Supporting information

An integrated proteogenomic approach identifying a protein signature of COPD and a new splice variant of SORBS1

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1 Detailed material and methods

1.1 Lung tissue samples

Left-over frozen lung tissue from 10 ex-smoker stage IV COPD patients and 8 ex-smoker non-COPD controls was used. All subjects were Caucasian. All samples were obtained from peripheral lung tissue according to national and local ethical guidelines and the research code of the UMCG[1]. Non-COPD control (referred throughout as 'control') was defined with lung function FEV1/FVC \geq 70% and FEV1 >90% predicted. Subjects in this category did not have a history of lung disease, apart from lung cancer for which the patients underwent surgery. For these cases, lung tissue samples were taken as distant from the tumor as possible. Thus, any possible effect of the tumor on the lung tissue was minimised. All COPD patients suffered from severe emphysema and underwent lung transplantation.

1.2 Sample preparation of frozen human lung tissue samples for proteomics analysis All chemicals were from Sigma Aldrich (St. Louis, Missouri, US) if not stated otherwise.

1.3 Transcriptomics analysis

Total RNA from parenchymal lung samples was isolated using TRIzol Reagent (Ambion, Foster City, CA), according to manufacturer's instructions. We extracted PolyA-tailed mRNA fraction from total RNA and prepared RNA-seq libraries using NEXTFlex Rapid Directional qRNA-Seq Kit (Bioo Scientific, Austin, TX) according to the manufacturer's protocol. Libraries were pooled and sequenced on HiSeq2500 sequencer (Illumina, San Diego, CA) using paired-end 2×110bp mode. Quality of resulting reads was evaluated by FastQC software (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Unique molecular identifiers (UMIs) and flanking T base were trimmed using custom Perl script. Quality/adaptor trimming was performed using Trimmomatic software v. 0.33[2]. Trimmed reads were mapped to reference genome GRCh38 either by STAR program v. 2.4.1d[3] or by TopHat2 (version 2.0.13)[4] using ensembl gene annotation release 80 (http://www.ensembl.org). The reason to use two reference genome alignment approaches is that the outcomes of TopHat2 and STAR aligner were significantly different. In the absence of the "ground truth for alignment" and in order to present the most complete set of transcriptome isoforms for peptide identification, we have used both alignment methods. Duplicate reads (reads mapping to the same genomic positions and having same combination of UMIs) were excluded using custom Perl script. Reads were quantified using HTSeq program, v. 0.6.1p1[5]. Transcript assembly was performed from alignments using StringTie v. 1.3.3[6] and Trinity v. 2.0.0[7]. The longest open reading frames of new transcripts were predicted using custom Perl script (all scripts are available upon request).

We used the BCFtools program (<u>http://htslib.org</u>) v. 0.1.17 to call variants on the combined set of RNA-seq alignments. Only high quality variants were retained (variant quality >50). They were annotated using snpEff tool (<u>http://snpeff.sourceforge.net</u>, version 4.1b) using gene annotation from aforementioned Ensembl release. Variants that change amino acid sequences were extracted and applied to reference protein sequences using a custom perl script. The resulting protein isoforms affected by non-synonymous variants were added to reference protein database.

Based on the Ensembl transcript annotation, RNA sequencing generated a total of 32,435 predicted translated non-redundant protein sequences (18,624-23,780 per sample, Table S6). These corresponded to 15,708 human protein-coding genes with detectable expression level in our samples and used for differential transcript expression analysis.

1.4 Lysis, protein extraction and reduction alkylation

Frozen lung tissue samples were lysed in 6 M urea and 50 mM ammonium bicarbonate buffer for 30 min on ice using thorough mixing. Samples were centrifuged at 10,000 g for 10 min and supernatants transferred into a new vial. Pierce BCA kit was used for protein determination. To reduce cysteine residues 10 mM DTT was added (1 h at 60 °C) followed by 50 mM iodoacetamide (30 min at room temperature in the dark) to disrupt native disulphide bonds and prevent their formation. Finally, 0.02 μ g/ μ L chicken lysozyme was added to the extracts (internal control)[8].

1.5 Buffer exchange and trypsin digestion

Hundred microliters of each sample was filtered on Amicon Ultra centrifuge filters (0.5 mL volume with 10 kDa filter cutoff, Millipore, Dublin, Ireland). Samples were washed three times in 50 mM

ammonium bicarbonate buffer (14,000 g for 20 minutes). The proteins remained on the filter were washed off with 50 mM ammonium bicarbonate buffer with centrifugation (3 minutes, 1,000 g). Trypsin digestion was performed overnight at 37°C after addition of 10 μ L trypsin (0.1 μ g/ μ L, Promega, Madison, Wisconsin, US) having 1:100 protein:enzyme ratio (w/w). The digestion was stopped with 24 μ L 10% formic acid (FA) and the samples were dried in a vacuum concentrator at room temperature for 9 hours. The samples were dissolved in 100 μ L water/0.1% FA providing a final protein concentration of 1 μ g/ μ L. The digests were centrifuged (5 minutes, 10,000×g) and 20 μ L of peptide retention time calibration mixture (PRTC, product # 88320, Pierce, Rockford, Illinois, US) was added to each sample (25 fmoL/ μ L).

1.6 Fractionation (SCX) and desalting

Fifty microliters of each protein digest was dried in a vacuum concentrator, and the samples was dissolved in 10 mM KH₂PO₄/20% acetonitrile (ACN). Strong cation exchange (SCX) columns (SEM HIL-SCX, 10-100 μ g capacity, The Nest group Inc., SouthBorough, Massachusetts, USA) were used for fractionation. Each column was washed with (1) 100 μ L MeOH, 100 μ L Milli-Q water, (2) 100 μ L 50:50 of 10 mM KH₂PO₄/20% ACN and (3) 1 M KCl and 10 mM KH₂PO₄/20% ACN at pH 2.8. Each washing step was followed by centrifugation (1 minute, 110 \times g). The columns were equilibrated and washed twice with 100 μ L 10 mM KH₂PO₄/20% ACN at pH 2.8 (1 minute, 200 \times g). Fifty microliters of each sample was loaded onto the column, centrifuged (1 minute, 110 \times g), and the flow-through (FT) was collected. The columns were then washed with 100 μ L 10 mM KH₂PO₄/20%ACN, and the eluent was combined with the FT of the previous step. Six fractions were collected using stepwise gradient of 20 (pooled with combined FT), 40, 60, 100, 500 and 1,000 mM KCl buffer resulting in ~8.3 μ g peptide per fraction. All fractions were dried in a vacuum concentrator and resuspended in 100 μ L 0.1% TFA in water. The resuspended solutions were desalted on C18 spin columns.

C18 spin columns (UltraMicroSpin Column C18, SUM SS18V, capacity 3-30 μ g, The Nest group Inc., South Borough, Massachusetts, USA) for desalting were conditioned with 100 μ L ACN/0.1% TFA and centrifugation (1 minute, 110 \times g). The spin columns were then flushed twice with 100 μ L 0.1% TFA and centrifuged (1 minute, 110 \times g). Fractions were loaded onto separate C18 spin columns and centrifuged (1 minute, 200 \times g), and washed twice with 100 μ L of 0.1% TFA (1 minute, 200 \times g). The desalted peptides were eluted with 2 \times 50 μ L of 50% ACN/0.1% TFA (1 minute, 200 \times g), and dried in a vacuum concentrator. The dried fractions were resolved in 17 μ L of 0.1% FA (peptide concentration of 0.5 μ g/ μ L). Finally, 17 μ L of each sample was mixed with PRTC mixture (12.5 fmoL/ μ L) in 1:1 v/v ratio.

1.7 Data-dependent LC-MS/MS analysis

The samples were first loaded onto a trap column (75 μ m × 2 cm, C18, 3 μ m and 100 Å, Thermo Fisher Scientific, San José, California, USA) at 3 μ L/min using solvent A (0.1% FA) and then separated using analytical column (75 μ m × 50 cm [or 25 cm], C18, 2 μ m, 100 Å, Thermo Fisher Scientific) using 300 nL/min flow rate and column temperature of 35°C. A linear gradient was applied for liquid chromatography separation, using solvent A and solvent B (0.1% FA in ACN). The gradient of non-fractionated samples went from 5 to 40% B in the first 120 minutes, followed by raise to 90% B in the next 5 minutes and maintained for 10 minutes. The fractionated samples were separated in a 90 minutes gradient (5 to 40% B). Samples injections were randomized for each analyzed data set (1D50, 1D25 and 2D25) separately and 45 minutes blank injections were applied after each sample analysis.

Two and 4 μ L of each sample equivalent to 1 μ g of protein content was injected onto the column (for unfractioned and fractioned samples, respectively).

Both unfractionated and fractionated samples were analysed using a Q-Exactive Plus Orbitrap mass spectrometer connected to an Easy-nLC 1000 pump (Thermo Fisher Scientific) in a data-dependent acquisition mode (DDA). Full MS scans were acquired over m/z range of 350–1800 Da with resolution of 70,000 (m/z 200 Da), target automatic gain control (AGC) was 10^6 and maximum ion injection time was 100 ms. The ten most intense peaks with charge state ≥ 2 were fragmented in a HCD collision cell with 30% normalised collision energy. Tandem mass spectra were acquired over m/z range of 200–2000 Da with resolution of 35,000 (at m/z 200 Da), target AGC value of 10^6 and 120 ms maximum injection time. The precursor ion selection threshold for fragmentation was set to $4.2 \cdot 10^4$ and dynamic exclusion of precursor ions was set to 20 seconds.

In total three datasets where acquired: 18 non-fractionated samples without replicate were analysed with 50 cm analytical column (1D50), all (19) unfractionated samples were analysed with 25 cm analytical column (1D25) in triplicate, and all (19) fractionated samples were analysed without replicate (except sample COPD5, which was analysed in triplicate) using 25 cm analytical column (2D25).

The synthetic peptides were analysed with the same type, but different instrument than the original analysis. We used an Q-Exactive Plus Orbitrap mass spectrometer connected to an Easy-nLC 1000 pump. Full MS scans were acquired with mass range of 300-1650 Da. The 23 synthetic peptides (non-reference peptides exclusively detected in control or COPD with length shorter than 30 amino acids) and the reference synthetic peptides of HWYITTGPVREK were pooled in three samples and analysed using 45 minutes of gradient instead of 90 minutes used to analyse the human lung tissue samples. All other settings were the same used to analyse human lung tissue samples.

1.8 Data pre-processing, peptide and protein identification

PEAKS Studio v8.0[9] (Bioinformatics solution Inc, Waterloo, Canada) was used for peptide and protein identification with false discovery rate (FDR) <1% at peptide-spectrum-match (PSM), peptide and protein level and using decoy approach for FDR calculation. PSM is a general term used in bottom-up proteomics and expresses the match between a fragment ion spectrum (MS/MS) and the (most probable) peptide sequence derived from the protein sequence database used in database search (PEAKS). FDR calculation was performed for each analysed sample for all three datasets (i.e. replicates and fractions were combined in one identification list). For each sample, a fasta file obtained from the RNAseq data of the same sample was used. The following settings were used for PSM search: precursor error tolerance 10 ppm; precursor mass search type: monoisotopic; fragment mass error tolerance: 0.02 Da; cleavage enzyme: trypsin with 2 missed cleavages and usage of only tryptic peptides. Carbamidomethylation was used for fixed modification, and methionine oxidation was used as variable modification, with 6 maximal variable post-translational modifications per peptide. Only high-quality MS/MS spectra were used for PSM (>0.65). PEAKS label-free analysis was performed with 10 ppm mass error tolerance and 5.0 minutes of retention time shift tolerance. PEAKS label-free ion count analysis has been performed individually only for 1D25 and 2D25 data sets, because in 1D50 dataset the retention time shift was larger than 5 minutes due to 2 times blockage and exchange of the 50 cm LC column, which prevent accurate analysis.

In house developed Perl scripts were used to: (1) calculate raw spectral counts (SPC, i.e. number of PSM) from peptides mapping to unique Ensembl genes (ENSG); (2) to identify peptides with a sequence that did not map to canonical sequences in Ensembl and Uniprot. The latter is called "non-

reference" in the manuscript. To compare protein and gene expression levels, quantitative data was aggregated (summed) to the Ensembl gene level using Ensembl identifiers.

1.9 Statistical analysis and proteogenomics data integration

The data pre-processing workflow used for raw RNAseq and LC-MS/MS pre-processing and proteogenomics data integration is shown in **Figure S1**.

In house developed R scripts were used for (1) differential protein and transcript analysis, (2) identification of non-reference peptides that are different between COPD patients and controls and (3) mapping differentially expressed genes at proteome and/or transcript level on a STRING protein-protein interaction network (https://string-db.org), (4) visualise annotated MS/MS and perform analysis with SpectrumAI R script.

(1) Differential protein and transcript analysis. Only genes having three PSM for proteomics occurring at least half of the samples in one of the sample groups (control or COPD) and at least one fragment per million (FPM) occurring at least half of the samples in one of the sample groups for transcriptomics was considered for the differential expression analysis. The data was normalized by upper quartile normalization and the RUVseq (k=1) package was used to remove unwanted (technical) variation according to the first principal component using residuals (RUVr approach) [10]. Next, the edgeR package [11,12] was used for linear regression of the data following a negative binomial distribution and correction for age, gender and age×gender interaction was included. The three proteomics datasets were analysed combined with two dummy variables using 1D25 dataset as reference using the following linear regression model:

 $Y_{protein} = a + b_{D_1D50} \cdot D_{1D50} + b_{D_2D25} \cdot D_{2D25} + b_{age} \cdot Age + b_{gender} \cdot Gender + b_{age \times gender} \cdot (Age \times Gender) + b_{disease} \cdot Disease + residuals.$

The independent variable D_{1D50} is corresponding with the dummy variable with value of 1 if the sample is from the 1D50 dataset and 0 otherwise, the independent variable D_{2D25} is corresponding with the dummy variable with value of 1 if the sample is from 2D25 dataset and 0 otherwise, *Age* reflects the age of the patient in year, *Gender* is the dummy for gender with 0 for male and 1 for female, the *Age×Gender* interaction and the *Disease* corresponding to dummy with 0 for control and 1 for COPD patients. The Y_{protein} is the dependent variable having the transcriptomics or proteomics data in counts and using linear regression model for negative binomial (count) data. P-values were corrected for multiple testing using Benjamini-Hochberg (FDR) method and threshold for adjusted (or nominal) p-value was 0.05.

The principal component analysis (PCA) plots with the raw and post-normalisation data using upper quartile normalization and removal of unwanted variation using residuals (RUVr approach) are shown in **Figure S2**. Plots were created with ggplot2, vennDiagram and heatmap2 R packages. Differential expression of spectral count data of non-reference differential peptides summed in all data sets were confirmed with separate heat maps in 1D25 and 2D25 data sets using label-free quantification module of PEAKS.

