

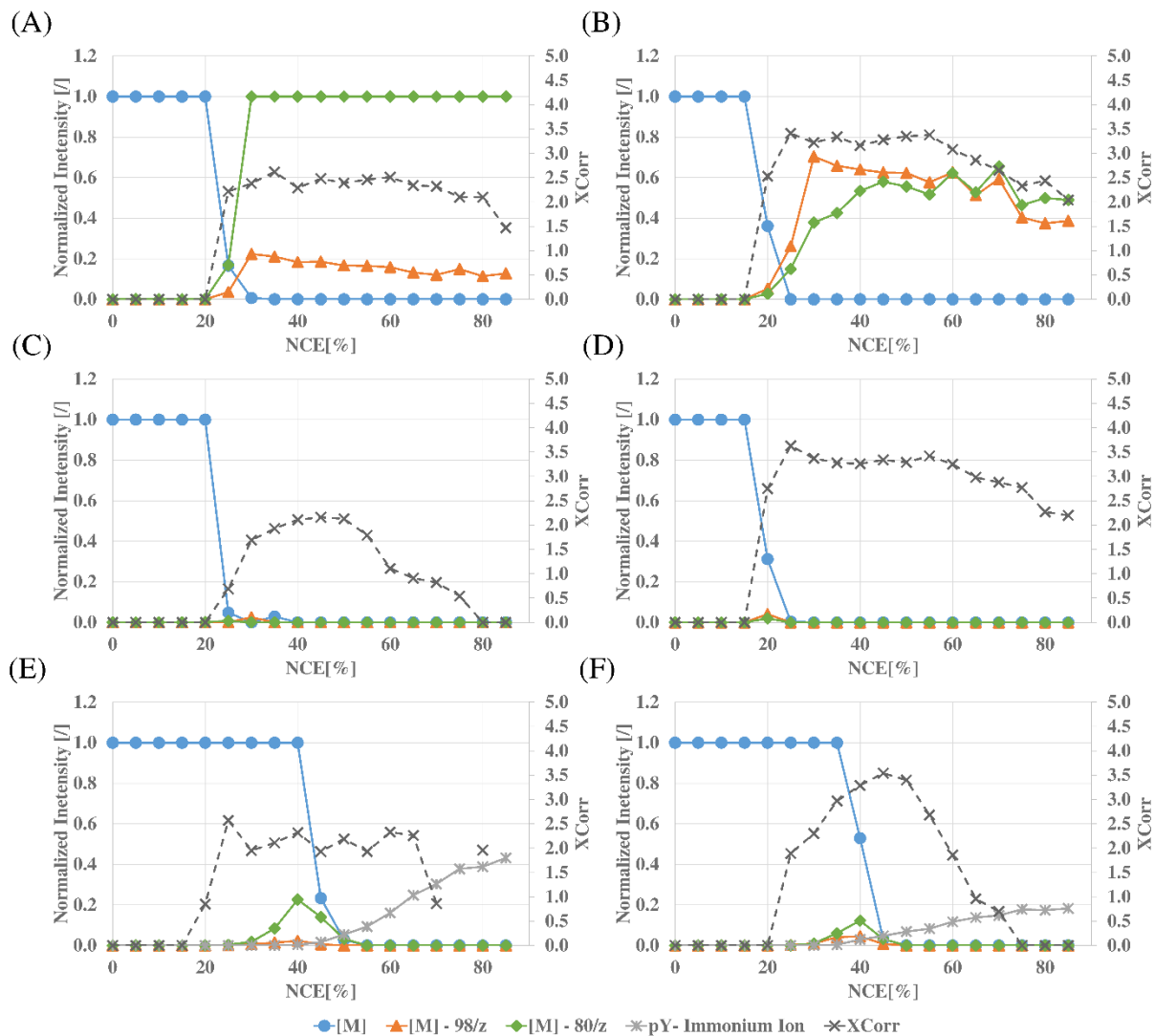
Supplementary Material to:

