Supporting Information for

CharmeRT: Boosting peptide identifications by chimeric spectra identification and retention time prediction

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Elutator features

Feature	Description
MS Amanda Score	The PSM score assigned by the MS Amanda algorithm.
Delta Score	Difference of the scores between 1st and 2nd rank matches. Nonzero for 1st rank matches only.
Delta Cn	Normalized score difference relative to the first best scoring PSM of the spectrum. Zero for 1st rank matches and non-zero for rank 2 and above.
Retention Time [min]	Measured peptide retention time.
Delta RT [min]	Deviation of the measured retention time (time of spectrum scan) from the predicted.
Absolute Delta RT [min]	Absolute value of the delta retention time.
Combined Score	Combined score of the MS Amanda score and retention time deviation.
% Isolation Interference	Fraction of ion current in the isolation width not attributed to the identified precursor.
MH+ [Da]	Singly charged mass of the peptide.
m/z	Measured m/z value.
Calibrated Delta m/z [Th]	Absolute calibrated deviation of the measured m/z from the theoretical value of the peptide.
Calibrated Delta Mass [ppm]	Calibrated deviation of the measured mass from the theoretical mass of the peptide in ppm.
Peptide Length	Length of the peptide in residues as a set of binary flags: length <= 6; length = 7; length = 8; length = 9; length = 10; length >= 11
Charge State	Precursor charge state, as a set of binary flags: z <= 2; z >= 3
# Missed Cleavages	Number of missed cleavages.
Log Peptides Matched	Logarithm of the number of candidates (search space) in the precursor mass window.
Log Total Intensity	Logarithm of the total ion current of the fragment spectrum.
Fraction Matched Intensity [%]	Fraction of the total ion current of the fragment spectrum that is matched by fragments of the PSM.
Log Total Intensity of Fragments	Similar to Log Total Intensity; peaks corresponding to precursor peaks (including isotopes) are excluded.
Longest Consecutive Series Y	Length of the longest consecutive matched sequence among the y fragment ion series peaks.

Longest Consecutive Series A+B+Y	Length of the longest consecutive matched sequence confirmed by a, b or y ions.
Mean Squared Delta m/z for Fragments	Average of squared mass errors of all fragments in Th, calculated as $\sqrt{\frac{\sum {\Delta m_i}^2}{n}}$, where Δm_i is the mass difference of measured and calculated masses of fragment i, and n is the number of identified fragments.
Top Y Fragment Delta m/z	Absolute calibrated deviation of the measured m/z from the theoretical value for the top-intense y fragment. y1 and y2 ions are not considered.
Second Top Y Fragment Delta m/z	Absolute calibrated deviation of the measured m/z from the theoretical value for the second top-intense y fragment. y1 and y2 ions are not considered.

Table S1. ***All features used in Elutator to validate PSMs and peptides.***

Retention time prediction model

Our retention time prediction model can be fully described as the following non-linear sequence dependent function, which has been described by Krokhin[31], 2006:

$$H = F + newIso(seq, F) + helices1(seq) + helices2(seq)$$

where *H* is the hydrophobicity, *newIso* is a function modeling the isoelectric charge, *seq* is the peptide sequence, *helices1* and *helices2* are adjustments for short and long helices, and *F* is defined by:

$$F = sumScale(lengthScale(length) * R))$$

with *sumScale* being a polynomial function over the argument, *lengthScale* a polynomial factor dependent on the length of the peptide, *length* the length of the peptides sequence and *R* defined as:

$$R = G + smallness\left(\frac{G}{length}\right) - undigested(sequence) \\ -clusterness(sequence) - proline(sequence)$$

with *smallness* being a correction factor depending on the length of the peptide, *undigested* a function to handle special positively charged amino acids (L/H/K), *clusterness* a function for handling clusters of hydrophobic amino acids, decreasing the hydrophobicity, *proline* a function to handle sequences with >=2 prolines in the peptide sequence, and *G* defined as:

$$G = baseSumOfRetentionCoefficients(sequence) + C(sequence)$$

with *baseSumOfRetentionCoefficients* being the sum of all retention time coefficients of all amino residuals of the peptide sequence and *C* modeling the impact of neighboring amino acids (see below).

Interactions between neighboring amino acids

We describe the cumulative contribution of neighbor residual's interactions C for peptide sequence s to the hydrophobicity index as

$$C(s) = \sum_{i} \sum_{k=0}^{4} \alpha_k * f(s,i)^k$$

summed over all residues i. $f(s,i)$ is defined as

$$f(s,i) = \sum_{i-9 \le j \le i+9, j \ne i} \lambda(j-i)\beta(s_i)\gamma(s_j)$$

The summation by i,j runs through all amino residuals in the sequence s with a maximal difference of ±9 amino acid positions. For each amino acid pair, we consider two coefficients, β for the amino residual at position i in sequence s and γ for the amino residual j in sequence s, such that the interaction between the amino residuals at positions i and j is described by the product $\beta(s_i)\gamma(s_j)$. Distance coefficients $\lambda(\delta) = \lambda(j-i)$ account for the contribution of residual pairs with a distance δ between them. All coefficients, including α_{0-4}, all lambda values, and the values of the lookup tables $\beta$and γ are optimized during training of the RT model.

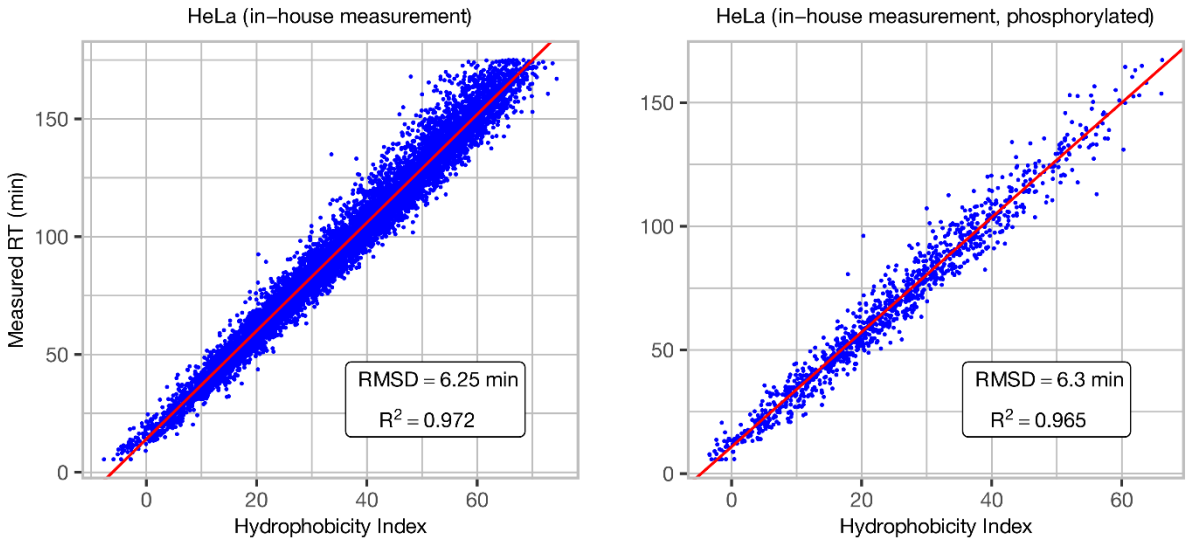

Figure S1 ***Correlation of theoretically calculated hydrophobicity index to the measured retention time for high confident matches (FDR=0.001) of in-house HeLa and TiO2 enriched data sets****. 70% of all matches in the TiO2 enriched data set contain one or more phosphorylated sites. Outliers were not removed.*

