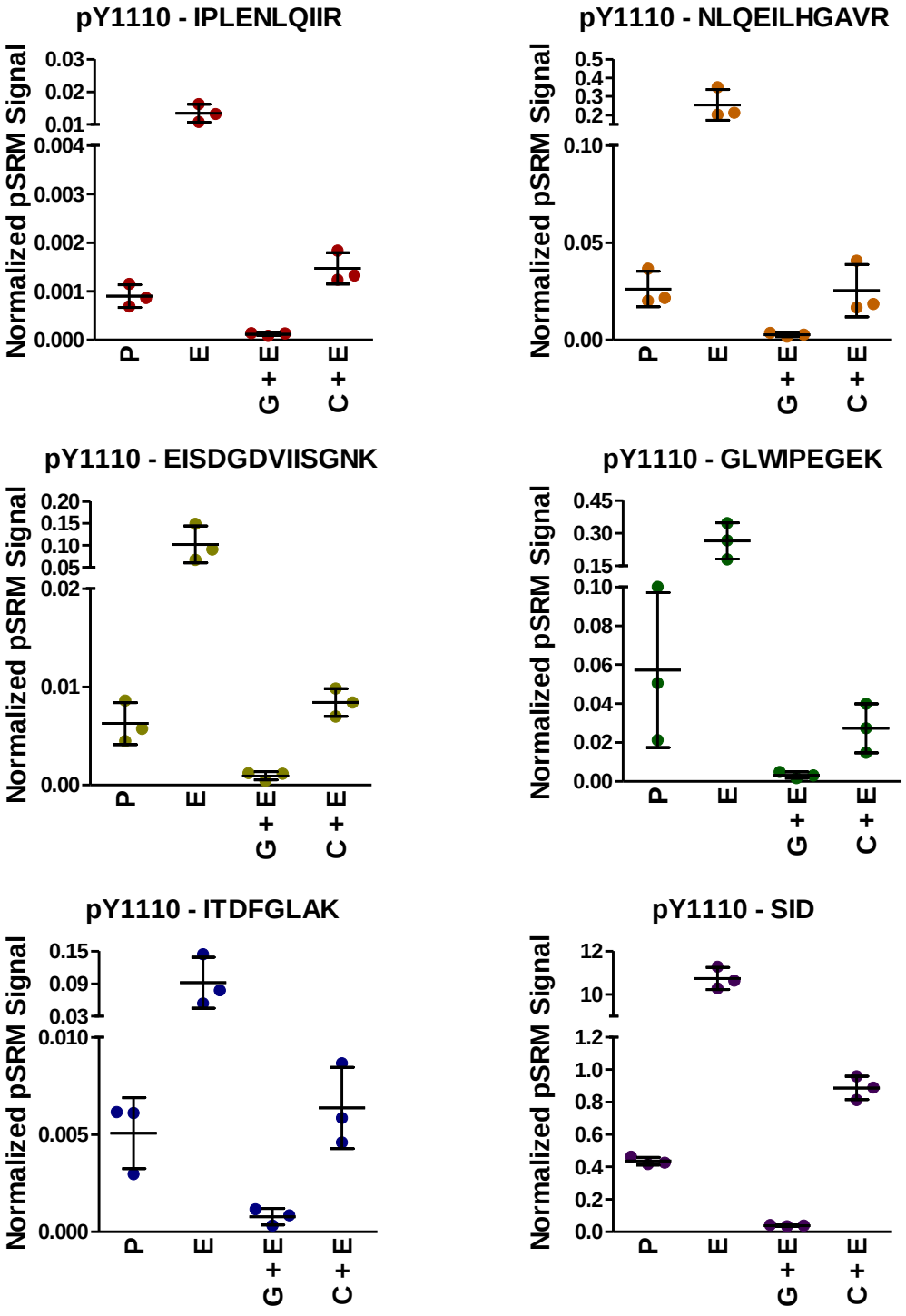
Supplemental figure S22. Time vs. ion injection time for five internal reference peptides and a stable isotope labeled peptide (GSTAENAE*p*YLR) for proliferating samples (biological replicate 3). The maximum allowable ion injection time in these experiments is 100ms. These data show the ion injection time for technical replicate 1 was similar to both technical replicates 2 and 3, unlike supplemental figure S17.