Optimized fragmentation conditions for iTRAQ labeled phosphopeptides

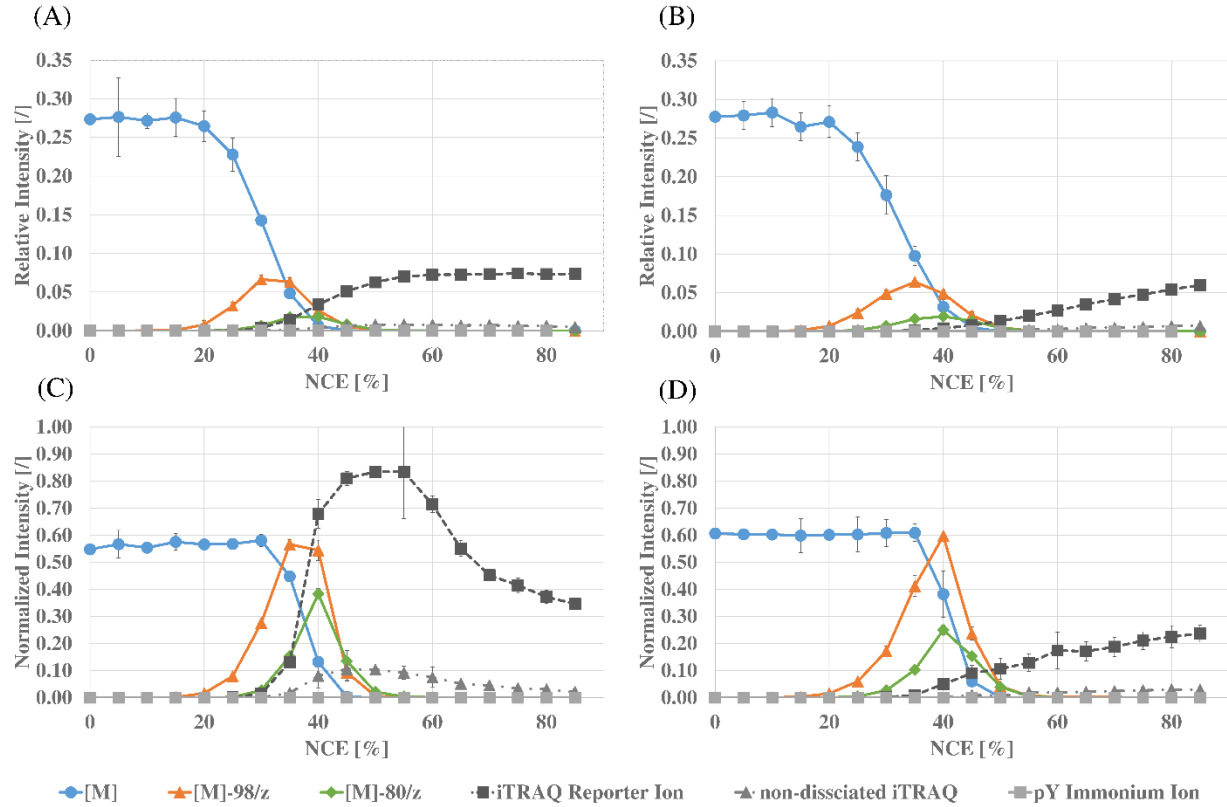
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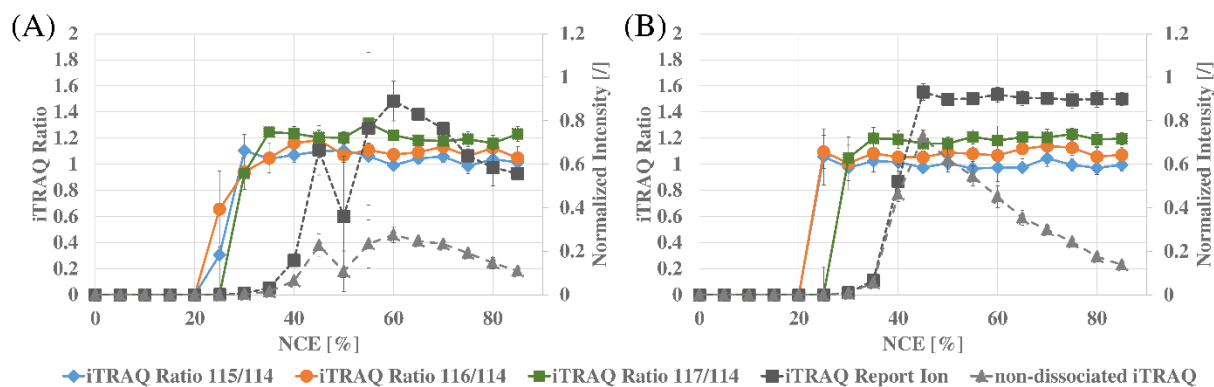


Supplementary Figure 1. Offline MS/MS measurements of two iTRAQ labeled, 2+ charged phosphotyrosine peptides i_{115} -VpYELMR (A,C,E), m/z 517.7; and i_{114} -VIEDNEpYTAR (B,D,F) at m/z 717.3. (A, B): CID; (C, D): CID/MSA; (E, F): HCD.