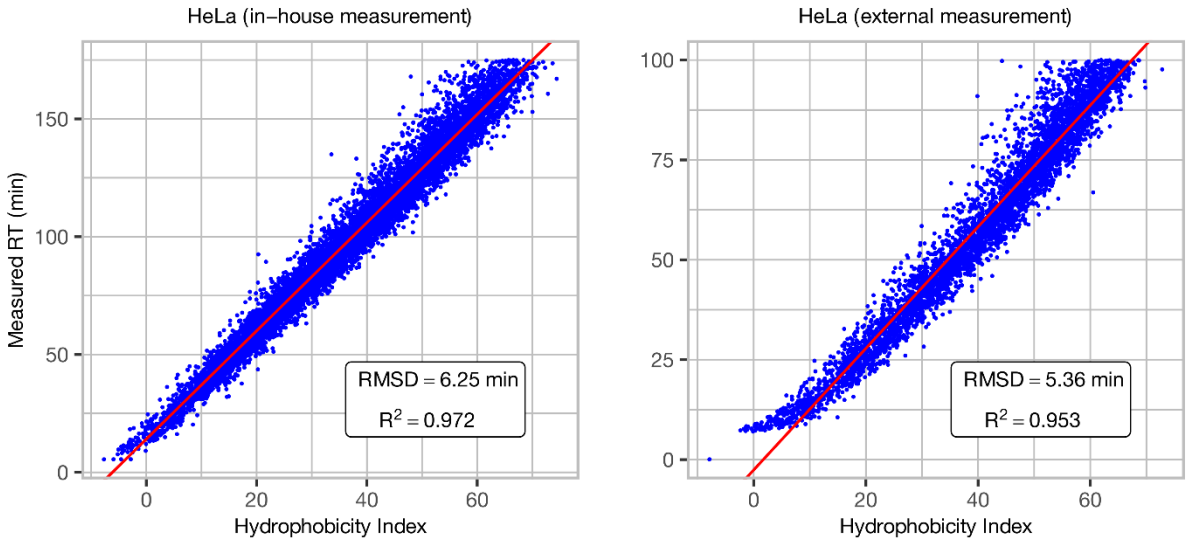

Figure S2 ***Correlation of theoretically calculated hydrophobicity index to the measured retention time for high confident matches (FDR=0.001) of in-house and external HeLa data set****. Outliers were not removed.*

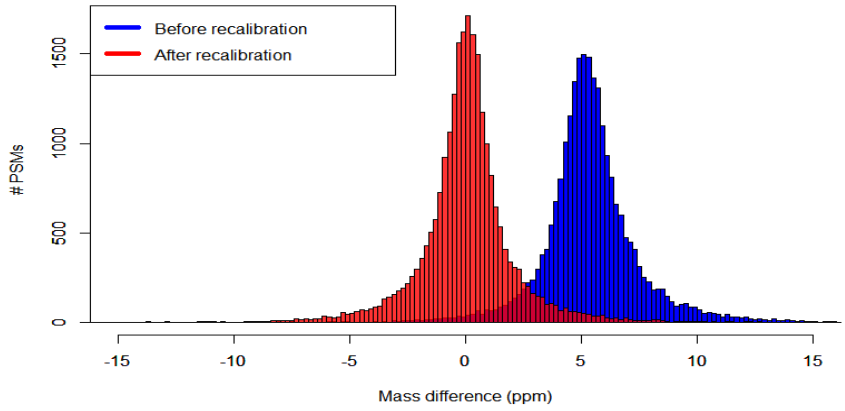

Figure S3 ***Histogram of mass deviations for highly reliable identifications before and after recalibration****, with disabled lock mass. External human dataset has been taken from Michalski et al.*[32]

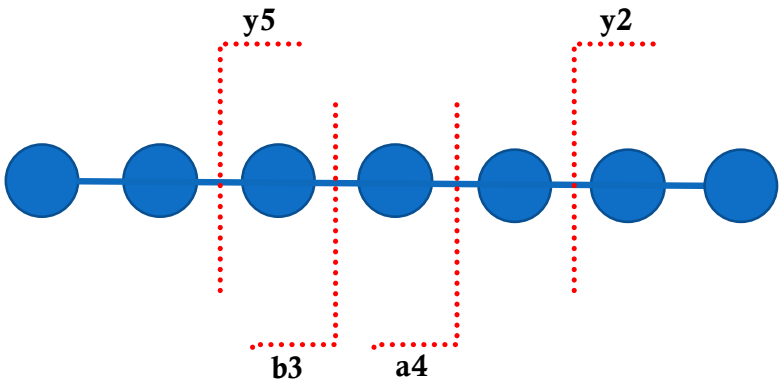

Figure S4 ***Longest Consecutive Series A+B+Y****. a4 and b3 ions confirm the sequence, filling gaps between y5 and y2 ions. The length of the Longest Consecutive Series A+B+Y is four in this case.*

		Average fragment ion overlap in % between	
Dataset	replicate	first and second peptides	second peptides
A (1h, 2m/z)	1	0.7131	0.4035
	2	0.7394	0.4054
	3	0.6850	0.4000
B (3h, 2m/z)	1	0.7182	0.3799
	2	0.7311	0.3917
	3	0.7137	0.3821
C (1h, 4m/z)	1	0.7015	0.6210
	2	0.6925	0.6876
	3	0.6945	0.6440
D (3h, 4m/z)	1	0.6830	0.6652
	2	0.6621	0.6914
	3	0.6860	0.7073
E (1h, 8m/z)	1	0.6510	0.9318
	2	0.6346	0.9333
	3	0.6232	0.9497
F (3h, 8m/z)	1	0.6620	1.0482
	2	0.6662	1.0300
	3	0.6569	1.0371

Table S2 ***Shared ions between first and second peptides.*** *Overlap of fragment ions given in percent between peptides identified in the first and in the second search or between peptides in the second search, when multiple precursors were identified in the second search.*