(2) Identification of non-reference peptides differing between COPD and control. Non-reference peptides that were detected with minimally 5 PSM in at least 4 COPD or control samples were combined. From this list non-reference peptides that were only identified in COPD or control samples were selected and the remaining non-reference peptides was subjected to Mann-Whitney U test to assess which non-reference peptides were different between COPD and controls samples. Differential

non-reference peptides mapping only to new gene models, were mapped to Swissprot (2017 July) using NCBI pBlast[13], to determine from which gene the peptide sequence was derived. Immunopeptides were further mapped with ANARCI[14] and IgBlast[15] tools to identify their mapping region in immunoglobulins. Visualisation was performed with qplot R package. Highest PEAKS PSM score (-10·log₁₀(p-value of PSM)) for the 61 differential peptides was extracted from the combined 1D50, 1D25 and 2D25 datasets and is summarized in section 6 "MS/MS spectra and identification parameters of 61 non-reference peptides" in this supporting information document. The MS/MS spectra are annotated with fragment ions and the fragment ion table of the best scoring PSM are reported. Gene ontology analysis was performed with GProfiler R package[16].

(3) STRING protein-protein interaction network visualisation. String analysis was performed for differentially expressed proteins and genes at an FDR<0.01 for either (1) proteomics only or (2) combined set of differential expressed genes at either proteomics or transcript level. The Igraph R library[17] was used for network visualisation and STRINGdb package[18] to interact with the STRING protein-protein interaction database. In the STRING network analysis we used only "experiments" and "database" links with a score larger than 399. The STRING database has in total 4 274 001 connections for 19 247 proteins, from which 16 837 have these two types of connections with scores larger than 399 from 4 685 proteins. Our STRING analysis was performed on 94 differentially expressed proteins at FDR 0.01 to avoid a highly dense network. These 94 proteins mapped to the STRING database with a median of 0 connections and an average of 1.75 connections per node, while the STRING network of our proteomics dataset showed a median connection of 2 and an average connection of 2.77. Connection degree distribution is, however, not normal and the density plot (Figure S8) of the connection distribution shows the overall larger connection degree distribution in our proteomics dataset compared to the distribution of the STRING database using the connections described above, i.e. what would be expected from STRING by selecting genes randomly.

(4) Visualisation of endogenous and synthetic peptide, and SpectrumAI validation of single amino acid variant non-referenced peptides was performed with R packages mzR and RforProteomics, while SpectrumAI was downloaded from https://github.com/yafeng/SpectrumAI. SpectrumAI is a MS/MS spectra analysis tool that was used to identify fragment ions evidence in MS/MS spectra for the presence of the altered amino acids among newly identified, non-reference, single amino acid variant (SAAV) peptides. mzR and RforProteomics R packages was used to visualise annotated MS/MS spectra. The excel sheet containing the SpectrumAI analysis results is available as supplementary file 2.

1.10 Data files.

mRNA and protein data is available for future collaboration on request.

2 Supporting information figures

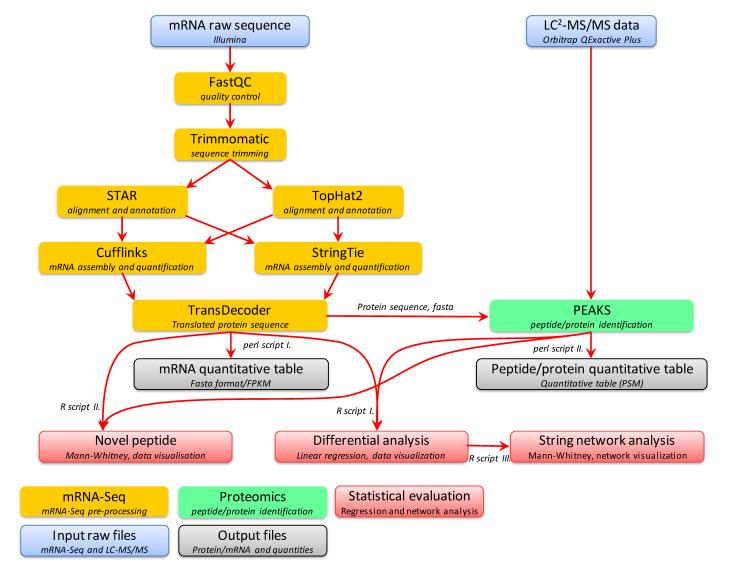


Figure S1. Scheme of the proteogenomics data integration workflow that was used for the joint pre-processing of the LC-MS/MS proteomics and RNAseq data obtained from the same human lung tissue samples.

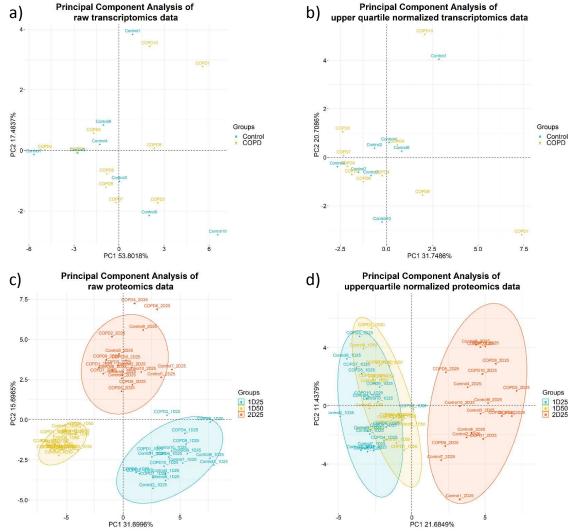
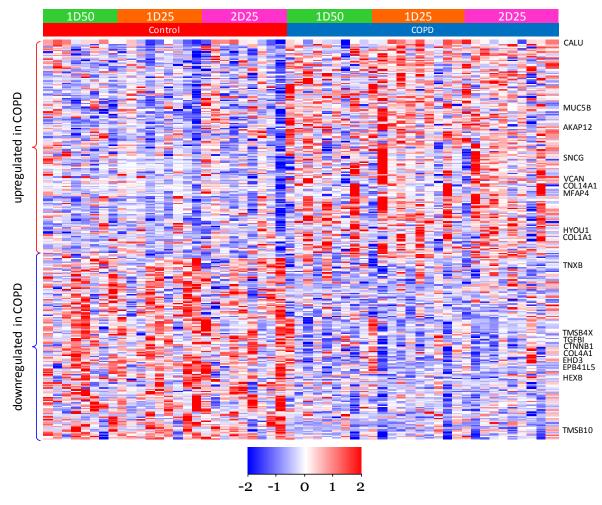


Figure S2. **Principal component score plots** showing the first two principal components of raw transcriptomics (a), raw proteomics data (c) and the same datasets (b, d) after upper quartile normalization and removal of unwanted variation using residuals (RUVr).



Combined proteomics Z-value normalized heat map (FDR = 0.05 Proteomics)

Figure S3. Heat map of differential protein expression in severe COPD lung tissue, showing upregulated proteins (top) and downregulated proteins (bottom). The heat map shows the Z-scores of the residuals of the linear regression with age, gender and age×gender interaction. Data generated from the 1D50, 1D25 and 2D25 data sets is given in the column headers and shows consistency across the data sets. The named genes are discussed in the text.

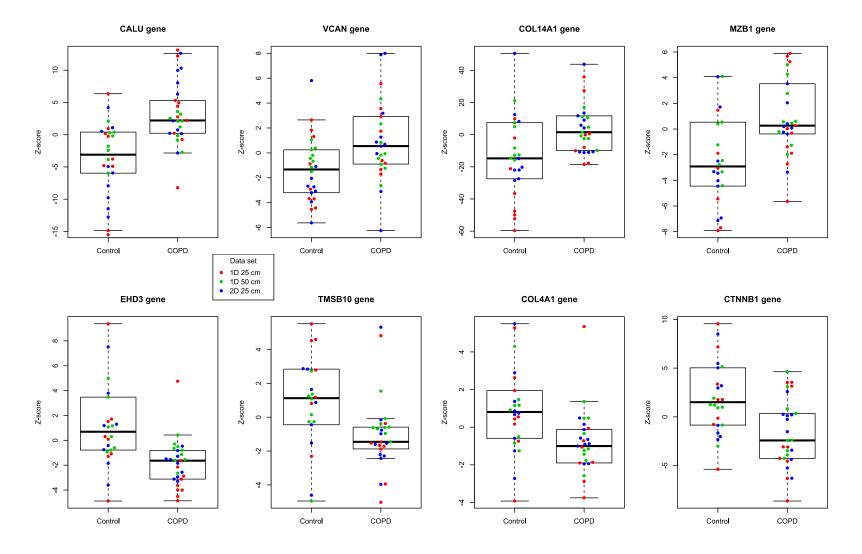
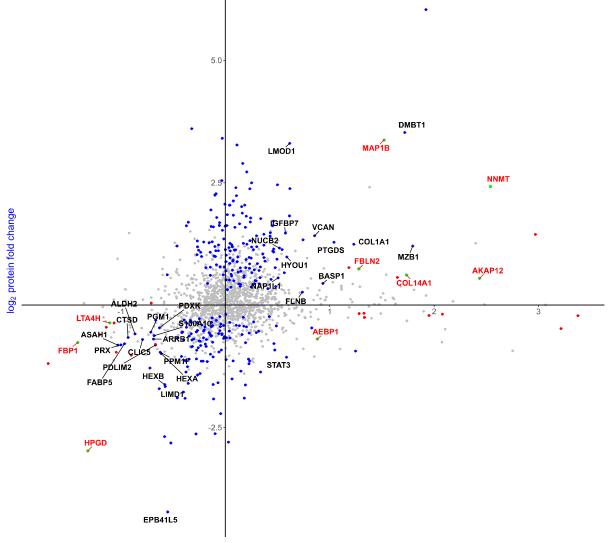


Figure S4. Bee swarm plots are shown for 4 proteins with higher protein levels in COPD (i.e. CALU, VCAN, COL14A1, MZB1) and 4 proteins with lower protein levels in COPD (i.e. EHD3, TMSB10, COL4A1, CTNNB1). Z-scores (residuals) are depicted for data generated in each proteomics dataset (1D 25cm in red, 1D 50 cm in green, 2D 25 cm in blue) after correction for confounders.



log₂ transcript fold change

Figure S5. Fold change scatter plot of transcript and protein expression. Fold change scatter plot of transcript (horizontal axis) and protein expression (vertical axis) of protein-coding human genes identified in control and COPD human lung tissue. Differentially expressed proteins at an FDR <0.05 (blue), differentially expressed transcripts at an FDR <0.05 (red), differential-expression on both the protein and transcript levels at an FDR <0.05 (green) with gene names (red text). All proteins that are differentially-expressed at the transcript level with a nominal p<0.05 are named (black text).

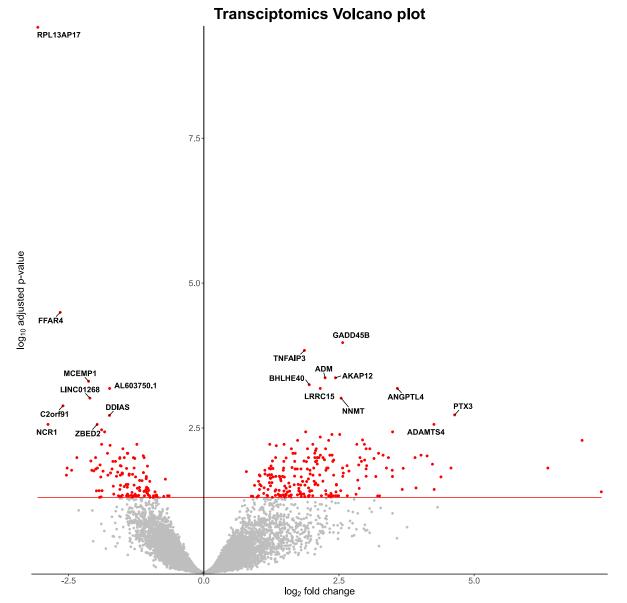


Figure S6. Differential gene expression in severe COPD lung tissue. Volcano plot of all genes consistently expressed in COPD and control lung tissue. Differentially expressed genes (FDR < 0.05) are shown in red. The most significantly differentially expressed genes are indicated with gene symbol.

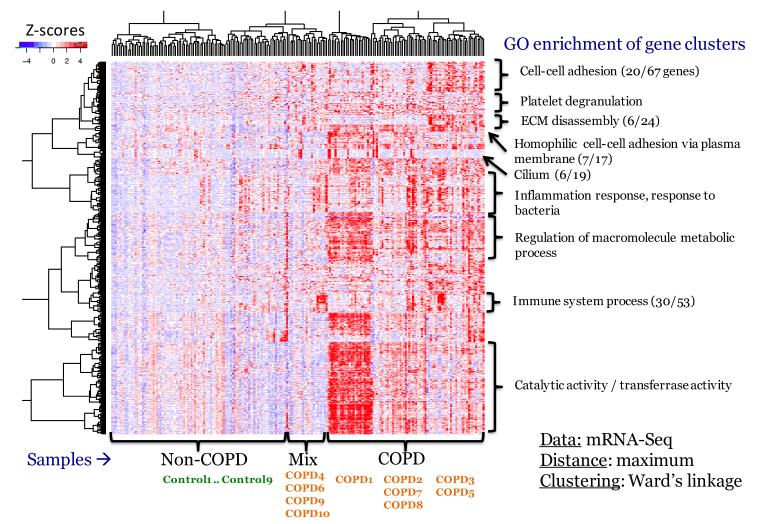


Figure S7. Heat map of 350 differential transcripts (FDR<0.05) in human lung tissue measured in this study (10 COPD and 8 controls) and in 189 samples from a South Korean study[19]. The heat map shows the Z-scores of the transcripts counts after upper-quartile normalization. Both samples and transcripts expression are submitted to hierarchical clustering using maximum Ward's linkage. The most significant gene ontology enrichment of clustered transcript expression is shown right. Samples are clustered in there groups as control, mixed control and COPD and COPD, and our samples are highlighted in the bottom.

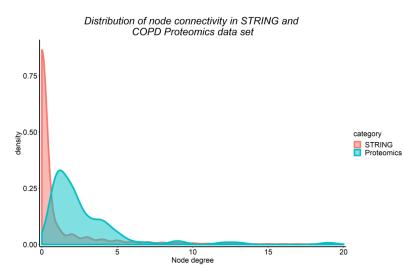


Figure S8. Density plot showing the connectivity (number of connections, degree) distribution in STRING and in differentially expressed proteins of combined COPD dataset (FDR 0.01) using "experiments" and "database" connections with scores higher than 399. STRING database contained 4 274 001 connections (any types) between 19 247 proteins, from which 16 837 were type defined above and which connections were between 4 685 proteins. The plot reflects the higher connections (degree distribution) of nodes between the differential proteins of COPD proteomics data set.