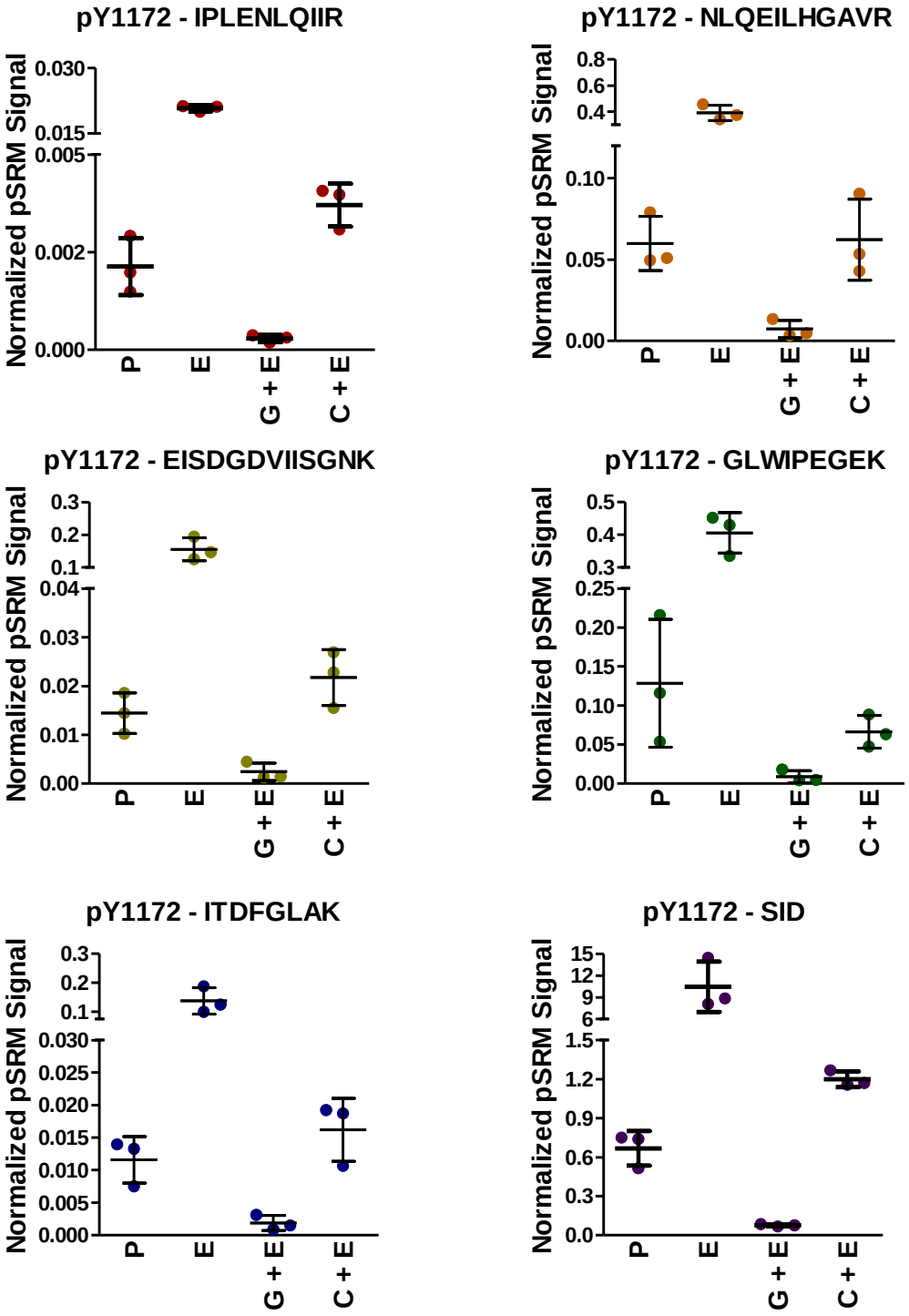
Supplemental figure S23. Extraction ion chromatograms (time vs. relative abundance (%)) for five internal reference peptides and a stable isotope labeled peptide (GSTAENAE*pY*LR) for proliferating samples (biological replicate 3). These data show that the relative abundance (%) for technical replicate 1 was similar to both technical replicates 2 and 3. The XICs for technical replicate 1 were similar to both technical replicates 2 and 3, unlike supplemental figure S18.