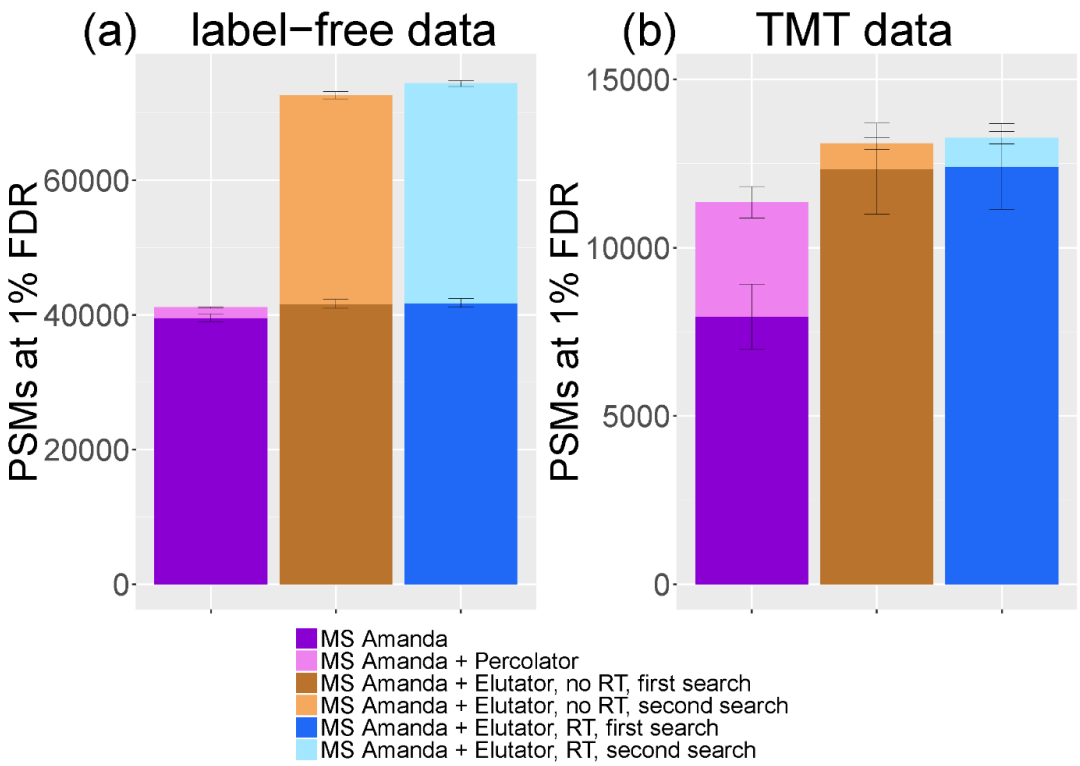

Figure S5 ***Results for data of O'Connell et al.[35]*** *Data have been analyzed with Protein Discoverer 1.4.using 10ppm(a)/50 ppm(b) precursor mass tolerance and 0.02Da/0.9Da fragment mass tolerance. a) Label –free data acquired at 1.4 m/z isolation width shows a high number of chimeric spectra that can be identified by CharmeRT. b) TMT data has been measured with an isolation window of 0.4 m/z showing a very low number of high confident interfering peptides. Still, the usage of CharmeRT is beneficial in this case as well, as it leads to the identification of more PSMs at the same FDR than the combination of MS Amanda and Percolator.*

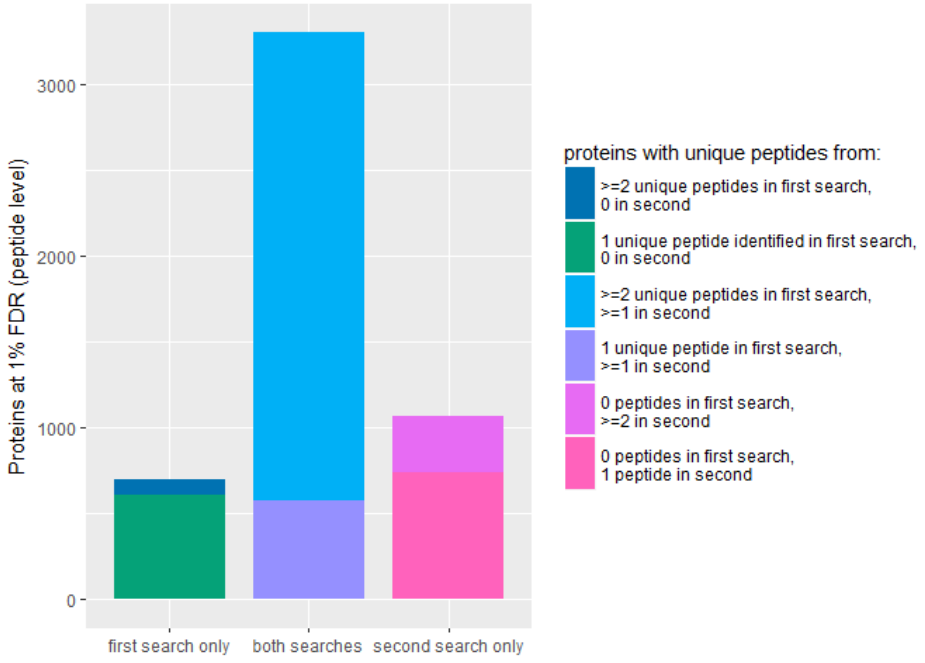

Figure S6 ***Protein evidence origin****. Proteins from a single HeLa run (4m/z, 3h gradient) are investigated and identified peptides are analyzed. The major part of proteins can be confirmed by peptide identifications from both searches, some proteins are only found in one of the two search iterations. Protein inference and grouping has been performed with Proteome Discoverer 1.4.*

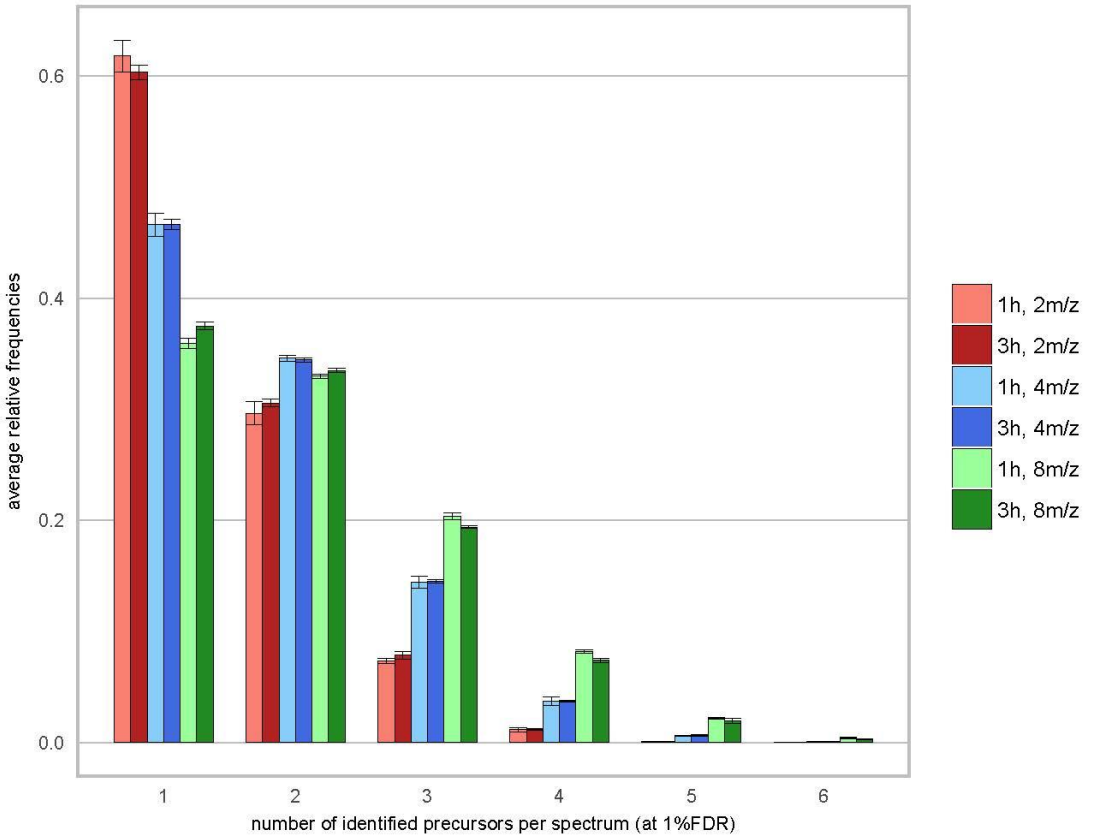

Figure S7 ***Presence of chimeric spectra in data sets with different isolation widths and gradient times****. All spectra having two or more reliably identified precursors are chimeric spectra. As expected, the presence of chimeric spectra rises with increasing isolation width.*