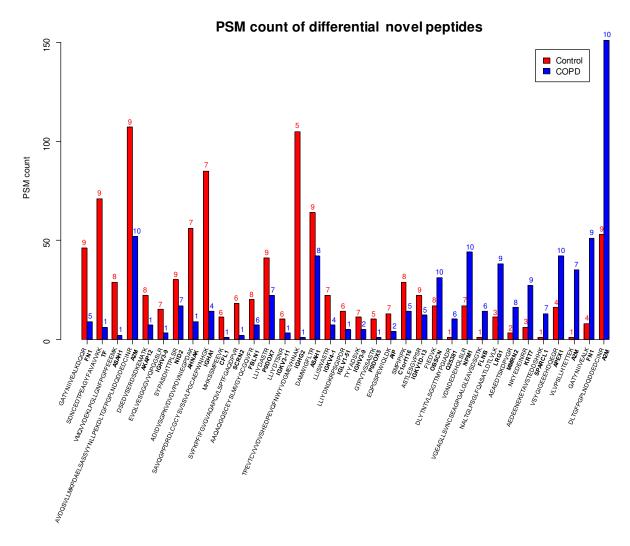


Figure S9. Differential non-reference sample specific peptides present in severe COPD and control lung tissue. Bar plot of the number of MS/MS spectra (PSMs) for non-reference sample specific peptides that were differentially-expressed based on Mann-Whitney U test (Figure 1D) but not exclusively present in one of the sample group. Only peptides with at least 5 PSMs and present in at least 4 COPD patients or controls were considered. The number of samples where the non-reference peptide was identified is indicated at the top of each bar.

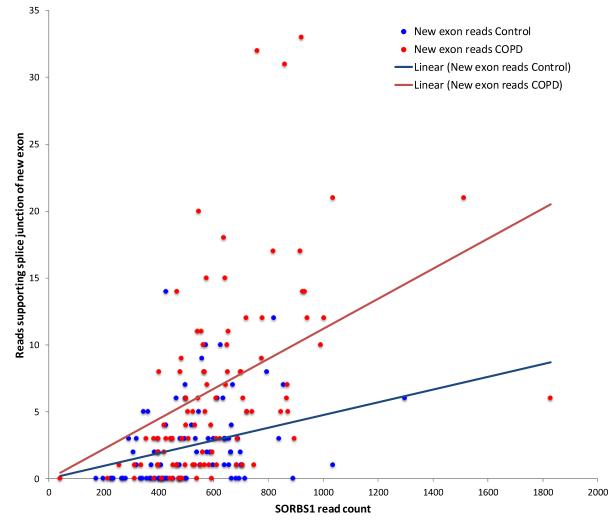
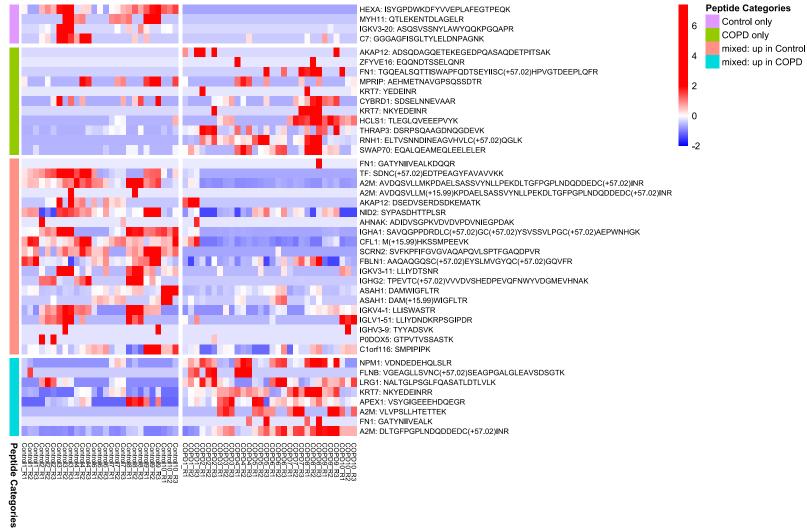
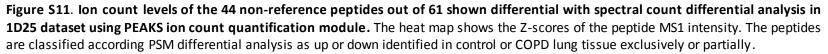


Figure S10. **Novel SORBS1 exon usage in independent lung tissue dataset.** The differential usage of the novel exon in SORBS1 in RNA sequencing data from an independent Korean dataset of COPD and control lung tissues is shown. The number of reads supporting the splice junction of the novel exon is plotted against the total read count for SORBS1. The reds dots and line representing COPD samples and the blue dots and line representing control samples. A higher slope of the line indicates a higher proportion of new exon usage.



Peptide ion counts in dataset COPD 1D 25cm



Thorax

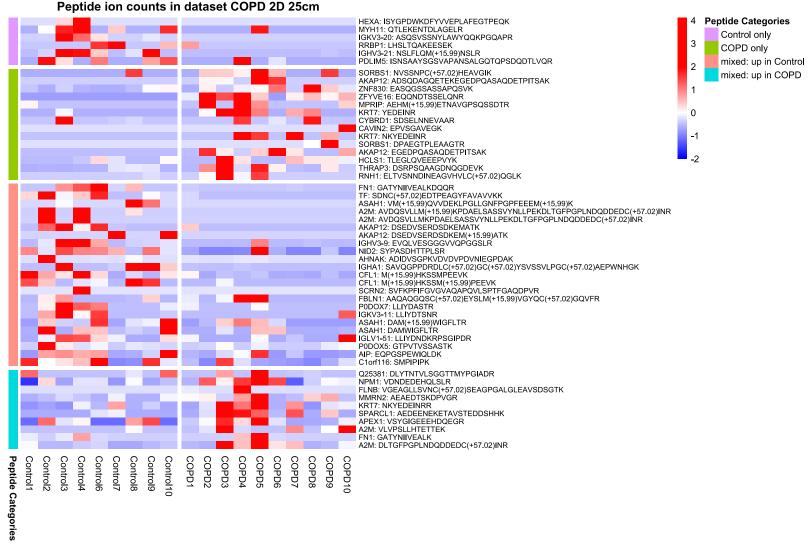


Figure S12. Ion count levels of the 49 non-reference peptides out of 61 shown differential with spectral count differential analysis in 2D25 dataset using PEAKS ion count quantification module. The heat map shows the Z-scores of the peptide MS1 intensity. The peptides are classified according PSM differential analysis as up or down identified in control or COPD lung tissue exclusively or partially.

3 Supporting information tables

Table S1. Complete list of differentially expressed transcripts and proteins with FDR < 0.05.</th>Separate Excel sheet (file name Protein_transcipt_differential_expression.xlsx).

Table S2. Top 15 enriched Gene Ontology terms based on differential protein expression with FDR
p<0.01 in severe COPD.

_		GO term id	GO term description	p-value	FDR p-value
=	1	GO:0030198	extracellular matrix organization	8.61·10 ⁻⁸	1.05.10-4
	2	GO:0043062	extracellular structure organization	8.95·10 ⁻⁸	1.05.10-4
	3	GO:1901575	organic substance catabolic process	$1.07 \cdot 10^{-7}$	$1.05 \cdot 10^{-4}$
	4	GO:0016192	vesicle-mediated transport	$1.96 \cdot 10^{-7}$	$1.44 \cdot 10^{-4}$
	5	GO:0007010	cytoskeleton organization	$2.16 \cdot 10^{-6}$	1.27·10 ⁻³
	6	GO:0044248	cellular catabolic process	4.26·10 ⁻⁶	1.98·10 ⁻³
	7	GO:0006897	endocytosis	4.71·10 ⁻⁶	1.98·10 ⁻³
	8	GO:0010941	regulation of cell death	6.44·10 ⁻⁶	2.13·10 ⁻³
	9	GO:0044712	single-organism catabolic process	6.52·10 ⁻⁶	2.13·10 ⁻³
	10	GO:0060548	negative regulation of cell death	1.02·10 ⁻⁵	2.58·10 ⁻³
	11	GO:1901564	organonitrogen compound metabolic process	1.03·10 ⁻⁵	2.58·10 ⁻³
	12	GO:0006915	apoptotic process	1.05·10 ⁻⁵	2.58·10 ⁻³
	13	GO:0012501	programmed cell death	1.21·10 ⁻⁵	2.68·10 ⁻³
	14	GO:0043066	negative regulation of apoptotic process	1.27·10 ⁻⁵	2.68·10 ⁻³
	15	GO:0043069	negative regulation of programmed cell death	1.39·10 ⁻⁵	2.74·10 ⁻³

GO: Gene Ontology, p-value represent the nominal p-value, FDR p-value was obtained after applying Benjamini-Hochberg procedure for multiple testing correction.

Peptide sequence	Quality score	Gene	Effect	lon support	Flanking ion support
ADSQDAGQETEKEGEDPQASAQDETPITSAK	134.83	AKAP12	E1600D amino acid substitution	×	×
DSRPSQAAGDNQGDE <mark>V</mark> K	132.86	THRAP3	A201V amino acid substitution	×	×
IGQEALSQTTISWAPFQDTSEYIISCHPVGTDEEPLQFR	131.44	FN1	Native	NA	NA
AEHMETNAVGPS <mark>Q</mark> SSDTR	129.43	MPRIP	P327Q amino acid substitution	×	×
EGEDPQASAQDETPITSAK	129.34	AKAP12	E1600D amino acid substitution	×	×
DPAEGTPLEAAGTR	118.25	SORBS1	Splice variant, exon extension	NA	NA
LTVSNNDINEAGVHVLCQGLK	115.80	RNH1	R188H amino acid substitution	×	×
TLEGLQVEEEPVY <mark>K</mark>	106.37	HCLS1	E361K amino acid substitution	×	×
ASQGSSASSAP <mark>Q</mark> SVK	105.60	ZNF830	H99Q amino acid substitution	×	×
DSELNNEVAAR	103.81	CYBRD1	S266N amino acid substitution	×	×
QALQEAMEQLEELELER	100.83	SWAP70	Q505E amino acid substitution	×	×
QQNDTSSELQNR	91.55	ZFYVE16	I192T amino acid substitution	×	×
GFQFLR	81.46	PCYOX1	S149F amino acid substitution	×	×
IKYEDEINR	70.58	KRT7	H186R amino acid substitution	×	×
PVSGAVEGK	62.17	CAVIN2	Q174E amino acid substitution	×	×
IVSSNPCHEAVGIK	56.53	SORBS1	Splice variant, exon extension	NA	NA
'EDEINR	49.80	KRT7	H186R amino acid substitution	×	×
Peptides only present in Control					
Peptide sequence	Quality score	Gene	Effect	lon support	Flanking ion support
SYGPDWKDFYVVEPLAFEGTPEQK	120.51	HEXA	I447V amino acid substitution	×	×
SNSA <mark>A</mark> YSGSVAPANSALGQTQPSDQDTLVQR	110.84	PDLIM5	T410A amino acid substitution	×	×
HSLTQAKEESEK	110.58	RRBP1	L1043H amino acid substitution	×	-
GGGAGFISGLTYLELDNPAGNK	108.34	C7	S389T amino acid substitution	×	×
ASQSVSS <mark>N</mark> YLAWYQQKPGQAPR	105.88	IGKV3-20	S52N amino acid substitution	×	×
QTLEKENTDLAGELR	77.52	MYH11	A1241T amino acid substitution	×	×
ISLFLQMNSLR	76.68	IGHV3-43	Y99F amino acid substitution	×	×
LIYWASAR	68.41	IGKV4-1	T79A amino acid substitution	×	×
LITWASAR	00.41	101(141			

Table S3. Properties of non-reference peptide sequences that were differentially expressed between COPD and Control. Peptides only present in COPD

Peptide sequence, identification score, mapping gene symbol and sequence variant type is shown. The quality scores (-10 log10(p-value of PSM)) reflect the quality of the peptide-to-spectrum-match, i.e., peptide identification by mass spectrometry. The effect 'native' indicates that the peptide sequence is now included in the most recent version of Ensembl, but was absent when proteogenomic data integration was performed. *×* indicates the presence, while – indicates the absence of Ion support or Flaking ion support provided by SpectrumAI assessment. The presence of Ion support indicates the presence of the altered amino acids, while the presence of Flanking ions providing evidence on the correct location of the altered amino acid in SAAV variants. NA indicates not applicable (variant other than SAAV). The altered amino acids in SAAV variants are highlighted in red.

Table S4. Properties of non-reference peptide sequences that were differentially expressed between COPD and Control. Pentides different between COPD and Control

Peptide sequence	Quality score	Gene	Effect	lon support	Flanking ion suppor
AVDQSVLLMKPDAELSASSVYNLLPEKDLTGFPGPLNDQDDEDCINR	200.00	A2M	N639D amino acid substitution	×	×
SAVQGPPDRDLCGCYSVSSVLPGCAEPWNHGK	200.00	IGHA1	E176D amino acid substitution	×	×
TPEVTCVVVDVSHEDPEVQFNWYVDG M EVHNAK	147.77	IGHG2	V161M amino acid substitution	×	-
SDNCEDTPEAGYFA <mark>V</mark> AVVKK	145.51	TF	I448V amino acid substitution	×	×
AAQAQGQSCEYSLMVGYQCGQVFR	140.99	FBLN1	Q179R amino acid substitution	×	-
ADIDVSGPKVDVDVPDVNIEGPDAK	138.93	AHNAK	Native	NA	NA
DLTGFPGPLNDQDDEDCINR	138.23	A2M	N639D amino acid substitution	×	×
VMQVVDEKLPGLLGNFPGPFEEEMK	134.27	ASAH1	1109V amino acid substitution	×	-
VGEAGLLSVNCSEAGPGALGLEAVSDSGTK	130.08	FLNB	D1157N amino acid substitution	×	×
SVFKPFIFGVGVAQAPQVLSPTFGAQDPVR	124.78	SCRN2	M331V amino acid substitution	×	×
NALTGLP <mark>S</mark> GLFQASATLDTLVLK	116.16	LRG1	P133S amino acid substitution	×	×
VSYGIGEEEHDQEGR	115.91	APEX1	D148E amino acid substitution	×	×
EVQLVESGGG <mark>V</mark> VQPGGSLR	111.36	IGHV3-13	L30V amino acid substitution	×	×
GATYNIIVEALKDQQR	109.33	FN1	V2230I amino acid substitution	×	×
DSEDVS <mark>E</mark> RDSDKEMATK	107.08	AKAP12	K117E amino acid substitution	×	-
LIYDNDKRPSGIPDR	105.39	IGLV1-51	N72D amino acid substitution	×	×
AEDEENEKETAVSTEDDSHHK	104.77	SPARCL1	A49D amino acid substitution	×	×
SYPASDHTTPLSR	98.27	NID2	G453D amino acid substitution	×	×
DAMWIGFLTR	95.37	ASAH1	V262A amino acid substitution	×	-
GTPVTVSSASTK	94.79	IGHG1	T140P amino acid substitution	×	×
EQPGSPEWIQLD <mark>K</mark>	93.79	AIP	Q228K amino acid substitution	×	×
VDNDEDEHQLSLR	89.48	NPM1	Paralog	NA	NA
AEAEDT <mark>S</mark> KDPVGR	86.10	MMRN2	G49S amino acid substitution	×	×
VLVPSLLHTETTEK	84.08	A2M	Native	NA	NA
NKYEDEINRR	82.99	KRT7	H186R amino acid substitution	×	×
DLYTNTVLSGGTTMYPGIADR	78.17	ACTB	Paralog	NA	NA
LIYDASTR	77.22	IGKV3D-20	S74T amino acid substitution	×	×
ASTLESGVPSR	75.55	IGKV1-5	S75T amino acid substitution	×	×
GATYNIIVEALK	73.04	FN1	V2261I amino acid substitution	×	×
LIYDTSNR	72.05	IGKV3-11	A71T amino acid substitution	×	×
LISWASTR	63.08	IGKV4-1	Y75S amino acid substitution	×	×
TYYADSVK	55.49	IGHV3-23	Native	NA	NA
SMPIPIPK	53.15	C10RF116	S444P amino acid substitution	×	×
FIEDVK	42.76	OBSCN	Native	NA	NA
MHKSSMPEEVK	36.09	CFL1	Paralog	NA	NA