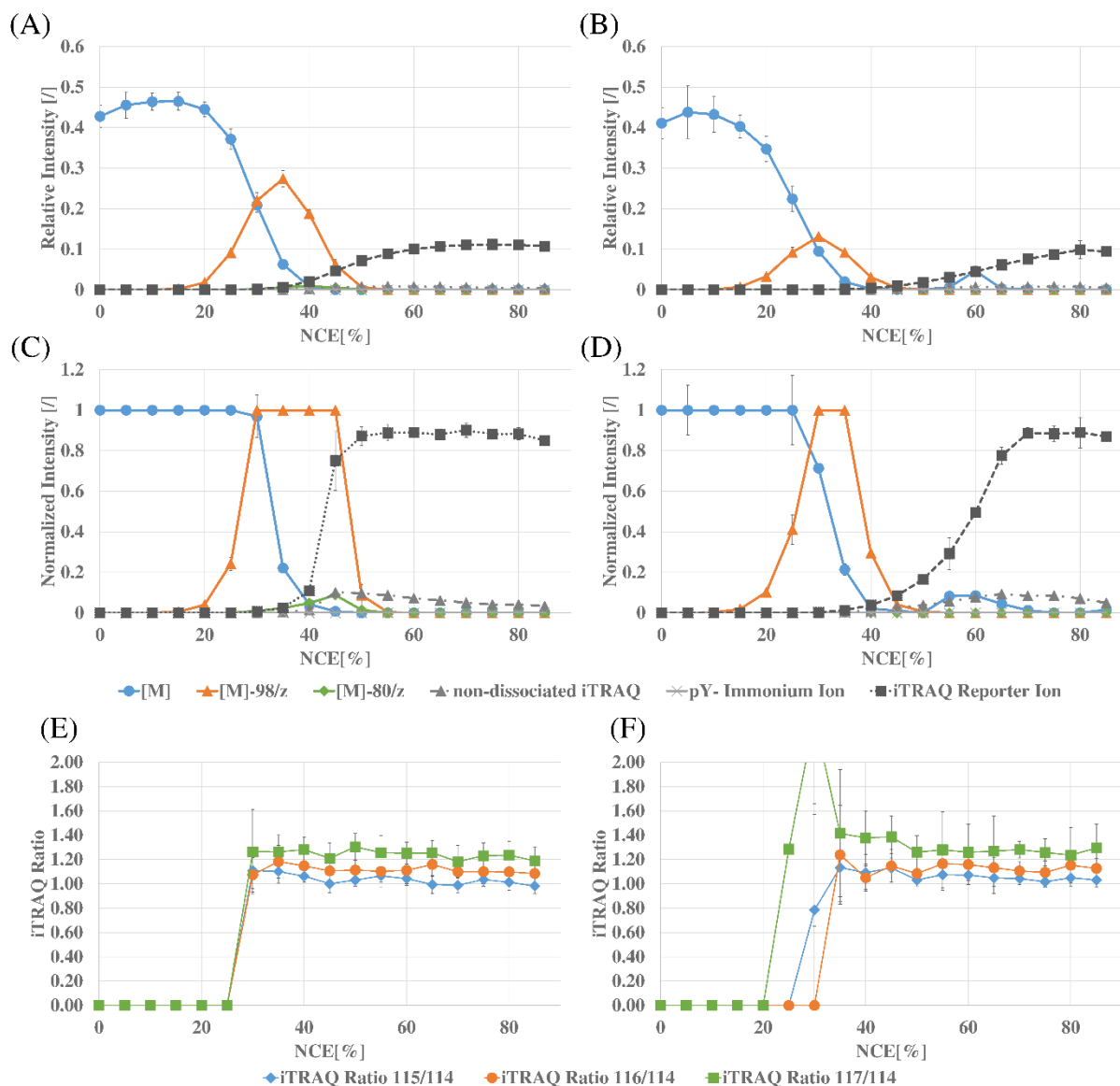


Supplementary Figure 2. Online LC-ESI-MS/MS measurements of the iTRAQ labeled phosphopeptide *i*₁₁₄₋₁₁₇-SpTFHAGQLR fragmented by HCD in dependence on the NCE.