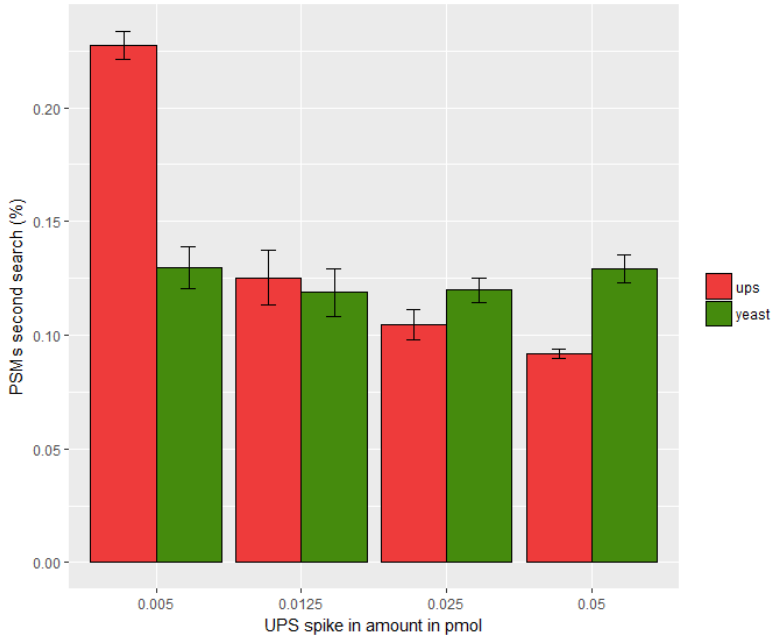

Figure S8 ***Proportion of second search PSMs for spike-in data****[40]. For low spike-in amounts, the proportion of UPS peptides is higher in the second search, as these originate from rare proteins and are therefore more likely to be coeluting peptides.*

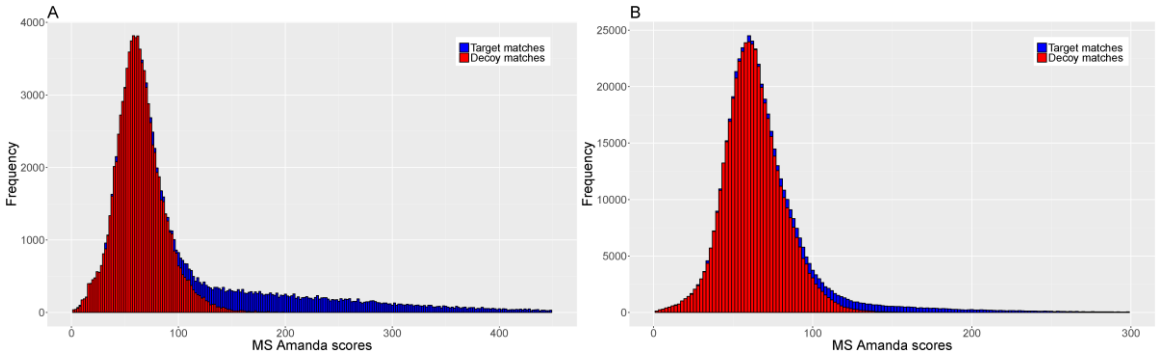

Figure S9 ***Score distributions of MS Amanda scores*** *for target (blue) and decoy (red) peptides identified in the first (A) or second (B) search. The spectrum quality for co-eluting peptides is lower and the score distributions of target matches of the second search look very similar to decoy matches, so the effect of including auxiliary information used in Elutator is higher.*

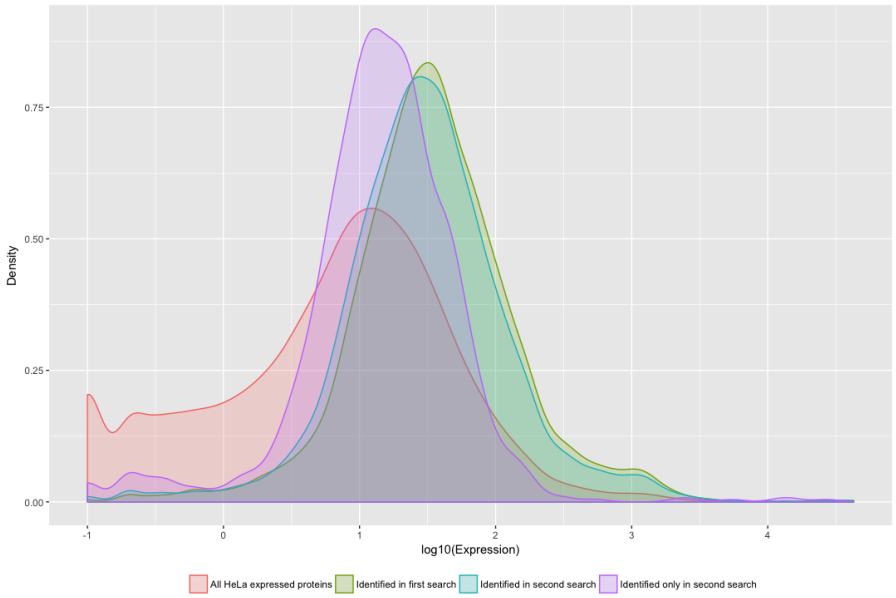

Figure S10 ***RNA abundance of HeLa proteins****. All HeLa proteins are depicted in red, all proteins identified in the first search in light green, all proteins identified in the second search in dark green and proteins solely identified in the second search are purple.*

Data set	Figure	Method	Ø PSMs first search	Ø PSMs second search	Ø PSMs added through validation	Ø Unique peptides first search	Ø Unique peptides overlap	Ø Unique peptides second search
A	3	CharmeRT	14506	10244		9445	3491	5360
B	3	CharmeRT	27340	20725		14716	8368	8262
C	3	CharmeRT	15918	17444		8308	5629	7715
D	3	CharmeRT	27234	32565		11131	11712	10295
E	3	CharmeRT	14219	25409		5064	7500	7874
F	3	CharmeRT	23138	44905		6266	13106	9178
G	4	Mascot + Percolator	4088		1371	2931		
G	4	MaxQuant	3525	398		2621		315
G	4	pParse + Mascot + Percolator	4379	109		3140		0
G	4	MS Amanda + Percolator	3709		704	2679		
G	4	MS Amanda + Elutator (no RT)	4511	247		3032	152	66
G	4	CharmeRT	5128	335		3371	199	88
H	4	Mascot + Percolator	17916		4996	14201		
H	4	MaxQuant	15488	1284		12111		960
H	4	pParse + Mascot + Percolator	16752	7089		13441		2999
H	4	MS Amanda + Percolator	18651		5313	14727		
H	4	MS Amanda + Elutator (no RT)	19720	15447		10330	5182	5948
H	4	CharmeRT	20199	18174		10107	5778	7062
I	2	Mascot + Percolator	21177		3047	17276		
I	2	MaxQuant	18973	1448		15095	1048	
I	2	pParse + Mascot + Percolator	20568	6012		16813	1994	
I	2	MS Amanda + Percolator	21203		1782	17230		
I	2	MS Amanda + Elutator (no RT)	22313		9770	13526	4614	3327
I	2	CharmeRT	22796		11970	13191	5346	4232

Table S3: ***Identified PSMs and unique peptides at 1% FDR*** *(PSM or peptide level) for all Figures presented in the manuscript*

	Total protein groups	RNA Expressed	Contaminants	No RNA Expression Data	Zero RNA Expression
First search only	3741	3550	24	85	82
First + Second searches	4696	4435	30	118	113

Table S4: ***Mapping grouped proteins identified in first and second searches to RNA HeLa protein expression data.***