Peptide sequence, identification score, mapping gene symbol and sequence variant type is shown. The quality scores (-10 log10(p-value of PSM)) reflect the quality of the peptide-to-spectrum-match, i.e., peptide identification by mass spectrometry. The effect 'native' indicates that the peptide sequence is now included in the most recent version of Ensembl, but was absent when proteogenomic data integration was performed. *×* indicates the presence, while – indicates the absence of Ion support or Flaking ion support provided by SpectrumAI assessment. The presence of Ion support indicates the presence of the altered amino acids, while the presence of Flanking ions providing evidence on the correct location of the altered amino acid in SAAV variants. NA indicates not applicable (variant other than SAAV). The altered amino acids in SAAV variants are highlighted in red.

Peptide	Gene	Effect	one representative entry	domain	subdomain	# of mRNA	tool	presence
NSLFLQMNSLR	IGHV3-43	Y->F	ENST00000434710_Y99F	VH	FR3 position 22	3	ANARCI, IPBlast	4 controls, 5 PSM
ASQSVSSNYLAWYQQKPGQAPR	IGKV3-20	S->N	ENST00000492167_S52N	VK	CDR1 position 6	1	ANARCI, IPBlast	4 controls, 11 PSM
LLIYWASAR	IGKV4-1	T->A	ENST00000390243_T79A	VK	FR3 position 1	1	ANARCI, IPBlast	4 controls, 8 PSM
EVQLVESGG <mark>GV</mark> VQPGGSLR	IGHV3-13	V->G, L->V	ENST00000390602_L30V	VH	FR1 positions 10, 11	3	ANARCI, IPBlast	7 control, 1 COPD, 15 and 3 PSM
LLIYDASTR	IGKV3D-20	G->D, N(S)->T	ENST00000390270_S74T	VK	CDR1 position 1, FR3 position 1	5	ANARCI, IPBlast	9 control, 7 COPD, 41 and 22 PSN
TPEVTCVVVDVSHEDPEVQFNWYVDGMEVHNAK	IGHG2	V->M, new gene mode	ls ENST00000390545_V161M	lg heavy gamma 2	CH3 domain (constant IgC_CH3 domain)	3	pBlast	5 control, 1 COPD, 105 and 1 PSN
LLIYDNDKRPSGIPDR	IGLV1-51	N->D	ENST00000390290_N72D	VL	CDR2 position 3	1	ANARCI, IPBlast	6 control, 1 COPD, 14 and 5 PSM
ASTLESGVPSR	IGKV1-5	S->T	ENST00000496168_S75T	VK	FR3 position 1	2	ANARCI, IPBlast	9 control, 5 COPD, 22 and 12 PSN
LLIYDTSNR	IGKV3-11	A->T	ENST00000483158_A71T	VK	CDR2 position 2	2	ANARCI, IPBlast	6 control, 1 COPD, 10 and 3 PSM
LLI <mark>S</mark> WASTR	IGKV4-1	Y->S	ENST00000390243_Y75S	VK	FR2 postion 17	1	ANARCI, IPBlast	7 control, 4 COPD, 22 and 7 PSM
TYYADSVK	IGHV3-23	Native	ENST00000390609	VH	peptide mapping last position of CDR2 and beginning of FR3	138	ANARCI, IPBlast	7 control, 2 COPD, 11 and 5 PSM
SAVQGPPDRDLCGCYSVSSVLPGCAEPWNHGK	IGHA1	E->D	ENST00000390547_E176D	lg heavy alpha 1	peptide mapping to CH2 domain (second constant Ig domain of the heavy chain) in immunoglobulin; IgC_CH2	1	pBlast	7 control, 4 COPD, 85 and 14 PSN
GTPVTVSSASTK	IGHG1	T->P	ENST00000612473_T140P	lg heavy gamma 1	peptide mapping the end of IgV-H domain (heterodimer interface)	2	pBlast	5 control, 1 COPD, 10 and 1 PSM

Table S5. Non-reference peptide sequences mapping to immunoglobulin proteins

Peptide sequence indicating single amino acid variants in red, immune gene symbol, the genetic variant effect, one representative entry of Ensembl transcript, human immunoglobulins structural domain and subdomains, number of matching mRNA entries, used mapping tools (ANARCI, IgBlast) and presence of the peptide in controls and COPD samples with corresponding PSMs.

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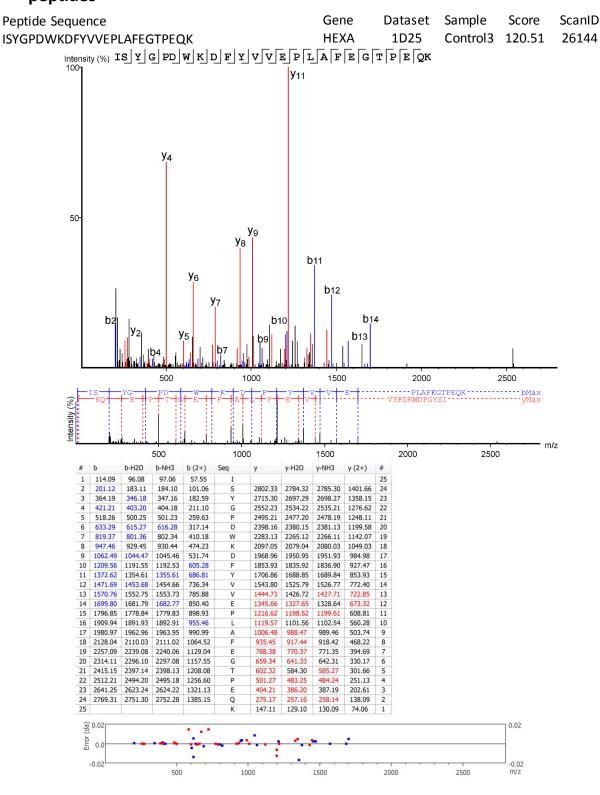
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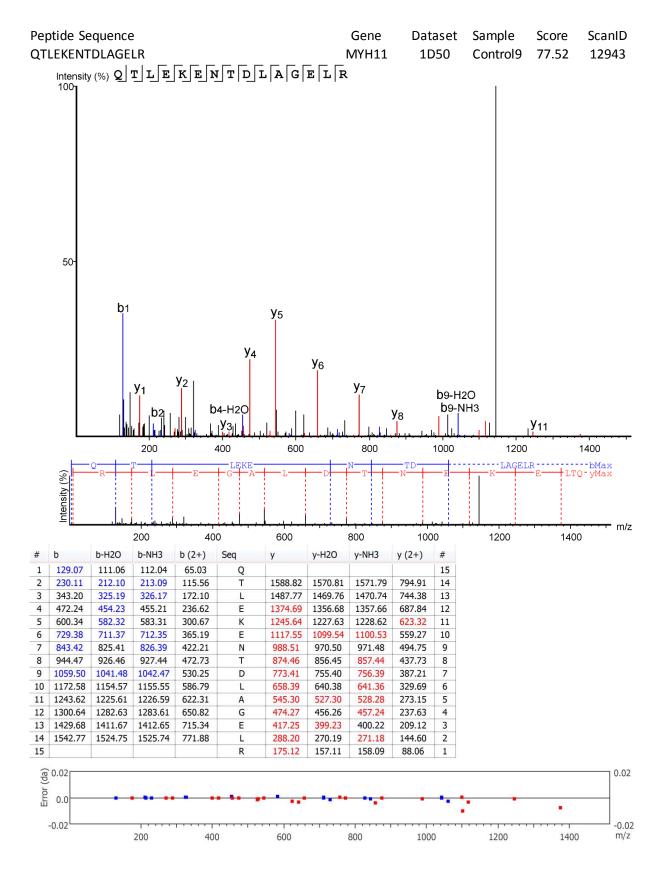
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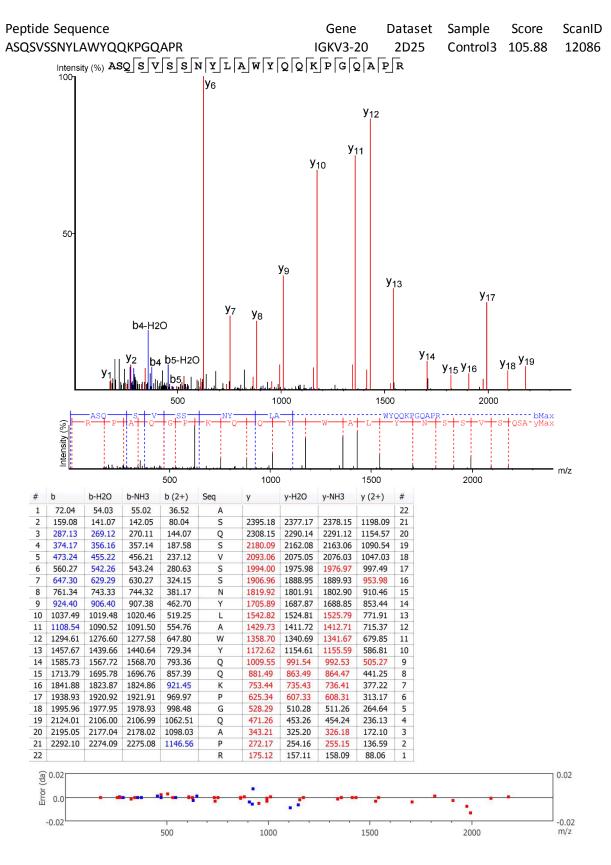
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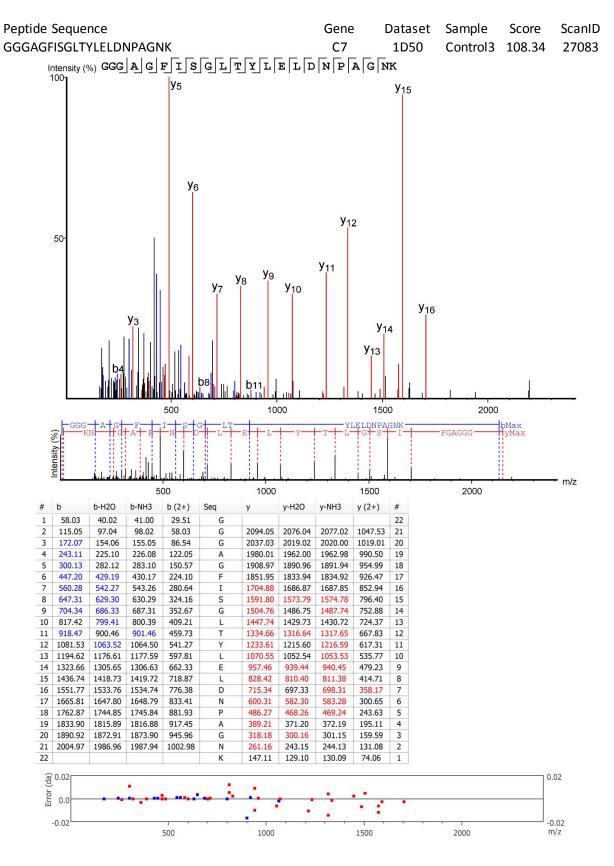
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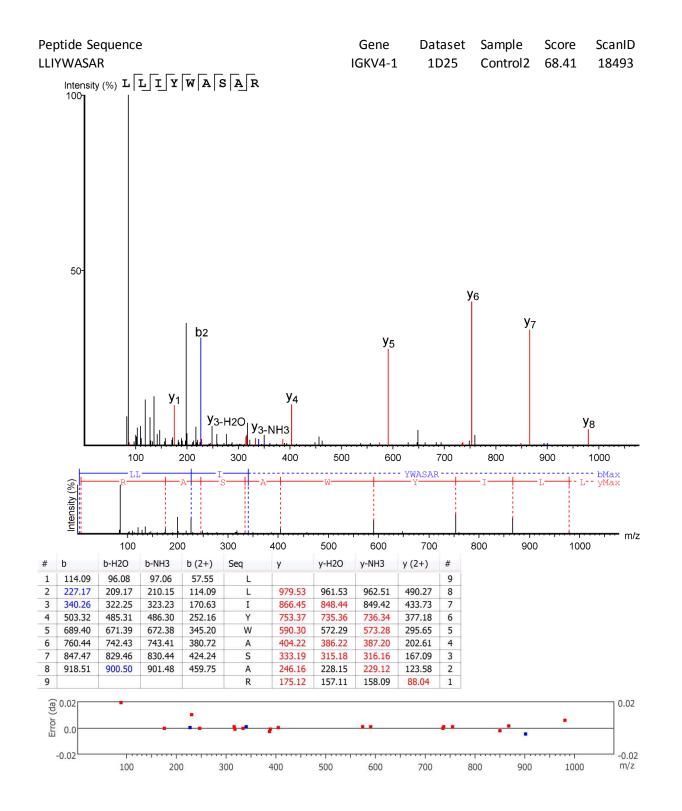