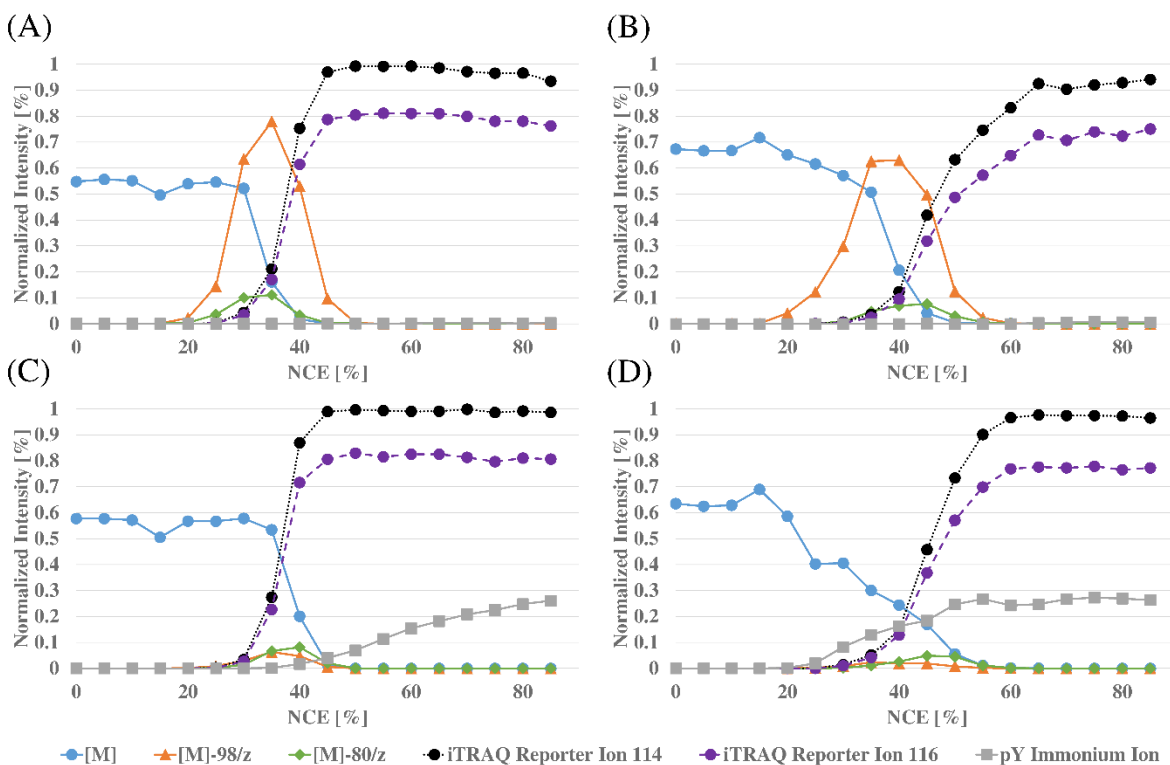
(A,B) relative intensities; (C,D) normalized intensities, for the 2+ charged precursor m/z 620.8 (A,C) and 3+ charged precursor (B,D) at m/z 414.2.