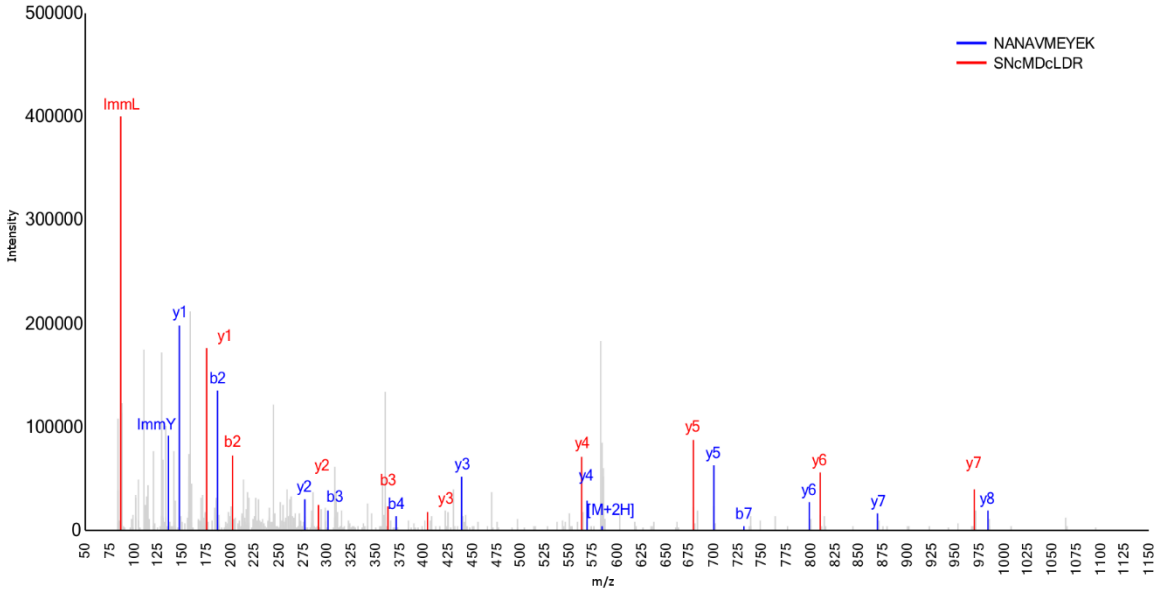

Figure S11 ***Chimeric spectrum example.*** *Spectrum is part of data set D (HeLa tryptic digest, Q Exactive Hybrid, 3h gradient, 4m/z isolation width).Matched ions of* ***p****eptide NANAVMEYEK are given in blue and ions of peptide SNcMDcLDR (with Cs being carbamidomethylated) are given in red.*

NANAVMEYEK				SNcMDcLDR		
matched ion	m/z	delta mass [ppm]		matched ion	m/z	delta mass [ppm]
[M+2H]	584.768	48.87		ImmL	86.096	6.39
ImmY	136.076	0.44		y1	175.119	0.17
y1	147.113	1.09		b2	202.082	0.45
b2	186.087	1.34		y2	290.146	2.41
y2	276.155	1.23		b3	362.113	3.07
b3	300.130	2.00		y3	403.230	8.06
y3	439.219	0.93		y4	563.261	1.70
b4	371.167	3.23		y5	678.288	1.39
y4	568.261	3.54		y6	809.328	2.29
y5	699.302	1.97		y7	969.359	4.18
y6	798.370	1.79				
b7	730.319	10.43				
y7	869.407	1.14				
y8	983.450	0.00				

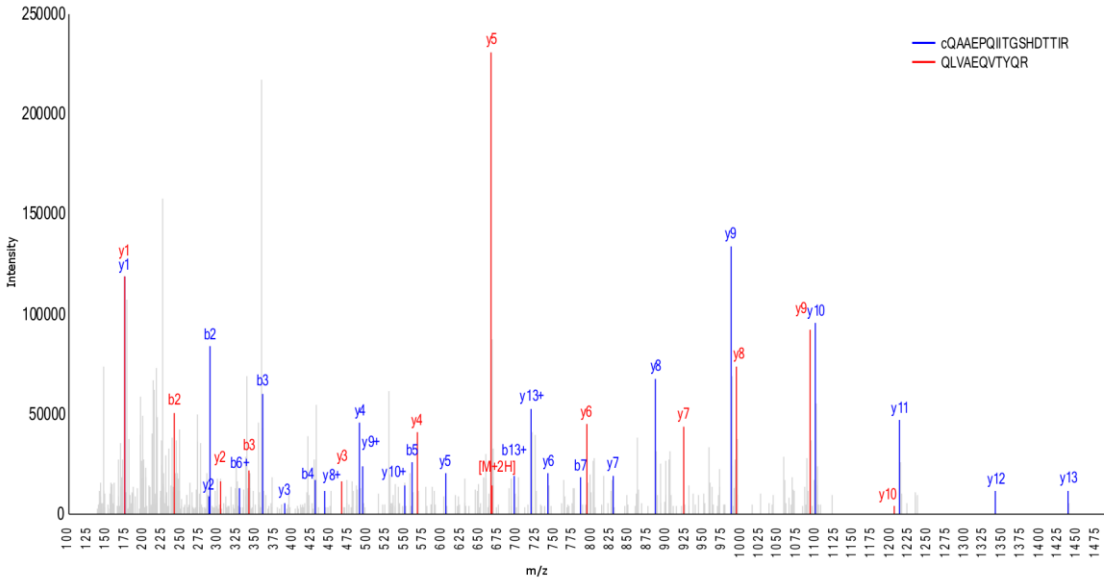

Figure S12 ***Chimeric spectrum example.*** *Spectrum is part of data set D (HeLa tryptic digest, Q Exactive Hybrid, 3h gradient, 4m/z isolation width).Matched ions of* ***p****eptide cQAAEPQIITGSHDTTIR (with C being carbamidomethylated) are given in blue and ions of peptide QLVAEQVTYQR are given in red.*

cQAAEPQIITGSHDTTIR				QLVAEQVTYQR		
matched ion	m/z	delta mass [ppm]		matched ion	m/z	delta mass [ppm]
y1	175.119	0.57		[M+2H]	667.857	16.40
b2	289.097	1.80		y1	175.119	0.57
y2	288.203	3.37		b2	242.150	1.36
b3	360.134	1.86		y2	303.178	1.25
y3	389.251	0.23		b3	341.218	3.25
b4	431.171	10.09		y3	466.241	0.62
y4	490.298	1.63		y4	567.289	1.53
b5	560.213	7.93		y5	666.357	53.08
y5	605.325	7.19		y6	794.416	0.35
b6+	329.137	24.82		y7	923.458	2.01
y6	742.384	0.15		y8	994.495	1.21
b7	785.325	14.91		y9	1093.564	1.26
y7	829.416	10.38		y10	1206.648	10.26
y8	886.438	1.74				
y8+	443.723	0.88				
y9	987.485	0.35				
y9+	494.246	7.30				
y10	1100.569	1.85				
y10+	550.788	0.20				
y11	1213.654	5.69				
y12	1341.712	0.48				
b13+	697.330	48.34				
y13	1438.765	1.01				
y13+	719.886	1.18				