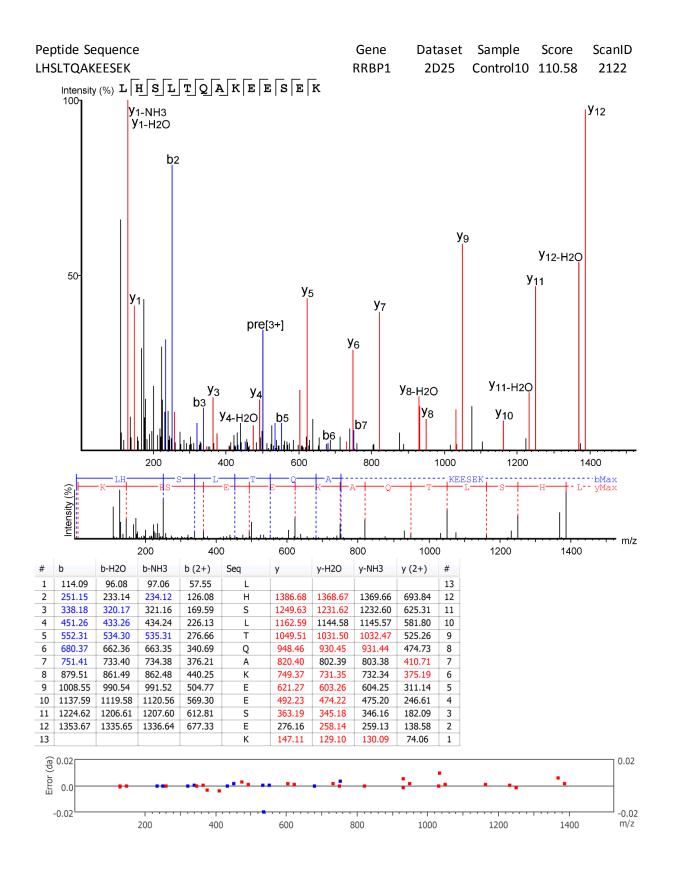


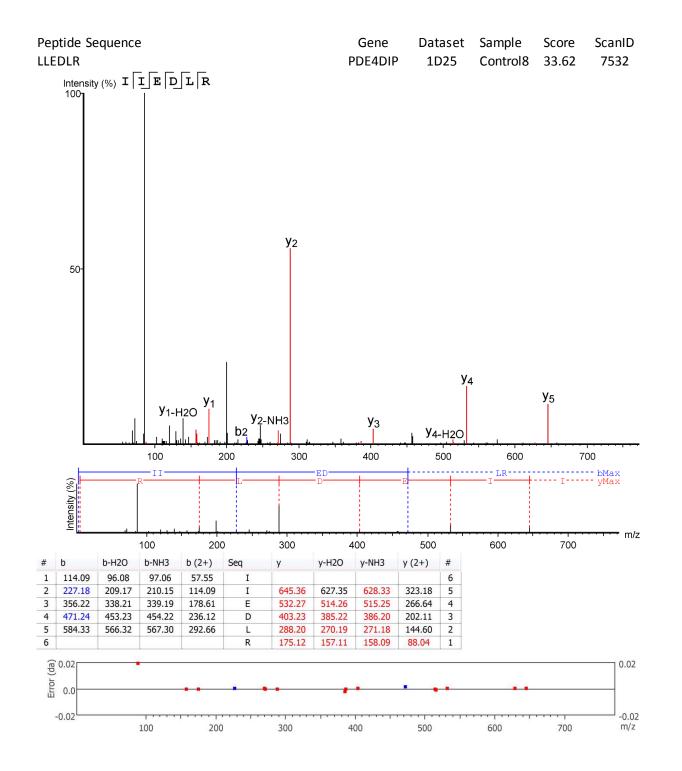


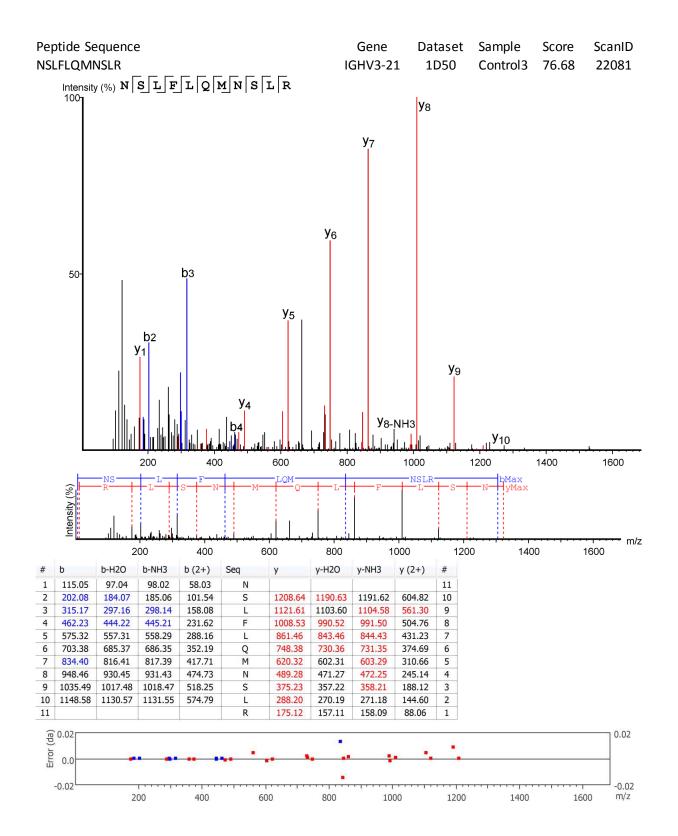


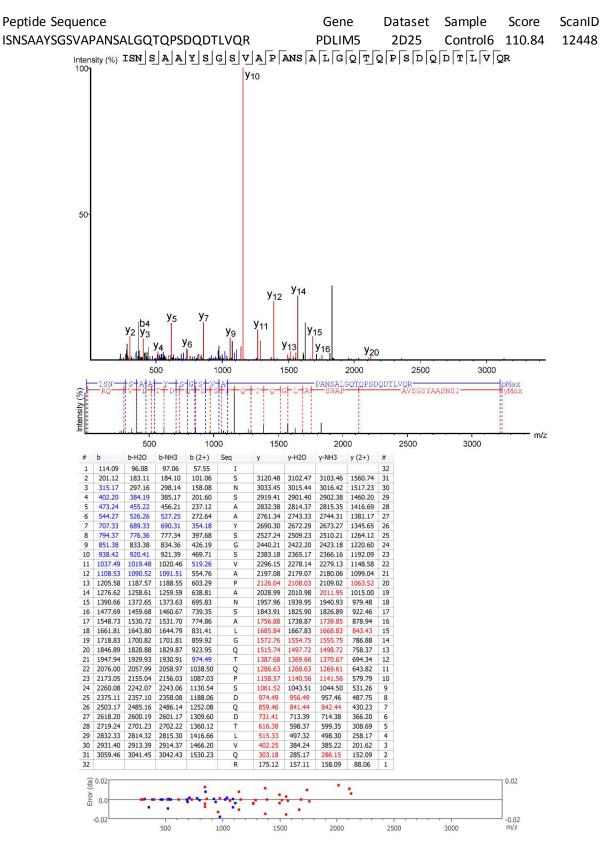


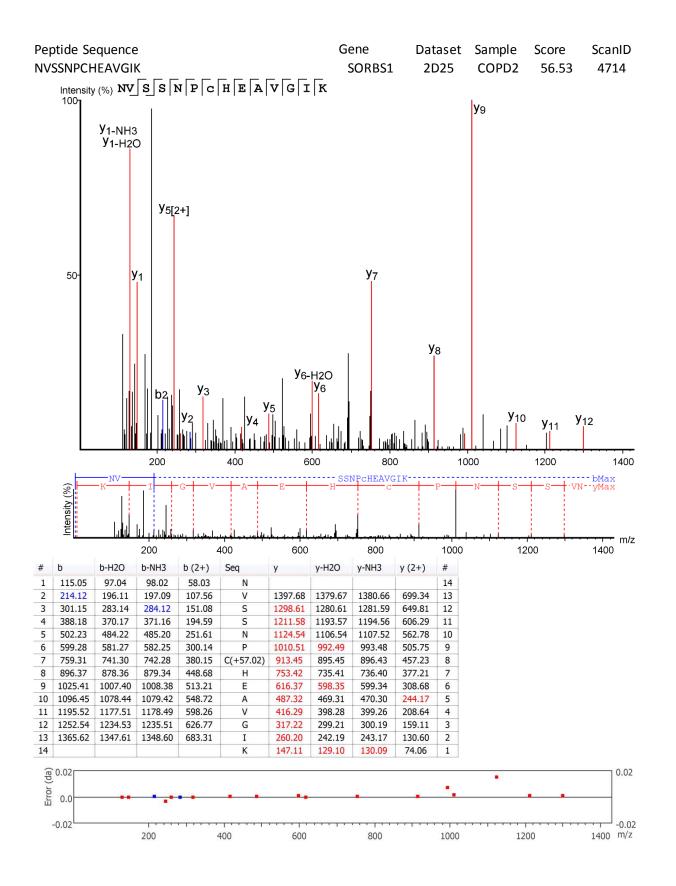


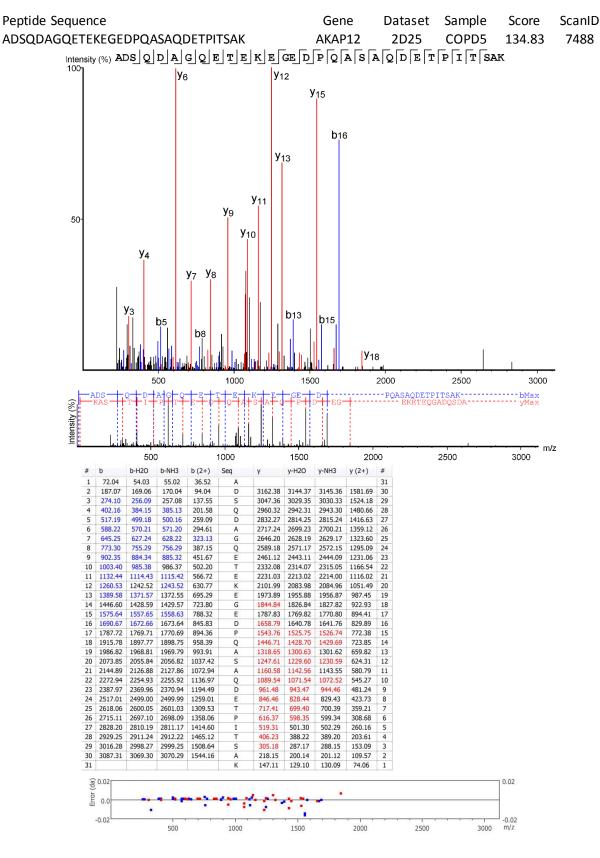


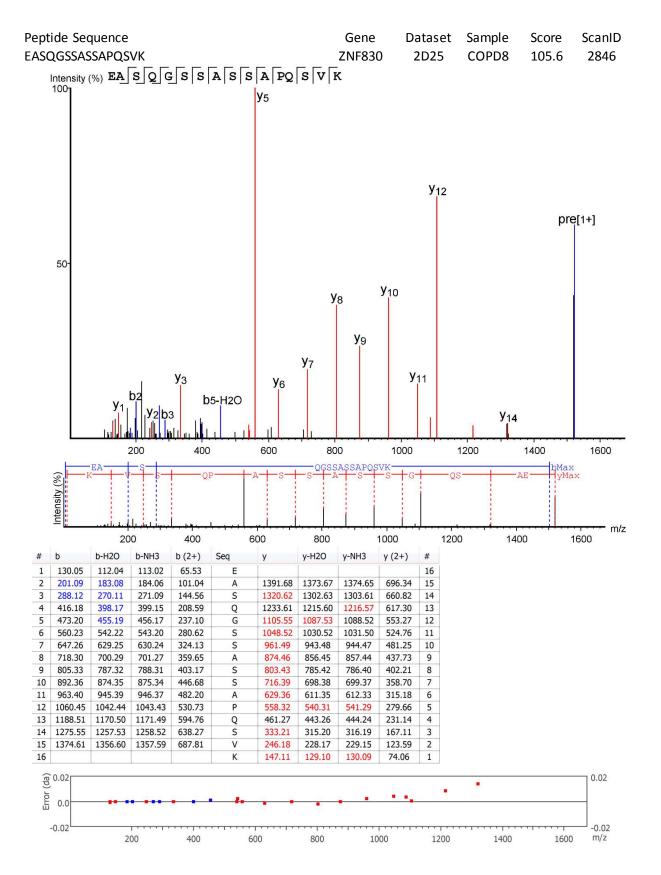


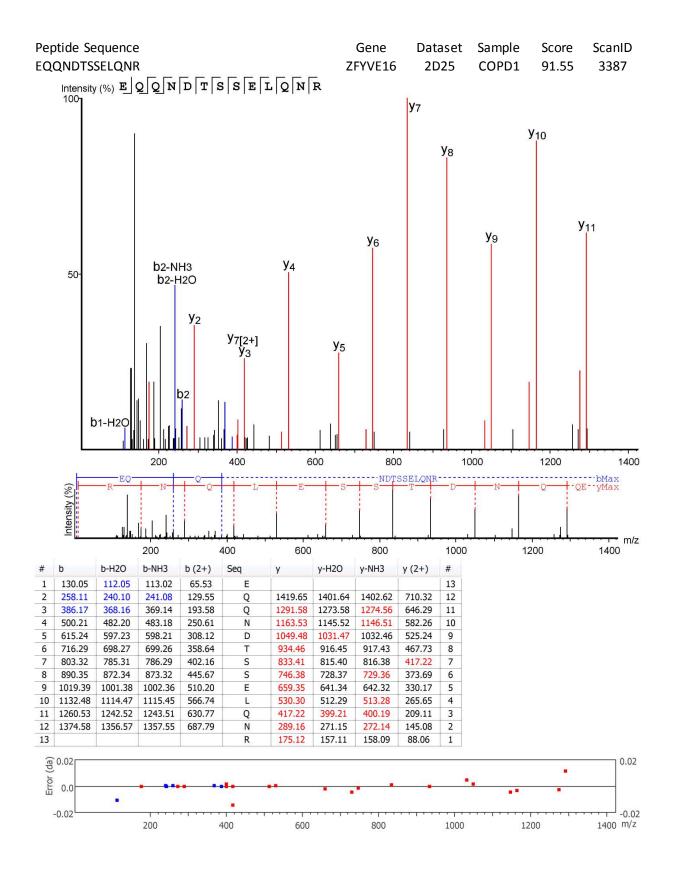