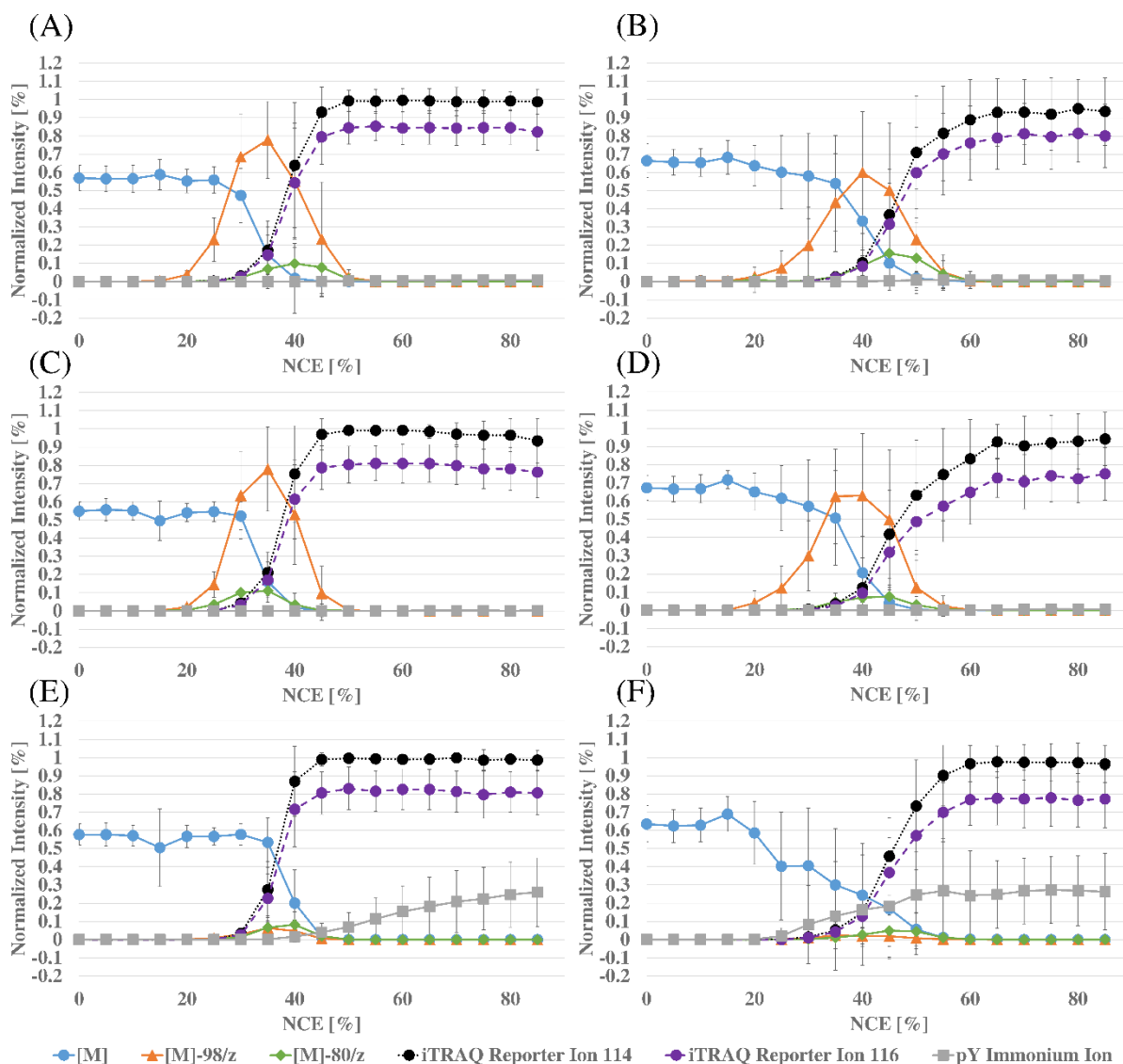
Supplementary Figure 3. Comparison of normalized iTRAQ reporter ion intensities, the protonated non-dissociated isobaric tag (iTRAQ + balancer group) at m/z 145.10 and the corresponding iTRAQ ratios (reporter ion 114 as denominator) depending on Normalized Collision Energy for HCD fragmentation for the 2+ charged precursors of peptides (A) $i_{114-117}$ -VpYELMR and (B) $i_{114-i117}$ -VIEDNEYTAR.



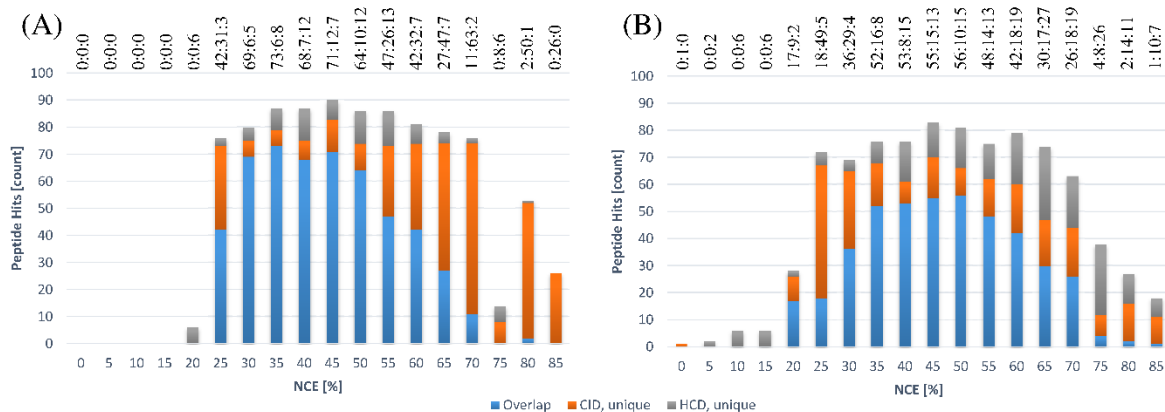
Supplementary Figure 4. Online LC-ESI-MS/MS measurements of the iTRAQ labeled phosphopeptide *i*₁₁₄₋₁₁₇-GHLpSEGLVTK fragmented by HCD. (A,B) relative intensities; (C,D) normalized intensities; (E,F) the corresponding iTRAQ ratios (reporter ion 114 as denominator), for the 2+ charged precursor at m/z 704.9.8 (A,C,E) and the 3+ charged precursor (B,D,F) at m/z 470.3.