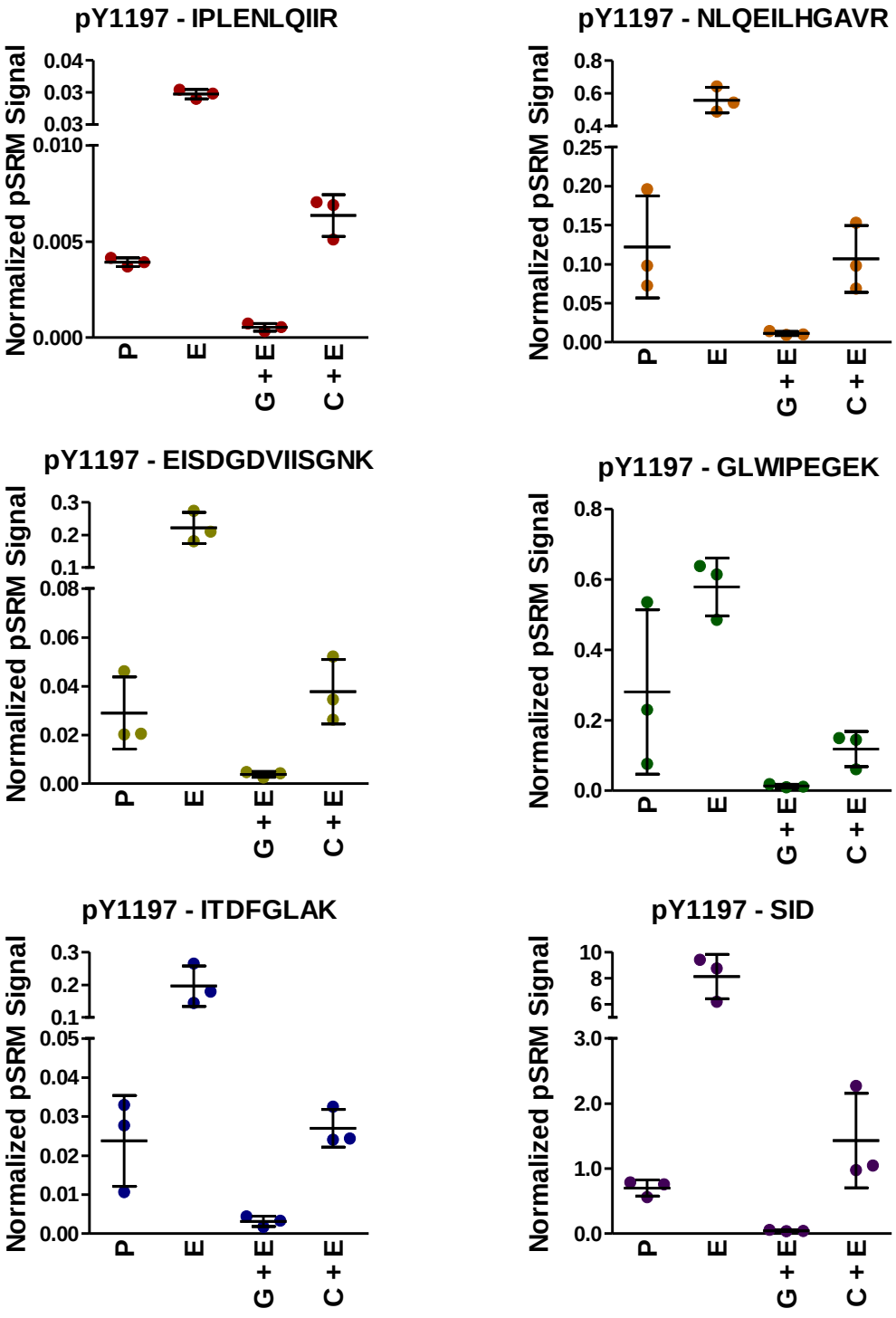
Supplemental figure S24. Normalized pSRM plots (MHLPSPTDSNF*pY*R) for each internal reference peptide and SID peptide for each sample type (P – proliferating, E – EGF stimulated, G + E – Gefitinib treatment followed by EGF and C + E – Cetuximab treatment followed by EGF). Data from biological replicate 1 (biological replicate 2 and 3 generate similar results), 3 technical replicates. Data was "not detected" for P and G + E samples.