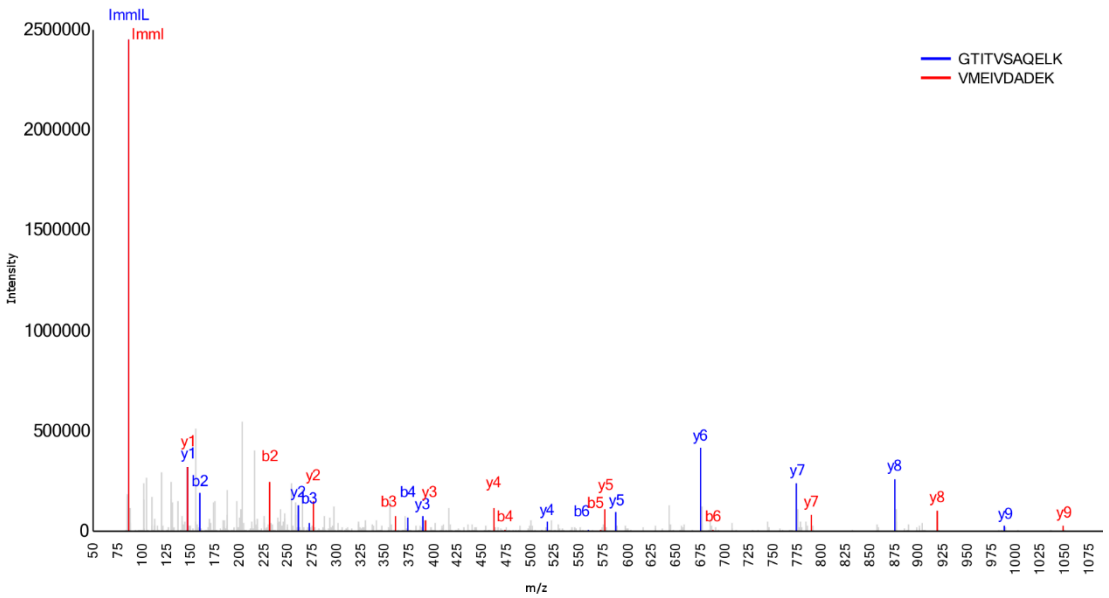

Figure S13 ***Chimeric spectrum example.*** *Spectrum is part of data set D (HeLa tryptic digest, Q Exactive Hybrid, 3h gradient, 4m/z isolation width).Matched ions of* ***p****eptide GTITVSAQELK are given in blue and ions of peptide VMEIVDADEK are given in red.*

GTITVSAQELK				VMEIVDADEK		
matched ion	m/z	delta mass [ppm]		matched ion	m/z	delta mass [ppm]
ImmIL	86.096	6.27		ImmI	86.096	6.27
y1	147.113	0.95		y1	147.113	0.95
b2	159.076	0.57		b2	231.116	2.60
y2	260.197	0.42		y2	276.155	0.80
b3	272.161	8.78		b3	360.159	5.14
y3	389.239	1.70		y3	391.182	0.05
b4	373.208	7.98		b4	473.243	3.61
y4	517.298	0.41		y4	462.220	3.07
y5	588.335	0.29		b5	572.311	12.34
b6	559.309	64.06		y5	577.246	7.73
y6	675.367	3.01		b6	687.338	1.82
y7	774.436	2.20		y7	789.399	2.51
y8	875.483	1.98		y8	918.442	2.23
y9	988.567	4.54		y9	1049.482	4.60

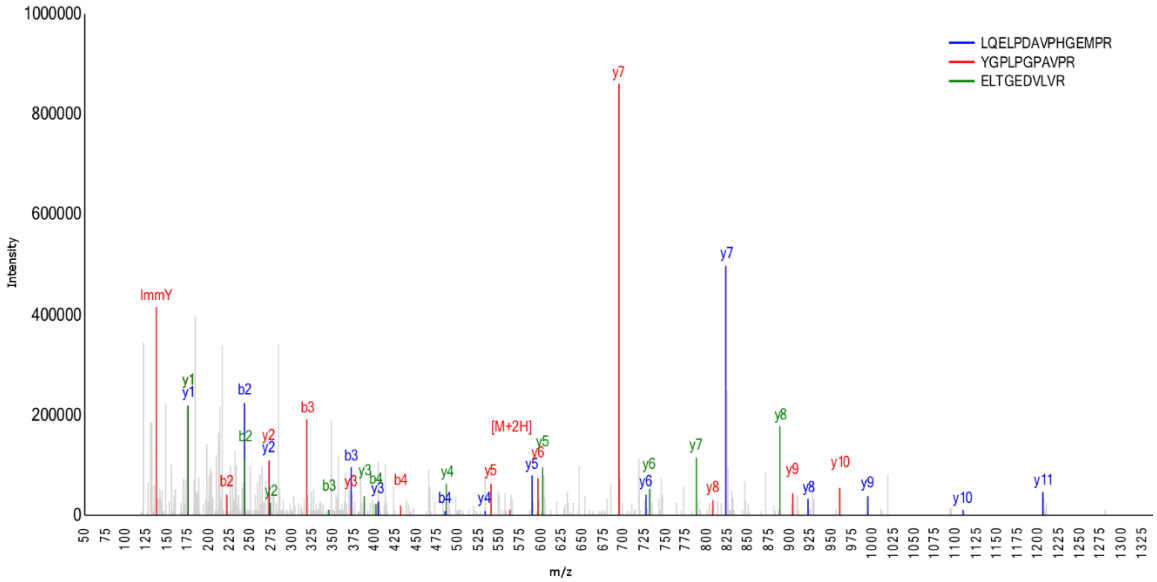

Figure S14 ***Chimeric spectrum example.*** *Spectrum is part of data set D (HeLa tryptic digest, Q Exactive Hybrid, 3h gradient, 4m/z isolation width).Matched ions of* ***p****eptide LQELPDAVPHGEMPR are given in blue, ions of peptide YGPLPGPAVPR are given in red, and ions of peptide ELTGEDVLVR are given in green.*

LQELPDAVPHGEMPR				YGPLPGPAVPR				ELTGEDVLVR		
matched ion	m/z	delta mass [ppm]		matched ion	m/z	delta mass [ppm]		matched ion	m/z	delta mass [ppm]
y1	175.119	0.17		[M+2H]	562.316	11.58		y1	175.119	0.17
b2	242.150	1.57		ImmY	136.076	0.37		b2	243.134	6.91
y2	272.172	0.66		y1	175.119	0.17		y2	274.187	0.15
b3	371.193	0.24		b2	221.092	6.06		b3	344.182	35.59
y3	403.212	49.75		y2	272.172	0.66		y3	387.271	0.49
b4	484.277	86.36		b3	318.145	0.97		b4	401.203	4.89
y4	532.255	3.70		y3	371.240	15.17		y4	486.340	0.41
y5	589.276	1.22		b4	431.229	0.60		y5	601.367	0.23
y6	726.335	0.73		y5	539.330	1.61		y6	730.409	2.12
y7	823.388	2.25		y6	596.351	0.99		y7	787.431	8.90
y8	922.456	2.07		y7	693.404	1.62		y8	888.479	1.56
y9	993.494	17.11		y8	806.488	2.00				
y10	1108.520	3.39		y9	903.541	0.76				
y11	1205.573	2.84		y10	960.563	17.09				