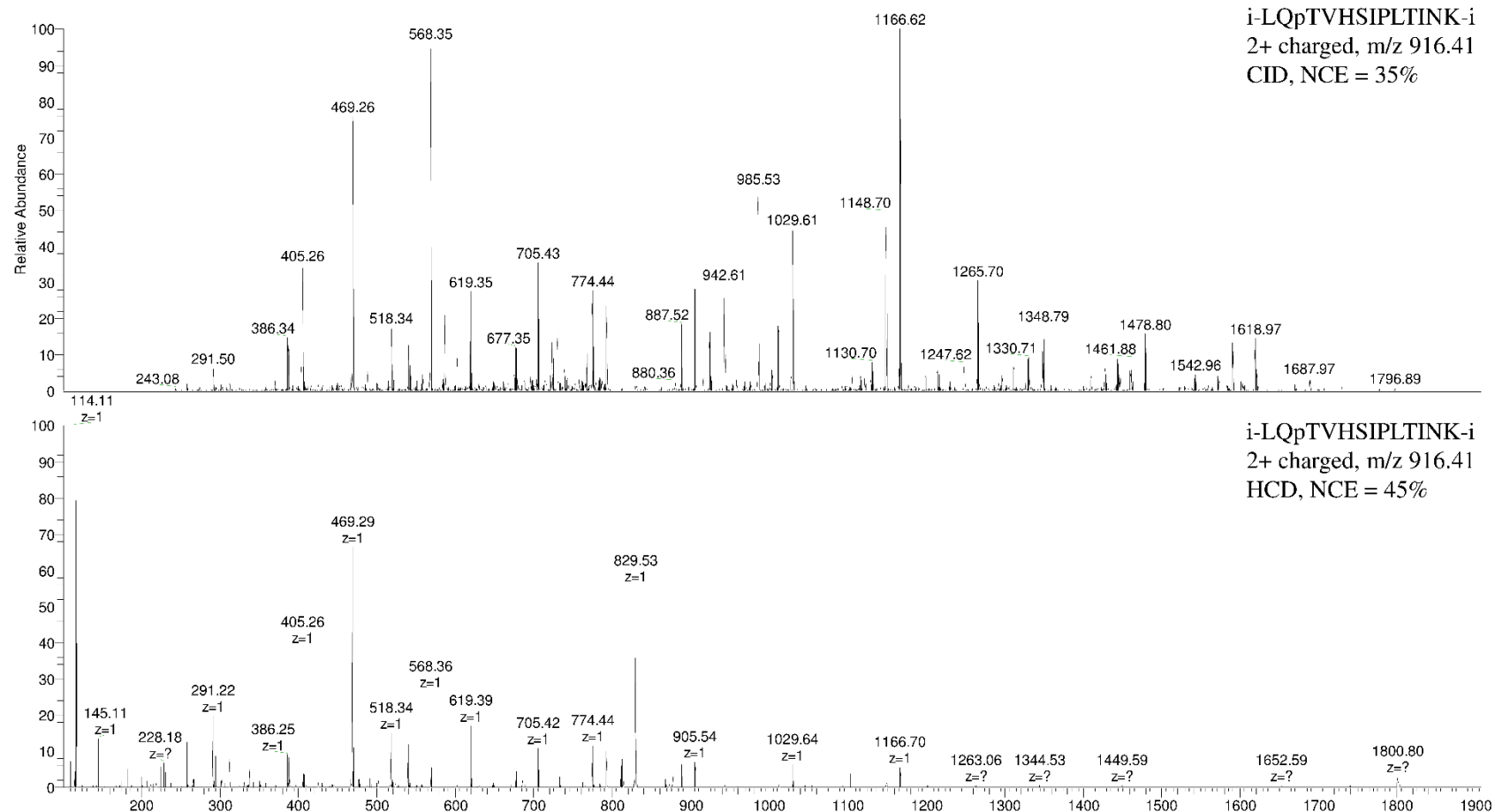
Supplementary Figure 5. Comparison of online LC-ESI-MS/MS measurements of the iTRAQ labeled $i_{114/i116}$ -phosphopeptide mix-2 fragmented by HCD at various NCE. Normalized intensities are shown, averaged over all identified peptides. (A): 14 phosphothreonine peptides, 2+ charged; (B) 15 phosphothreonine peptides, 3+ charged. (C): 30 phosphotyrosine peptides, 2+ charged; (D) 25 phosphotyrosine peptides, 3+ charged.



Supplementary Figure 6. Comparison of online LC-ESI-MS/MS measurements of the iTRAQ labeled $i_{114/i116}$ -phosphopeptide mix-2 fragmented by HCD at various NCE. Normalized intensities are shown, averaged over all identified peptides including the standard deviation. (A): 51 phosphoserine peptides, 2+ charged; (B): 52 phosphoserine peptides, 3+ charged; (C): 14 phosphothreonine peptides, 2+ charged; (D) 15 phosphothreonine peptides, 3+ charged. (E): 30 phosphotyrosine peptides, 2+ charged; (F) 25 phosphotyrosine peptides, 3+ charged.