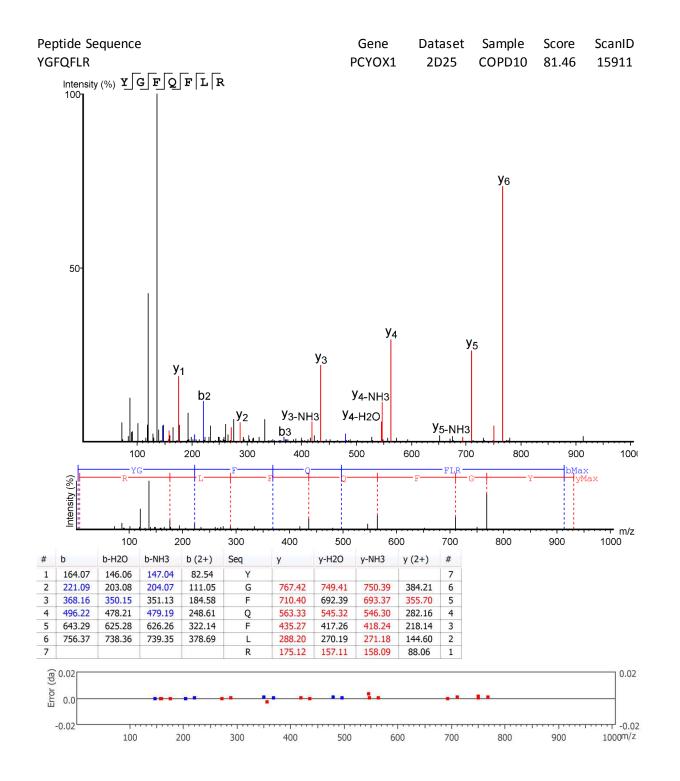


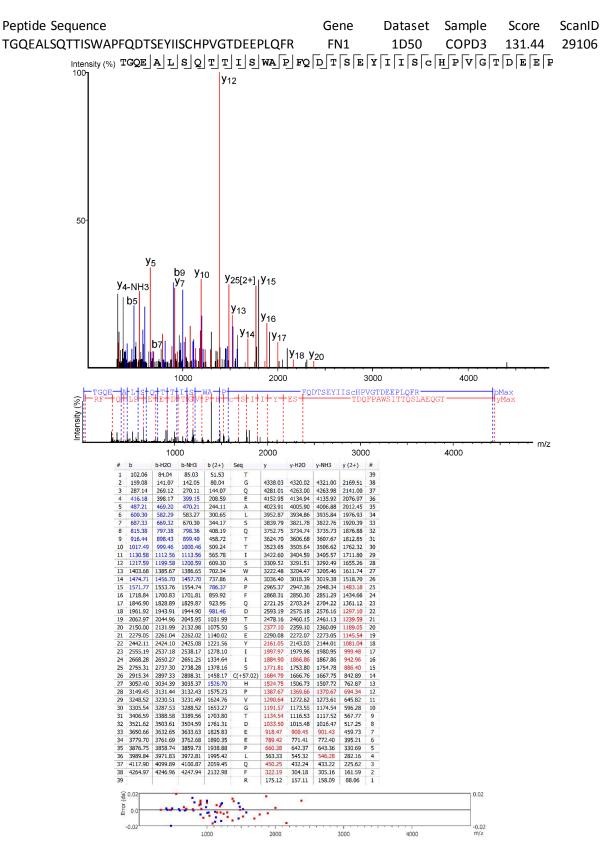


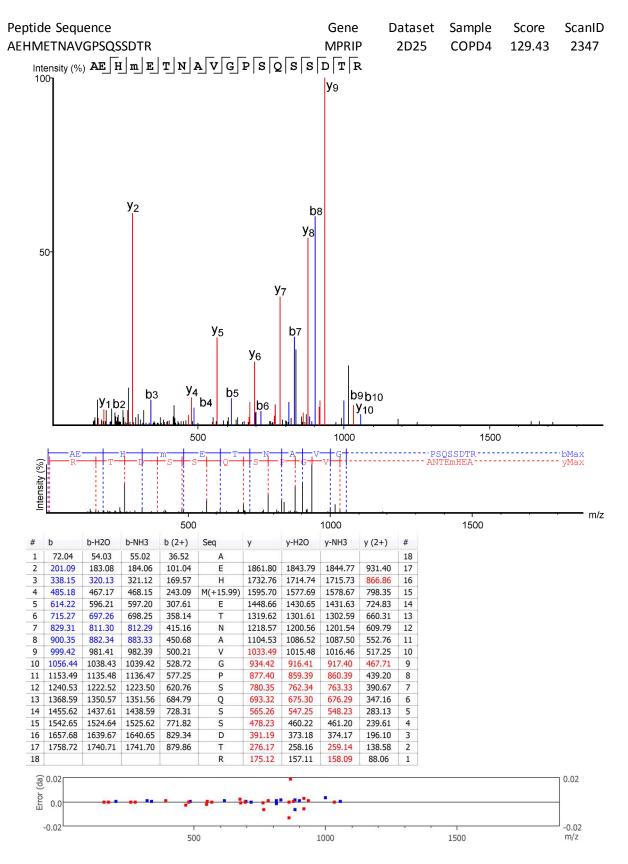




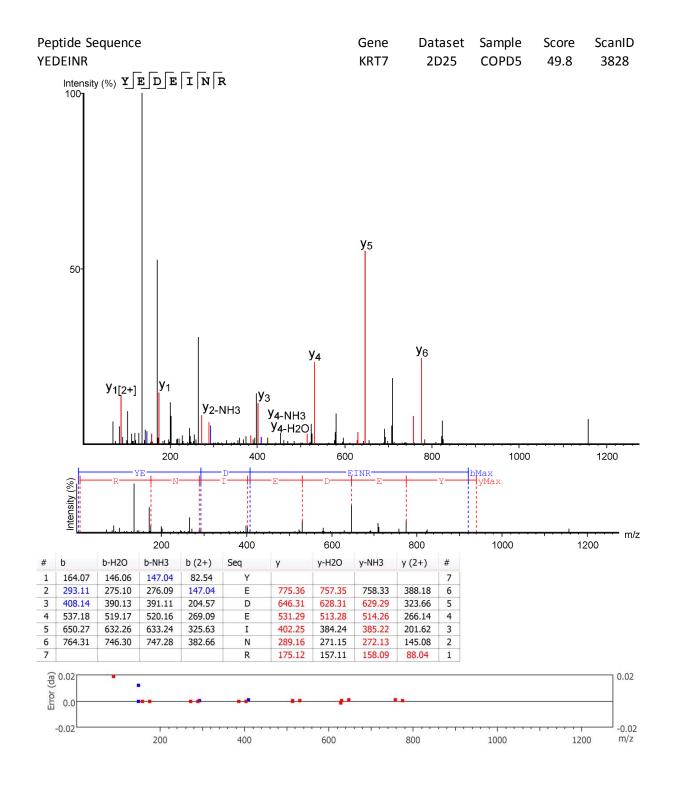


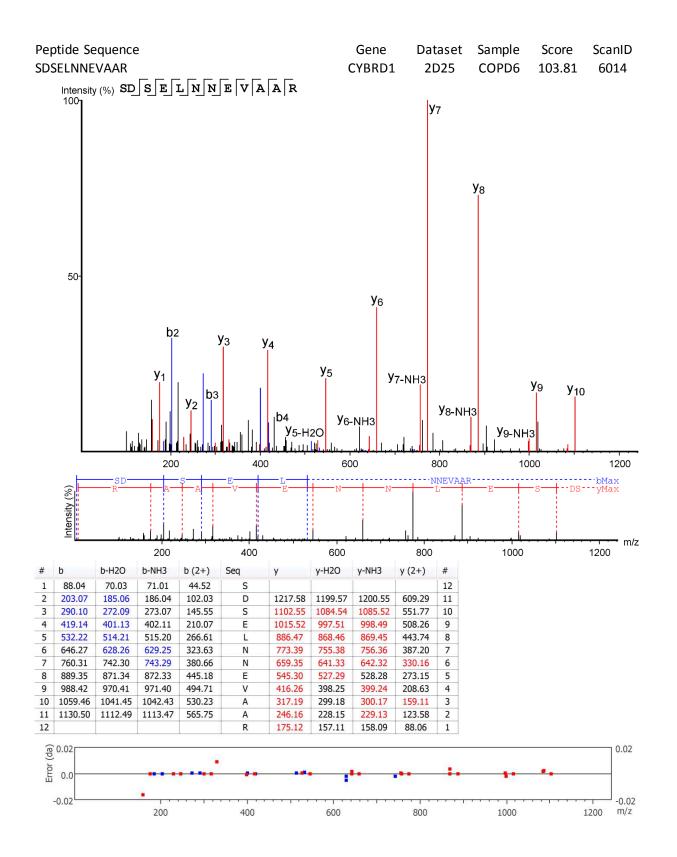


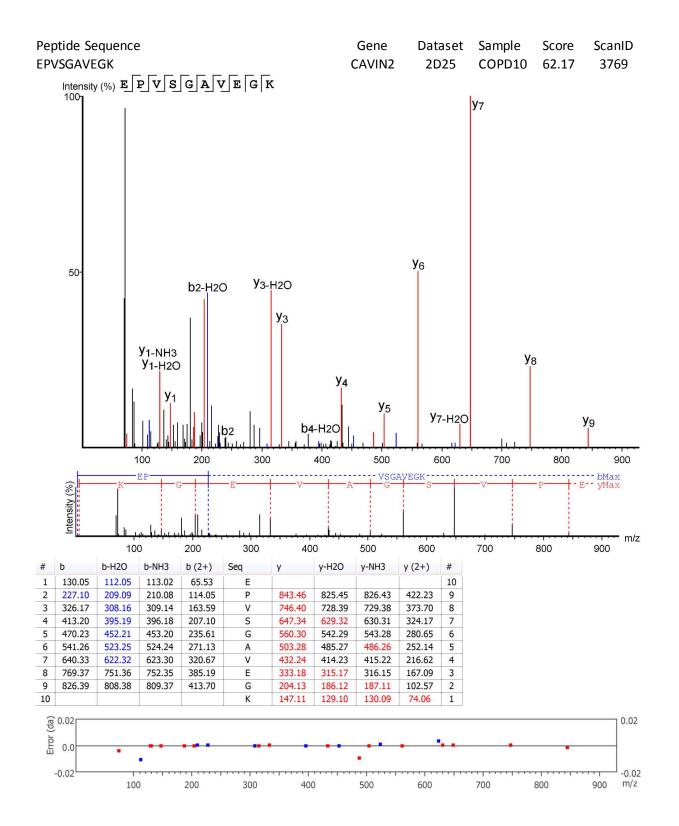


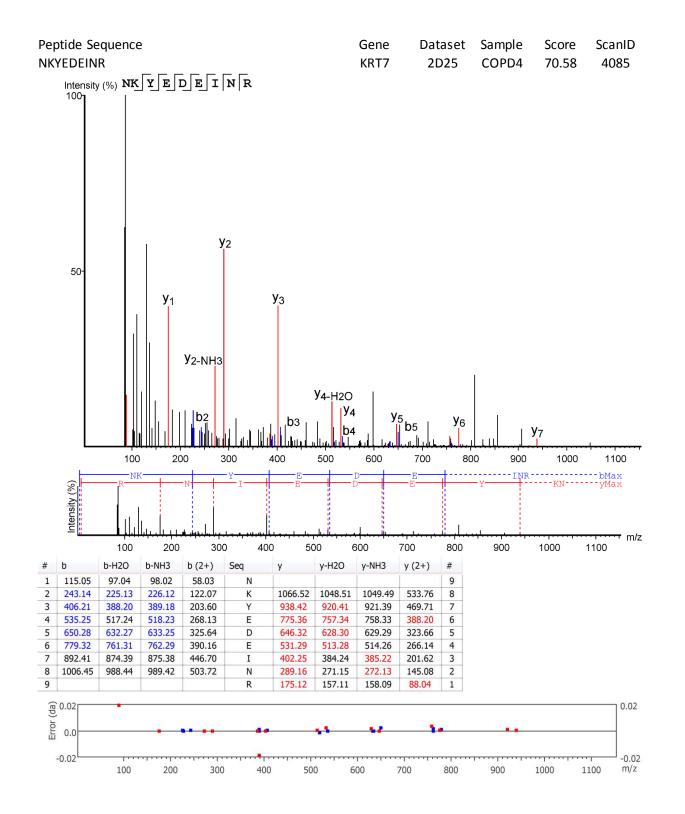


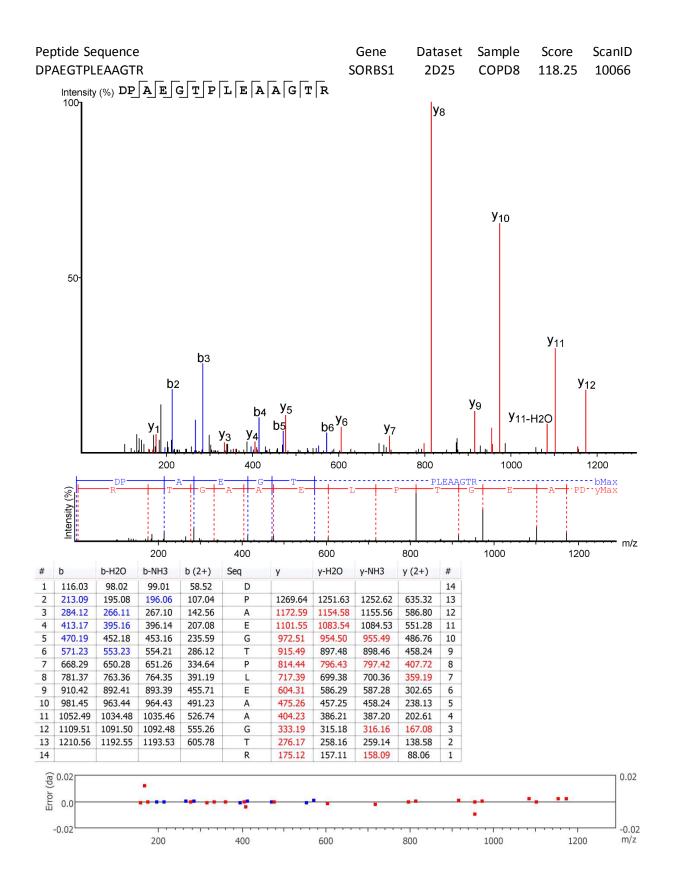


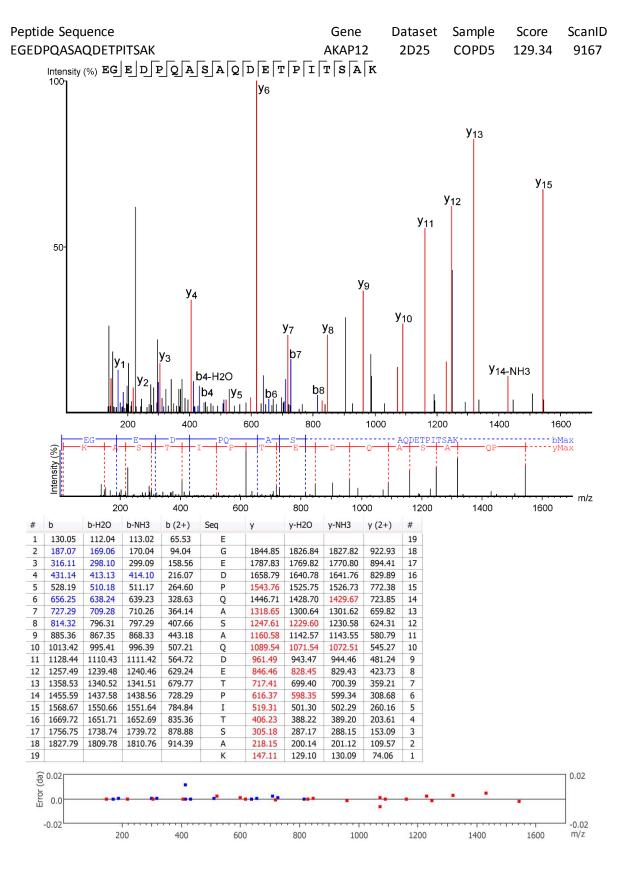