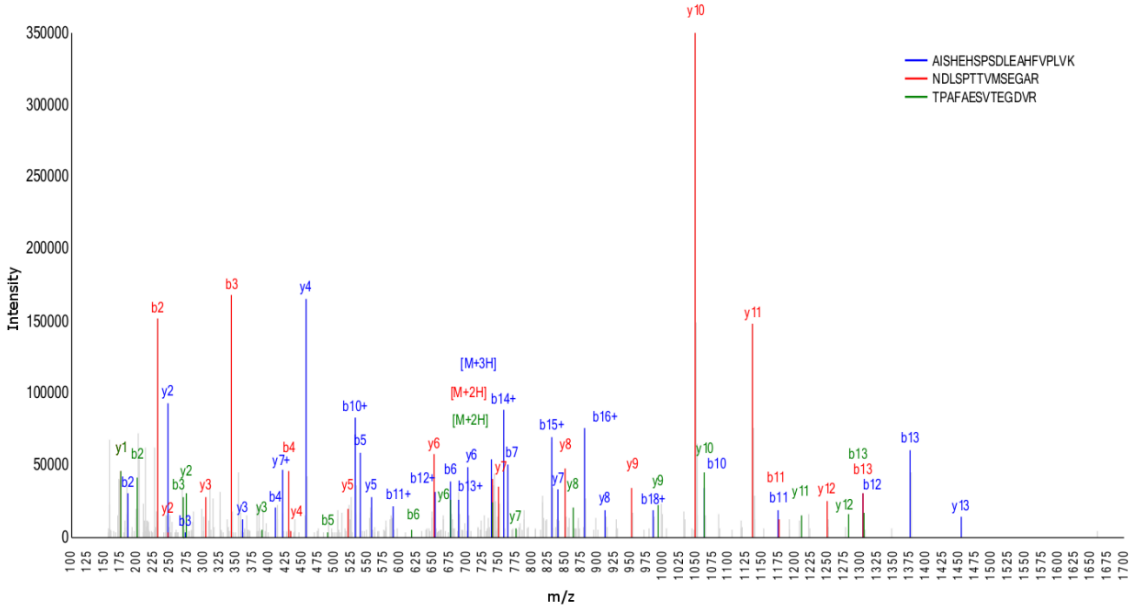

Figure S15 ***Chimeric spectrum example.*** *Spectrum is part of data set D (HeLa tryptic digest, Q Exactive Hybrid, 3h gradient, 4m/z isolation width).Matched ions of* ***p****eptide AISHEHSPSDLEAHFVPLVK are given in blue, ions of peptide NDLSPTTVMSEGAR are given in red, and ions of peptide TPAFAESVTEGDVR are given in green.*

AISHEHSPSDLEAHFVPLVK				NDLSPTTVMSEGAR				TPAFAESVTEGDVR		
matched ion	m/z	delta mass [ppm]		matched ion	m/z	delta mass [ppm]		matched ion	m/z	delta mass [ppm]
[M+3H]	738.383	16.92		[M+2H]	739.352	15.70		[M+2H]	739.854	16.37
b2	185.128	2.70		y1	175.119	0.06		y1	175.119	0.06
y2	246.181	1.18		b2	230.077	0.26		b2	199.108	0.95
b3	272.161	25.10		y2	246.156	13.69		y2	274.187	2.52
y3	359.265	1.39		b3	343.161	1.08		b3	270.145	0.44
b4	409.219	0.73		y3	303.178	1.65		y3	389.214	9.71
y4	456.318	2.59		b4	430.193	1.12		b5	488.250	34.57
b5	538.262	1.97		y4	432.220	6.13		b6	617.293	7.27
y5	555.386	5.64		y5	519.252	6.89		y6	676.326	8.32
b6	675.321	1.05		y6	650.293	2.34		y7	775.394	10.38
y6	702.455	0.23		y7	749.361	3.23		y8	862.427	5.61
b7	762.353	2.53		y8	850.409	0.36		y9	991.469	11.50
y7	839.514	5.86		y9	951.456	5.75		y10	1062.506	18.51
y7+	420.261	0.14		y10	1048.509	0.44		y11	1209.575	4.79
y8	910.551	9.70		b11	1175.525	16.32		y12	1280.612	8.43
b10	1061.465	0.42		y11	1135.541	0.74		b13	1304.600	0.58
b10+	531.236	0.08		y12	1248.625	5.18				
b11	1174.549	3.58		b13	1303.583	0.66				
b11+	587.778	9.41								
b12	1303.591	5.48								
b12+	652.299	0.69								
b13	1374.629	1.18								
b13+	687.818	5.73								
y13	1451.789	4.42								
b14+	756.347	0.38								
b15+	829.882	5.45								
b16+	879.416	0.10								
b18+	984.484	11.78								

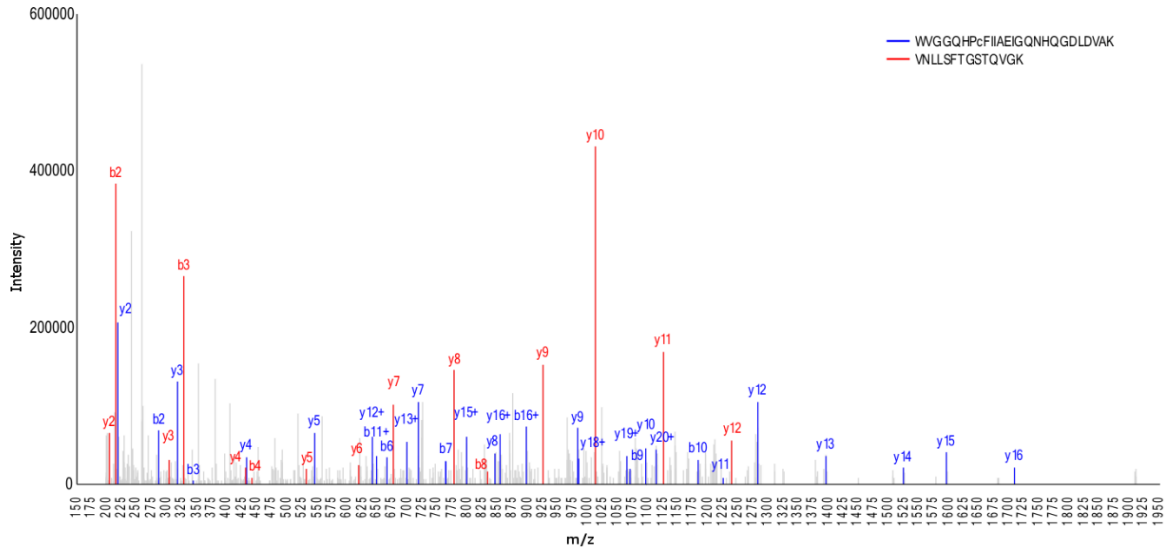

Figure S16 ***Chimeric spectrum example.*** *Spectrum is part of data set D (HeLa tryptic digest, Q Exactive Hybrid, 3h gradient, 4m/z isolation width).Matched ions of* ***p****eptide WVGGQHPcFIIAEIGQNHQGDLDVAK (with C being carbamidomethylated) are given in blue and ions of peptide VNLLSFTGSTQVGK are given in red.*

WVGGQHPcFIIAEIGQNHQGDLDVAK				VNLLSFTGSTQVGK		
matched ion	m/z	delta mass [ppm]		matched ion	m/z	delta mass [ppm]
b2	286.155	0.63		b2	214.119	0.05
y2	218.150	0.50		y2	204.134	1.22
b3	343.177	31.44		b3	327.203	0.18
y3	317.218	0.06		y3	303.203	4.22
y4	432.245	5.92		b4	440.287	1.91
y5	545.329	4.57		y4	431.261	0.93
b6	665.315	3.71		y5	532.309	6.42
b7	762.368	9.88		y6	619.341	14.32
y7	717.378	1.60		y7	676.362	5.46
y8	845.436	4.96		b8	832.456	58.05
b9	1069.467	1.07		y8	777.410	2.97
y9	982.495	2.59		y9	924.479	0.12
b10	1182.551	7.66		y10	1011.511	2.55
y10	1096.538	2.31		y11	1124.595	0.74
b11+	648.321	56.56		y12	1237.679	1.52
y11	1224.597	1.56				
y12	1281.618	0.41				
y12+	641.313	2.96				
y13	1394.702	2.52				
y13+	697.855	0.50				
y14	1523.745	0.32				
y14+	762.376	0.62				
y15	1594.782	5.22				
y15+	797.895	1.20				
b16+	897.443	41.53				
y16	1707.866	1.48				
y16+	854.437	2.25				
y18+	984.513	5.47				
y19+	1064.528	27.45				
y20+	1113.055	4.11				