Supplementary Figure 7. Comparison and overlap of the number of peptide identifications of mix-2 at different NCE. Blue bars: peptide identified by both, CID/MSA and HCD; orange bars: peptide only identified by CID; grey bars: peptide only identified by HCD. Numbers of identified by both (CID/MSA + HCD); identified by CID only and by HCD, respectively, displayed above the bars. (A) 2+ charged precursors; (B) 3+ charged precursors.



Supplementary Figure 8. Comparison of the 2+ charged peptide iTRAQ labeled *i*_{114/116}-LQpTVHSIPLTINK fragmented by (A) CID/MSA with an NCE of 35% and (B) HCD with and NCE of 45%. Online LC-ESI-MS/MS (mix-2).

Supplementary Table 1. Peptides used for the semi-complex mix-1 (online measurement) with a tandem CID/MSA (iontrap) and HCD (Orbitrap) method. After labeling, the ratio of the four different iTRAQ channels was 1:1:1:1.3 for 114:115:116:117. Peptides in bold were used for offline measurements with CID, CID/MSA and HCD in Orbitrap

Sequence	Phosphorylation	Phosphorylation Position	iTRAQ labeling applied	Usage
SRNSPLLER	pS	1	114	offline/online
VYELMR	pY	2	115	offline/online
VIDNEYTAR	pY	7	114	offline/online
STFHAGQLR	pT	2	115	offline/online
ATSLPSLDTPGELR	pS	6	116	offline/online
ADENYYK	pY	6	-	online
YMEDSTYYKASK	pYpY	7&8	-	online
GHLSEGLVTK	pS	4	-	online
GTVTPPPR	pT	3	-	online
VQTTPPPAVQGQK	pT	3	-	online
SRNSPLLER	pS	1	-	online
VYTHEVVTWLWYR	pT	3	-	online

Supplementary Table 2. Peptides in complex mixture (mix-2) used for online measurements with a tandem CID/MSA (iontrap) and HCD (Orbitrap) method. After labeling, the ratio of the two different iTRAQ channels was 1:0.75 for 114:116.

Sequence	Phosphorylation	Sequence	Phosphorylation
AGGKPSQSPSQEAAGEAVLGAK	pS/ 6	ADENYYK	pY/ 6
ALQKSPGPQR	pS/ 5	DGSLNQSSGYR	pY/ 10
AQSFPDNR	pS/ 3	DIYSTDYR	pY/ 7
ATSLPSLDTPGELR	pS/ 3	EALPMDTEVYESPYADPEEIRPK	pY/ 10
ATSPEAGGGGGALK	pS/ 3	EDAANNYAR	pY/ 7
AVGMPSPVSPK	pS/ 9	EPPPVVNYEEDAR	pY/ 8
DKSPSSLLEDAK	pS/ 3	EVGDYQQLHETEVLK	pY/ 5
DSPGIPPSAGAHQLFR	pS/ 8	FSDQAGPAIPTSNSYSK	pY/ 15
DSPGIPPSANAHQLFR	pS/ 2	GHGQPGADAIEKPFYVNVEFHHER	pY/ 14
ESKSSPRPTAEK	pS/ 4	GLPSDYGR	pY/ 6
ETTSPKKYYLAEK	pS/ 5	GQEYLILEK	pY/ 4
FGESDTENQNNK	pS/ 4	HSWYHGVPVSR	pY/ 4
FGSLTMDGGLR	pS/ 3	HTDDEMTGYVATR	pY/ 9
FTNSETAEHIAQGLR	pS/ 4	IKSYSFPAK	pY/ 4
GGFFSSFMK	pS/ 6	KYSLTVAVK	pY/ 2
GHLSEGLVTK	pS/ 4	LIEDNEYTR	pY/ 7
GRGSRDALVSGALESTK	pS/ 4	LSYYEYDFER	pY/ 4
GRRSPSPGNPSGR	pS/ 4	NSFNNPAYVLEGVPHQLLPPEPPSPAR	pY/ 8
HSIAGIIRSPK	pS/ 9	SISLRYEGR	pY/ 6
ILSDVTHSAVFGVPASK	pS/ 3	TAGTSFMMPYVVR	pY/ 11
IPLIKSHNDFVAILDLPEGEHQYK	pS/ 6	TIYVRDPTSNK	pY/ 3
IQPAGNTSPR	pS/ 8	VIDNEYTR	pY/ 7
IQPSSPPNHPNNHLFR	pS/ 4	VKEEGYELPYNPATDDYAVPPPR	pY/ 17
ISSLGSAQAMQMER	pS/ 3	VSPSPTTYR	pY/ 8
KTSPLNFK	pS/ 3	VYELMR	pY/ 2
LPLTRSHNNFVAILDLPEGEHQYK	pS/ 6	VYHYR	pY/ 2
LQPQEISPPPTANLDR	pS/ 7	WTAPESLAYNK	pY/ 9
LRSADSENAISVQER	pS/ 3	YATPQVIQAPGPR	pY/ 1
LTEERDGSLNQSSGYR	pS/ 8	YELTGLPEQDR	pY/ 1
MMSLSQSR	pS/ 5	YIEDEDYYK	pY/ 7
MPSHEAR	pS/ 3	YMEDSTYYK	pY/ 7
NFSAAKSLLNK	pS/ 7	YSLTVAVK	pY/ 1
NGSLKPGSSHR	pS/ 8	YVLDDDEYTSSVGSK	pY/ 7
NIDQSEFEGFSFVNSEFLKPEVK	pS/ 11	YVLDDQYTSSSGAK	pY/ 7