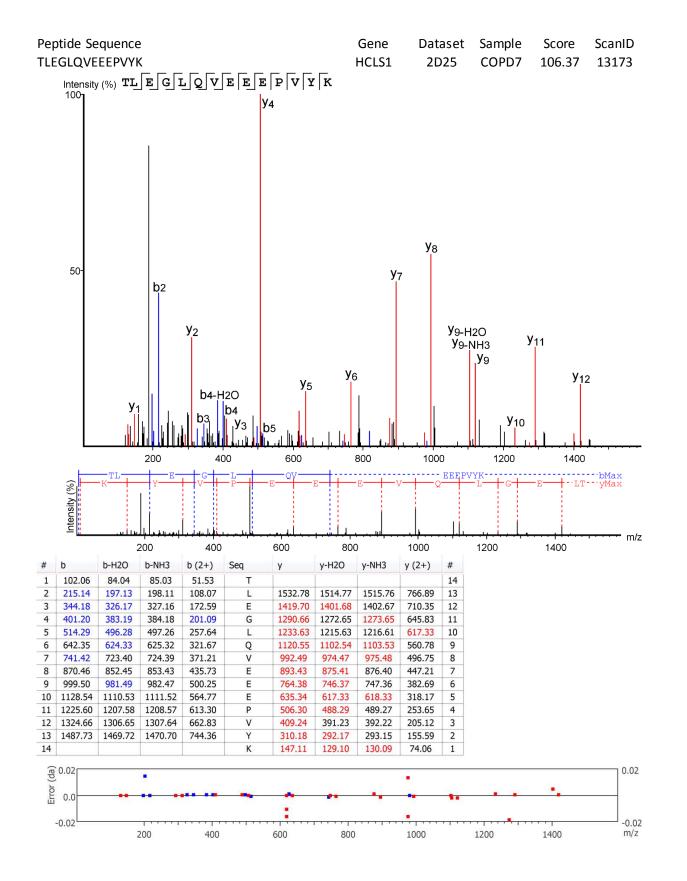


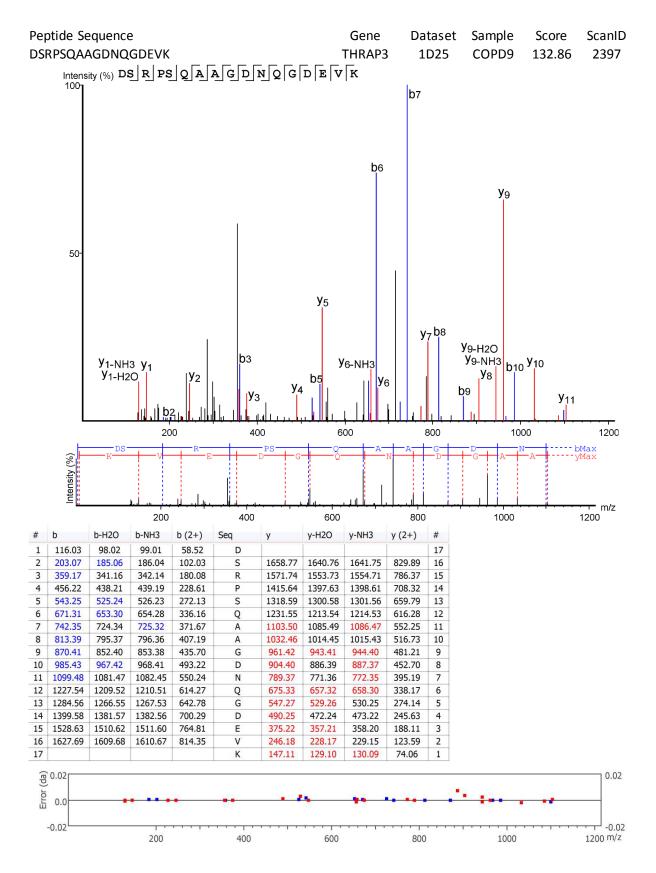


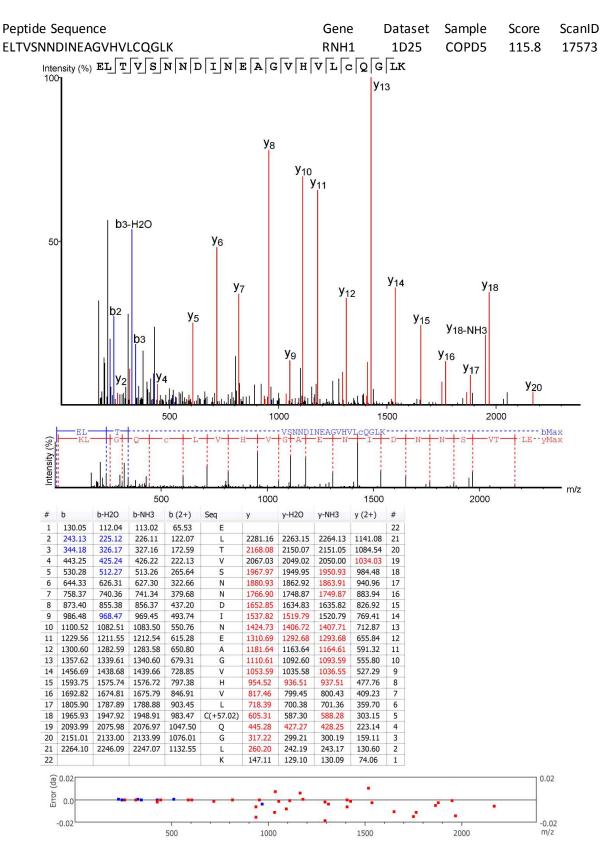


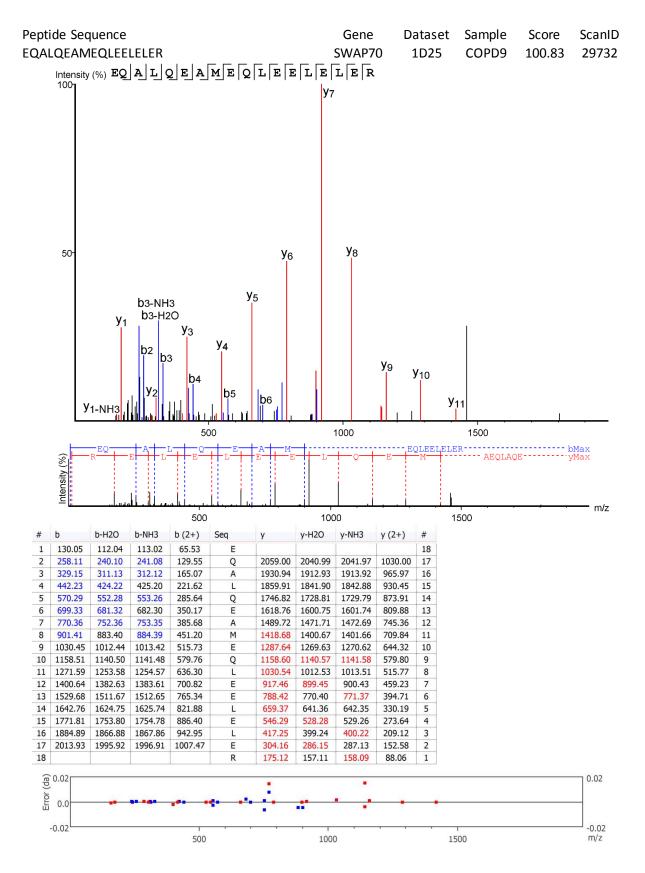


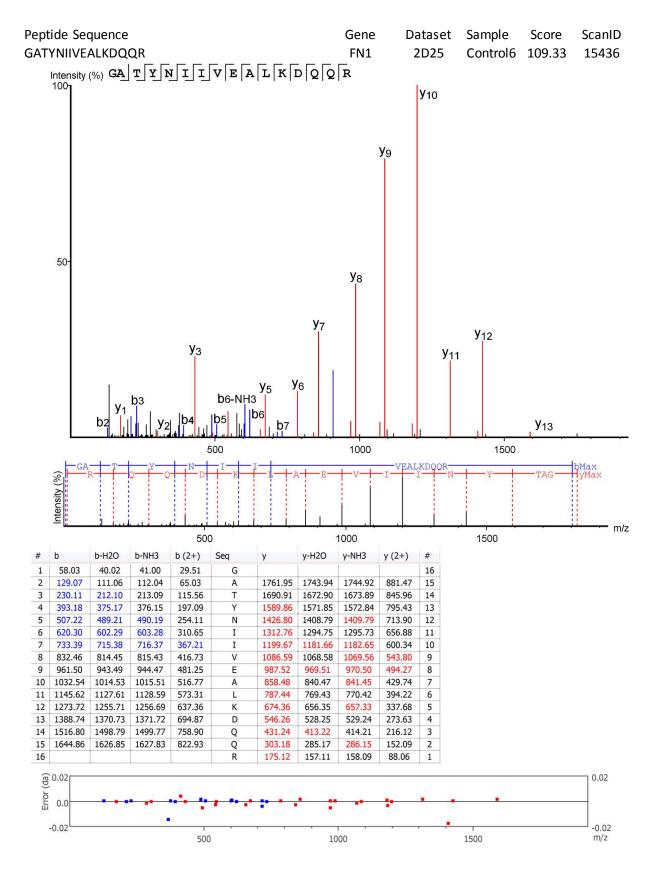


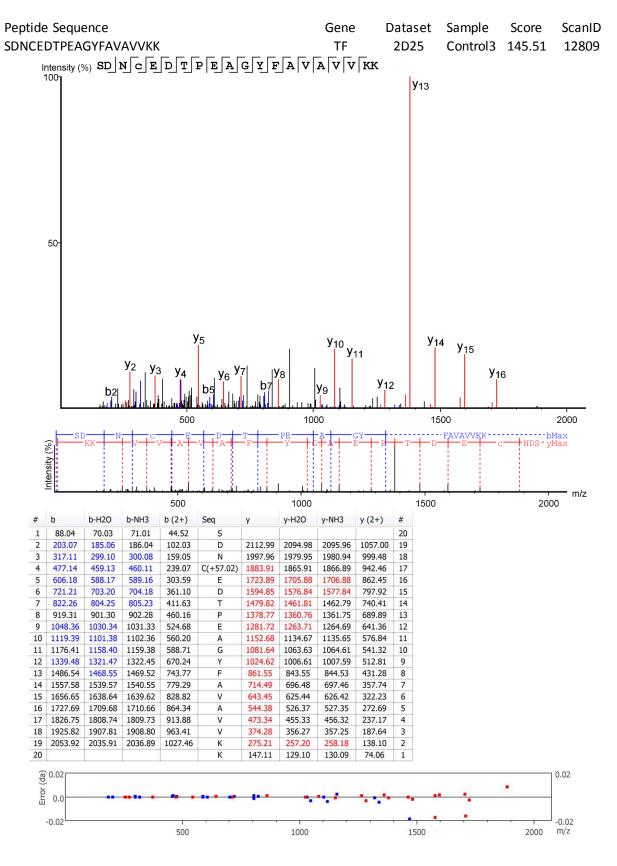


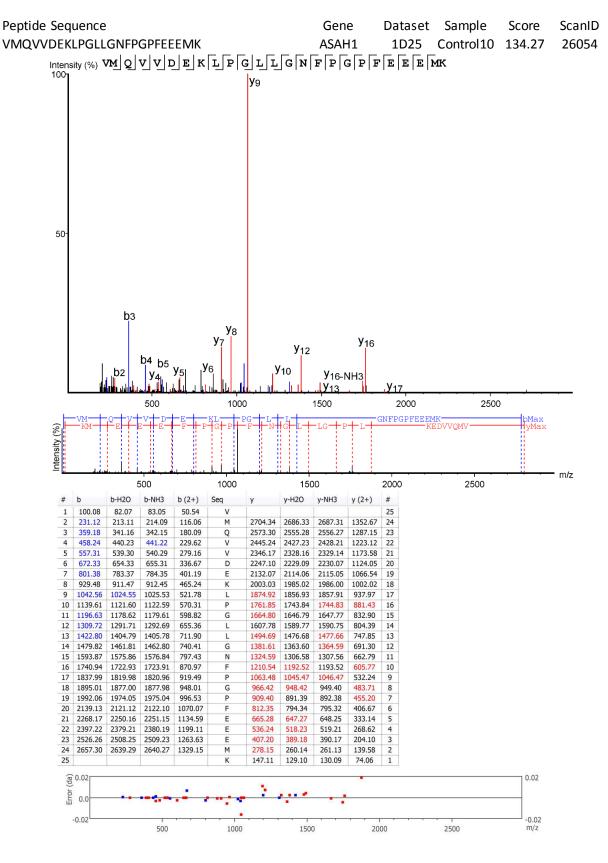


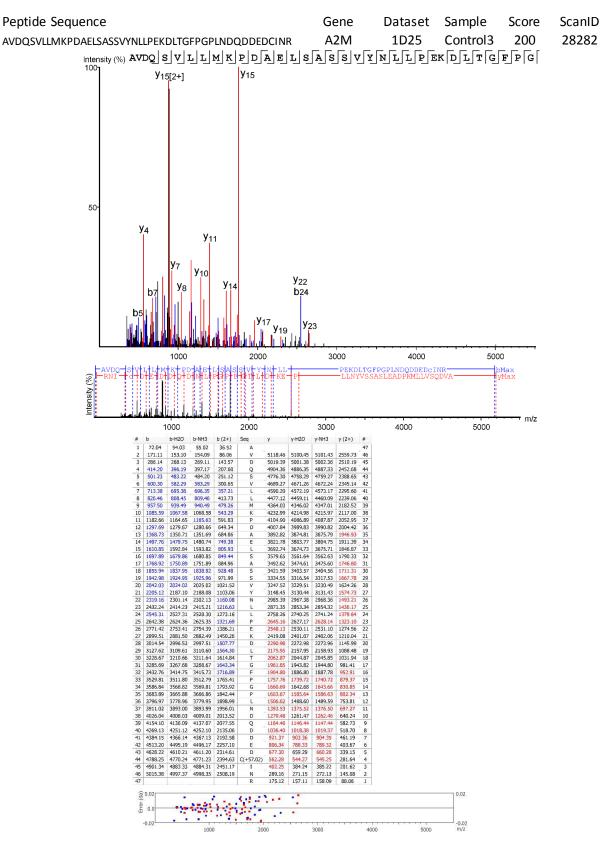