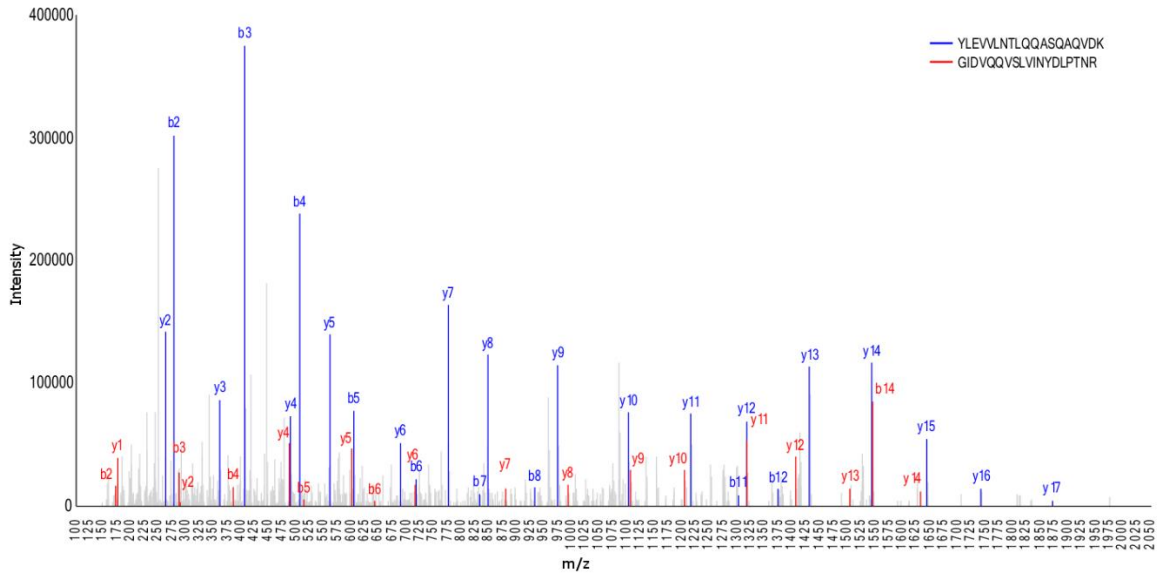

Figure S17 ***Chimeric spectrum example.*** *Spectrum is part of data set D (HeLa tryptic digest, Q Exactive Hybrid, 3h gradient, 4m/z isolation width).Matched ions of* ***p****eptide YLEVVLNTLQQASQAQVDK are given in blue and ions of peptide GIDVQQVSLVINYDLPTNR are given in red.*

YLEVVLNTLQQASQAQVDK				GIDVQQVSLVINYDLPTNR		
matched ion	m/z	delta mass [ppm]		matched ion	m/z	delta mass [ppm]
b2	277.155	0.47		y1	175.119	0.06
y2	262.140	0.42		b2	171.113	1.58
b3	406.197	0.00		y2	289.162	6.22
y3	361.208	2.60		b3	286.140	1.29
b4	505.266	0.10		b4	385.208	1.38
y4	489.267	4.50		y4	487.262	1.89
b5	604.334	6.47		b5	513.267	7.05
y5	560.304	3.77		y5	600.346	2.20
b6	717.418	11.76		b6	641.325	5.40
y6	688.362	4.74		y6	715.373	1.47
b7	831.461	13.11		y7	878.437	13.98
y7	775.395	0.27		y8	992.480	5.34
b8	932.509	7.75		y9	1105.564	3.84
y8	846.432	0.26		y10	1204.632	10.48
y9	974.490	25.20		y11	1317.716	17.92
y10	1102.549	1.60		y12	1404.748	2.99
b11	1301.710	28.26		y13	1503.816	12.93
y11	1215.633	0.26		b14	1544.796	10.60
b12	1372.747	36.05		y14	1631.875	0.00
y12	1316.681	0.39				
y13	1430.724	0.51				
y14	1543.808	0.86				
y15	1642.876	2.73				
y16	1741.944	2.58				
y17	1870.987	2.45				

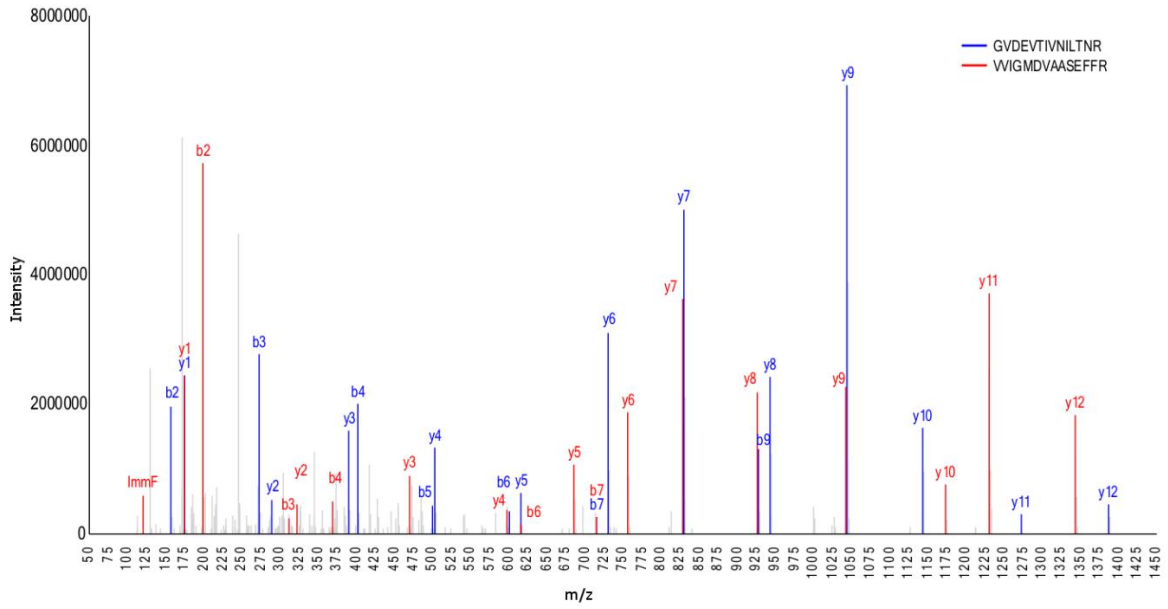

Figure S18 ***Chimeric spectrum example.*** *Spectrum is part of data set D (HeLa tryptic digest, Q Exactive Hybrid, 3h gradient, 4m/z isolation width).Matched ions of* ***p****eptide GVDEVTIVNILTNR are given in blue and ions of peptide VVIGMDVAASEFFR are given in red.*

GVDEVTIVNILTNR				VVIGMDVAASEFFR		
matched ion	m/z	delta mass [ppm]		matched ion	m/z	delta mass [ppm]
y1	175.119	0.17		ImmF	120.081	3.08
b2	157.097	0.95		y1	175.119	0.17
y2	289.162	0.24		b2	199.144	0.90
b3	272.124	3.42		y2	322.187	0.99
y3	390.210	2.18		b3	312.228	1.02
b4	401.167	0.72		y3	469.256	0.13
y4	503.294	1.35		b4	369.250	1.33
b5	500.235	6.08		y4	598.298	5.03
y5	616.378	4.10		y5	685.330	3.05
b6	601.283	2.79		b6	615.317	4.73
y6	730.421	0.29		y6	756.368	1.63
b7	714.367	15.58		b7	714.385	10.46
y7	829.489	3.70		y7	827.405	1.62
y8	942.573	1.10		y8	926.473	3.21
b9	927.478	2.64		y9	1041.500	0.82
y9	1043.621	2.78		y10	1172.541	1.58
y10	1142.689	0.57		y11	1229.562	0.78
y11	1271.732	0.05		y12	1342.646	0.45
y12	1386.759	7.77				