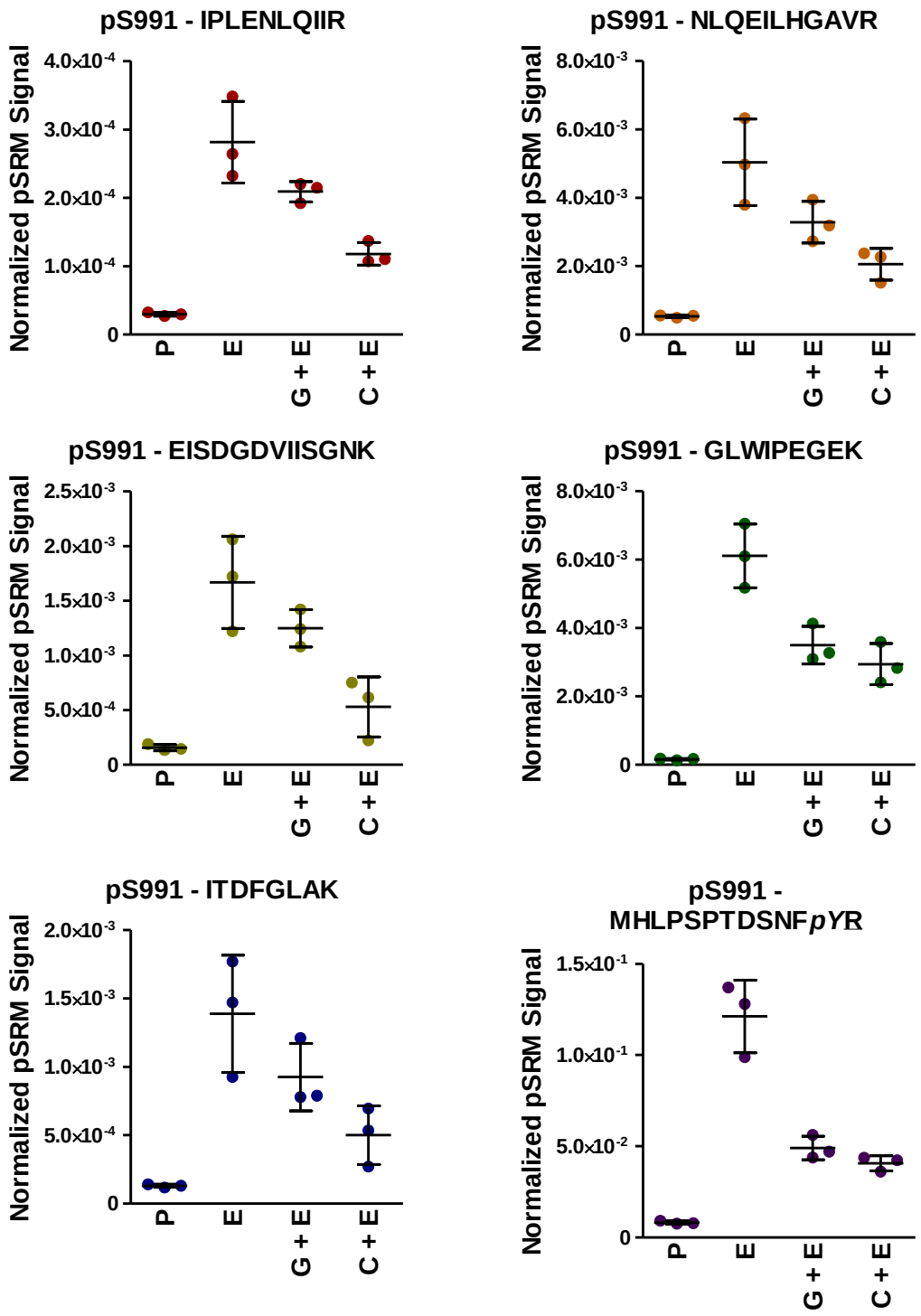
Supplemental figure S25. Normalized pSRM plots (RPAGSVQNPV*pY*HNQPLNPAPSR) for each internal reference peptide and SID peptide for each sample type (P – proliferating, E – EGF stimulated, G + E - Gefitinib treatment followed by EGF and C+ E – Cetuximab treatment followed by EGF). Data from biological replicate 1 (biological replicate 2 and 3 generate similar results), 3 technical replicates.



Supplemental figure S26. Normalized pSRM plots (GSHQISLDNPD*pY*QQDFFPK) for each internal reference peptide and SID peptide for each sample type (P – proliferating, E – EGF stimulated, G + E - Gefitinib treatment followed by EGF and C + E – Cetuximab treatment followed by EGF). Data from biological replicate 1 (biological replicate 2 and 3 generate similar results), 3 technical replicates.