RDSPPPPAR	pS/ 3	AYTHQVVTR	pT/ 3
RLSIIGPTSR	pS/ 3	ETTTSPKKYYLAEK	pT/ 2
RLSSFVTK	pS/ 3	GTVTPPPR	pT/ 4
RLSSTSLASGHSVR	pS/ 3	LMTGDTYTAHAGAK	pT/ 6
RPGAAASGERDDRGPPASVAALR	pS/ 7	LQTVHSIPLTINK	pT/ 3
RSMSPFRGPK	pS/ 4	NSQPNRYTNR	pT/ 8
RSPRPDHPGTPPHK	pS/ 2	RLEEPEEPKVLTPEEQLADKLR	pT/ 12
SDGGVKPQSNNK	pS/ 9	STFHAGQLR	pT/ 2
SEGSPVLPHEPAK	pS/ 4	STGDPQGVIR	pT/ 2
SESPPLSDPK	pS/ 3	STLVLDLLK	pT/ 2
SEVAVLSPEKAENDDTYKDDVNHQK	pS/ 7	TAPTTPKR	pT/ 4
SFNGSLKNVAVDELSR	pS/ 1	THLGTGMERSPGAMER	pT/ 1
SGGQRHSPLSQR	pS/ 7	TSPLNFK	pT/ 1
SINEKDYHSR	pS/ 1	TYTHEVVTLWYR	pT/ 3
SLESVLSLGR	pS/ 1	VGSLTPSSPK	pT/ 5
SLPAPQDNDFLSR	pS/ 1	VQTTPPAVQGQK	pT/ 4
SNSTSSMSSGLPEQDR	pS/ 3	VYTHEVVTLWYR	pT/ 3
SPGPSSPKEPLLSR	pS/ 1	ARSRTPPSAPSQSR	pSpS/ 3&8
SQERPTFYR	pS/ 1	GISRSLESVLSLGRPTGGGSSPPEIR	pSpS/ 3&5
SQSDIFSR	pS/ 1	RLSSTSLASGHSVR	pSpS/ 3&4
SQSNPILGSPFFSHFDGQDSYAAAVR	pS/ 3	SFGSPNRAYTHQVVTR	pSpT/ 4&10
SQSTSEQEK	pS/ 1	TSSFAEPGGGGGGGGGGPGGSASGPGGTGGGK	pSpS/ 2&3
SRNSPLLR	pS/ 4	TVSTSSQPEENVDR	pSpS/ 3&5
SRTPPSAPSQSR	pS/ 1	IGEGTYGVVYK	pTpY/ 5&6
SSSFREMDGQPER	pS/ 2	NSFNNPAYYVLEGVPHQLLPPEPPSPAR	pYpY/ 8&9
SSSFREMENQPHK	pS/ 3	PKPSNPYNEPDEPIAFYAMGR	pYpY/ 8&18
SSSPTQYGLTK	pS/ 3	PTTGVLPSGNTLRVK	pTpT/ 2&3
STVASMMHR	pS/ 1	YIEDEDYYKASVTR	pYpY/ 7&8
SVTLPRDLQSTGR	pS/ 1	YMEDSTYYKASK	pYpY/ 7&8
SVTSMERK	pS/ 4	-	-
TGMGSGSAGKEGGPFK	pS/ 5	-	-
TVSTSSQPEENVDR	pS/ 3	-	-
VPASPLPLER	pS/ 4	-	-
VSGRTSPPLDR	pS/ 6	-	-