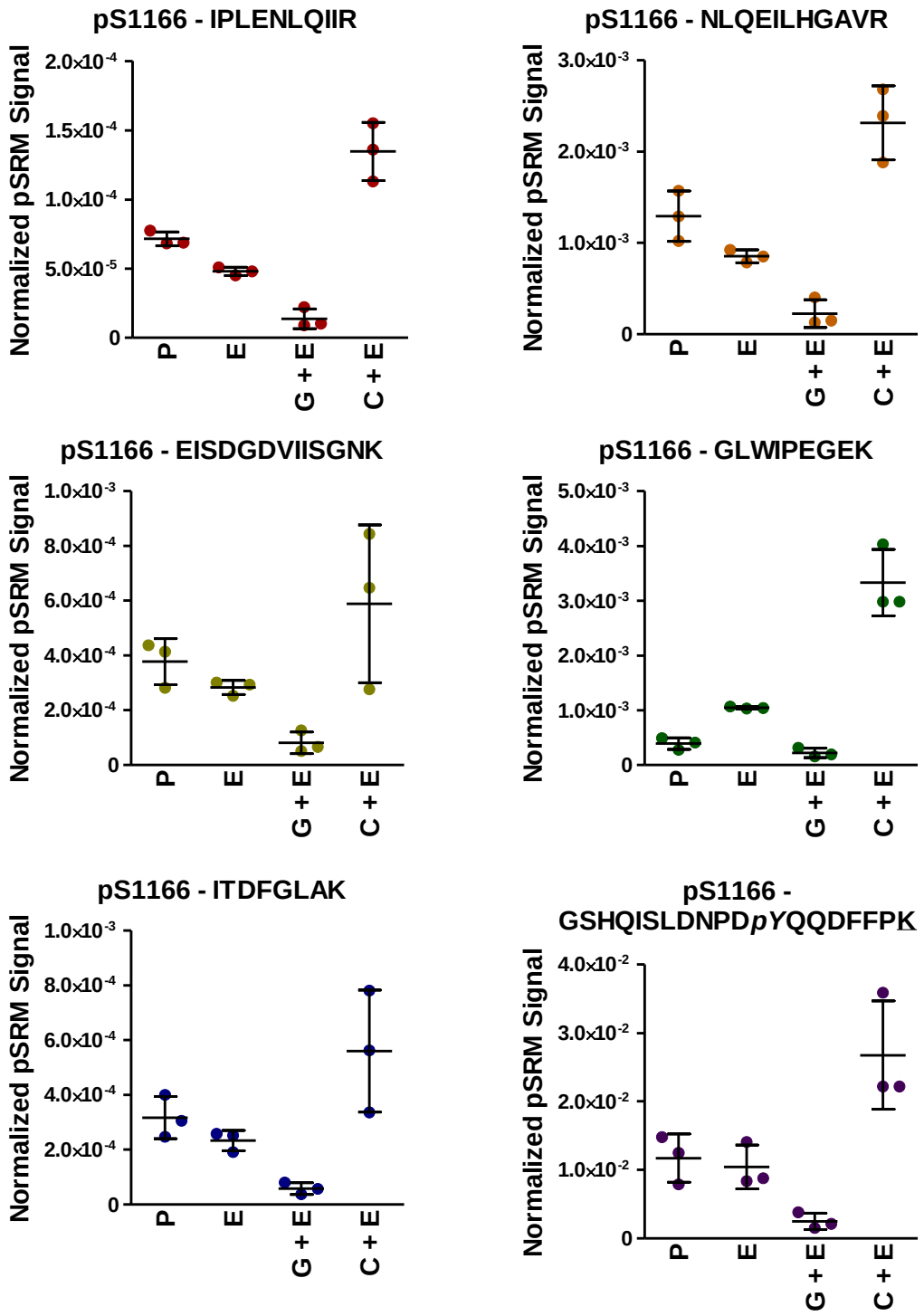


Supplemental figure S27. Normalized pSRM plots (GSTAENAE*pY*LR) for each internal reference peptide and SID peptide for each sample type (P – proliferating, E – EGF stimulated, G + E - Gefitinib treatment followed by EGF and C + E – Cetuximab treatment followed by EGF). Data from biological replicate 1 (biological replicate 2 and 3 generate similar results), 3 technical replicates.



Supplemental figure S28. Normalized MS^3 pSRM plots (MHLP*p*SPTDSNFYR) for each internal reference peptide and *pY* peptide complement for each sample type (P – proliferating, E – EGF stimulated, G + E - Gefitinib treatment followed by EGF and C + E – Cetuximab treatment followed by EGF). Data from

biological replicate 2, three technical replicates. The underlined amino acid indicates which amino acid was stable isotope labeled.



Supplemental figure S29. Normalized MS^3 pSRM plots (GSHQI*pS*LDNPDYQQDFFPK) for each internal reference peptide and *pY* peptide complement for each sample type (P – proliferating, E – EGF stimulated, G + E -

Gefitinib treatment followed by EGF and C + E – Cetuximab treatment followed by EGF). Data from biological replicate 2, three technical replicates. The underlined amino acid indicates which amino acid was stable isotope labeled.