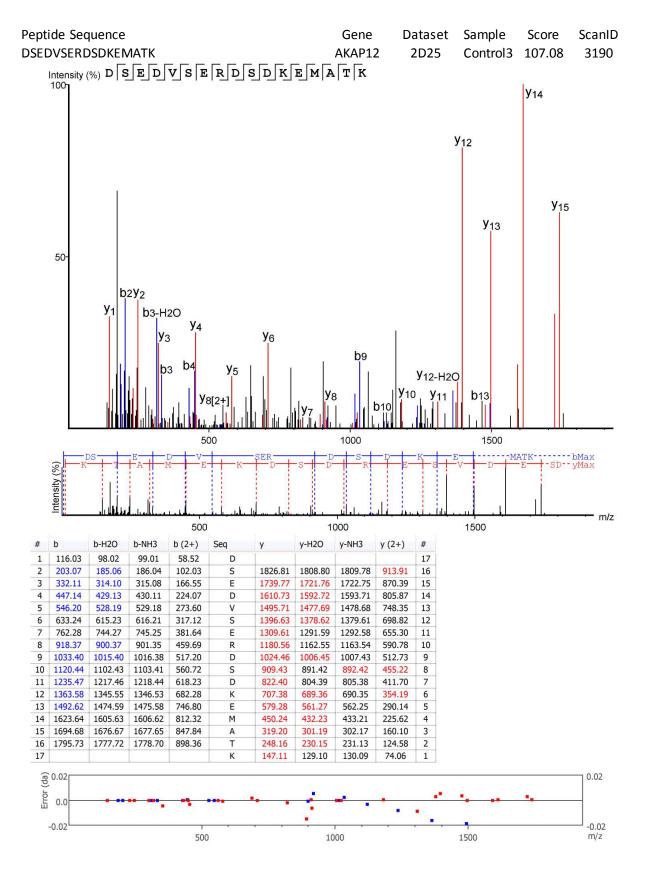


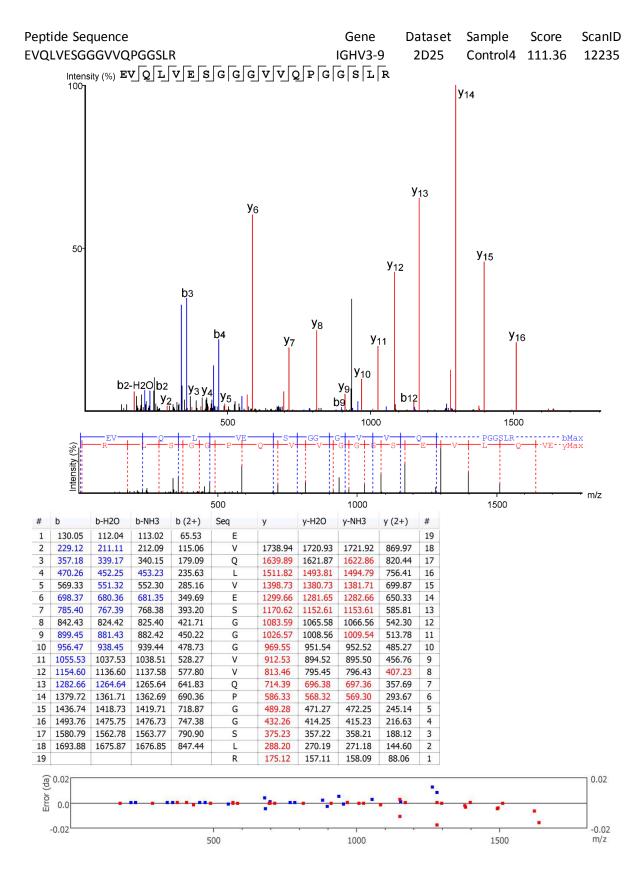


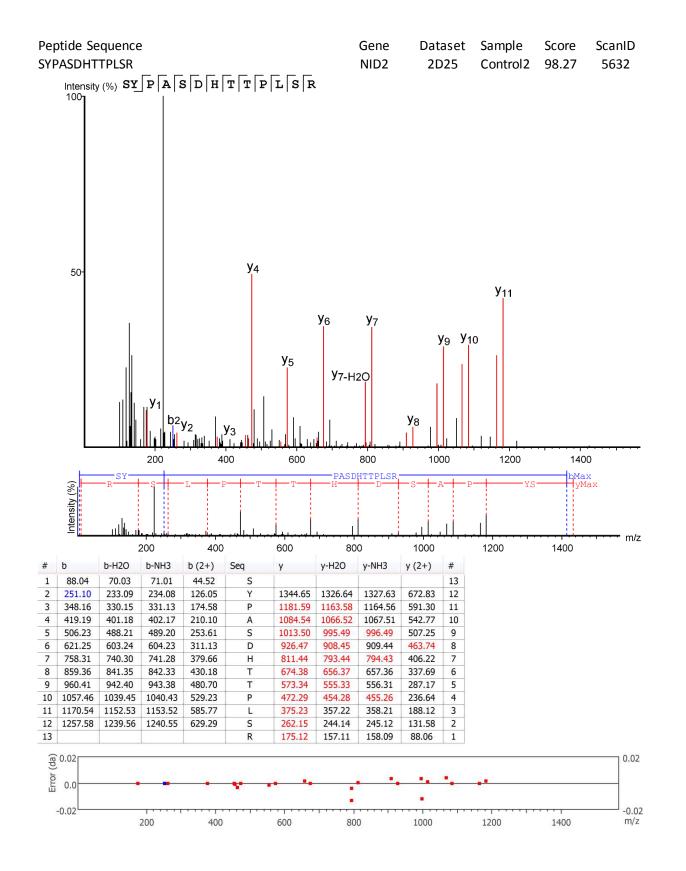


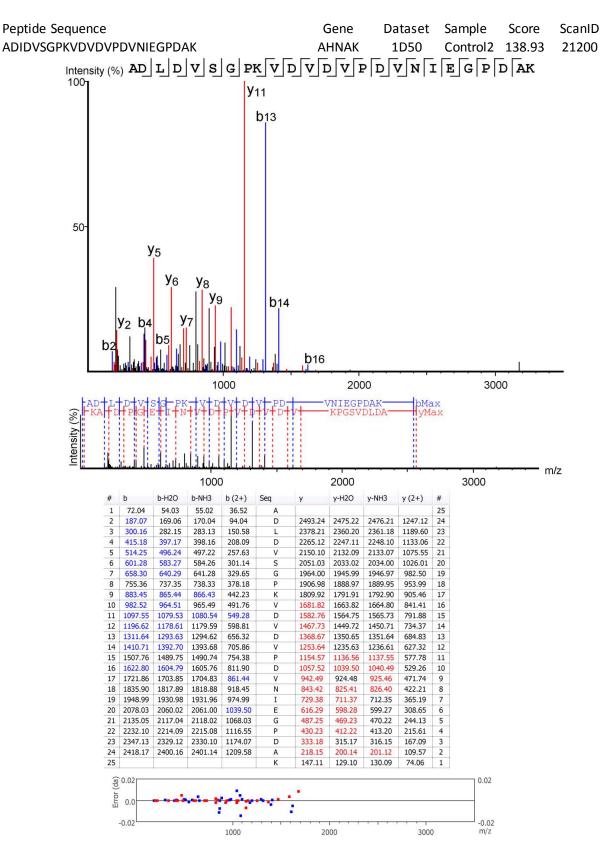




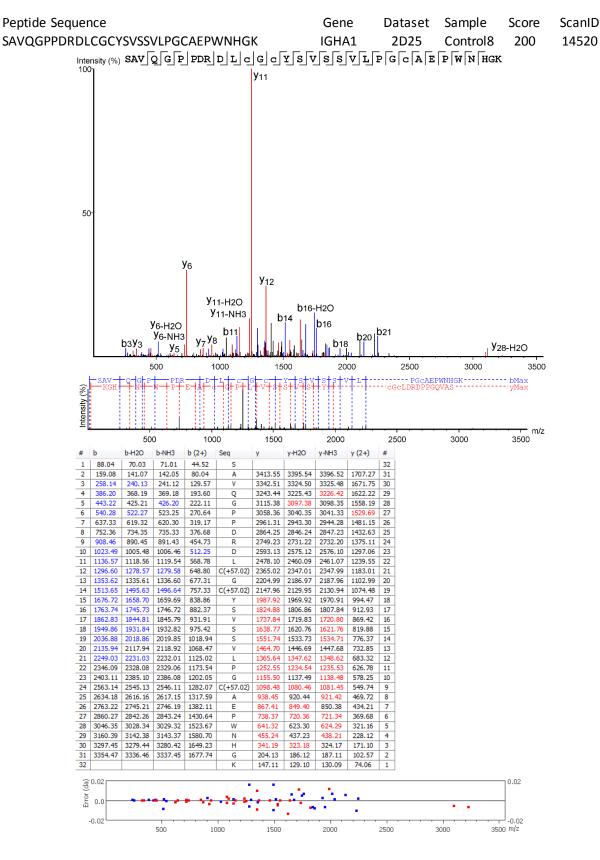


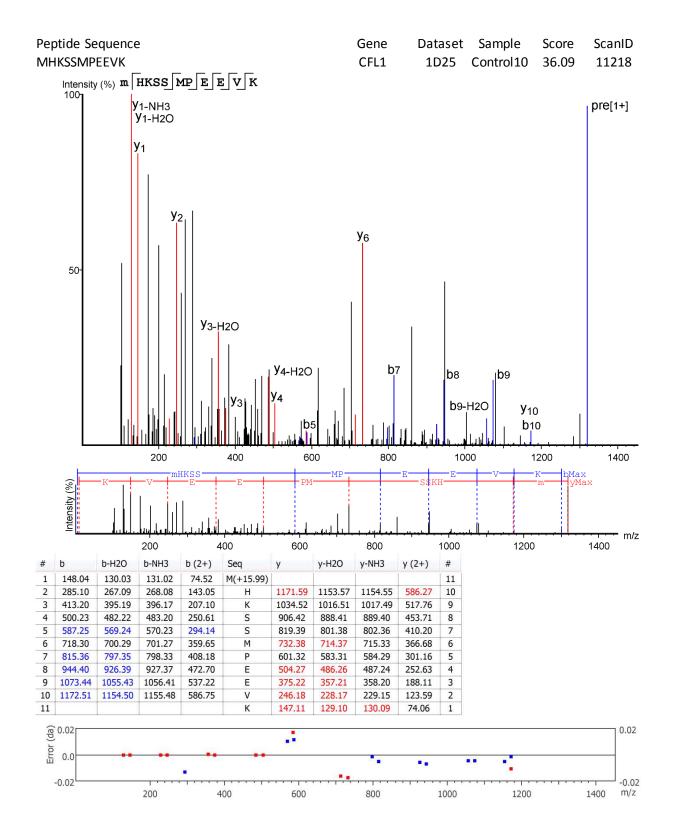


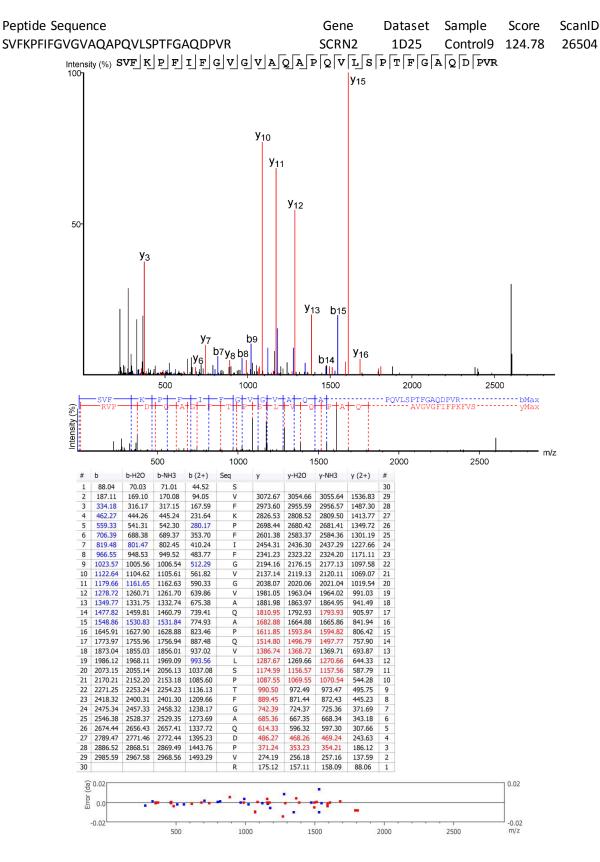


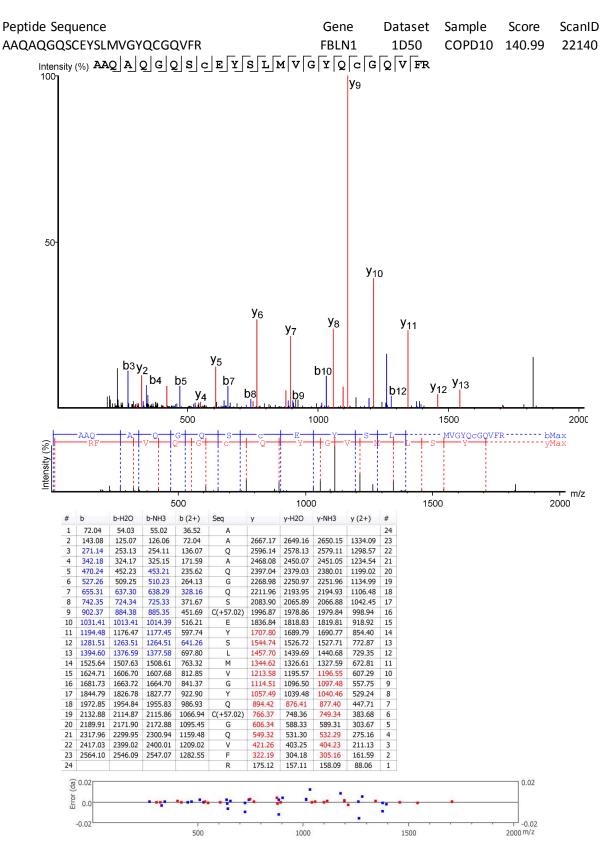




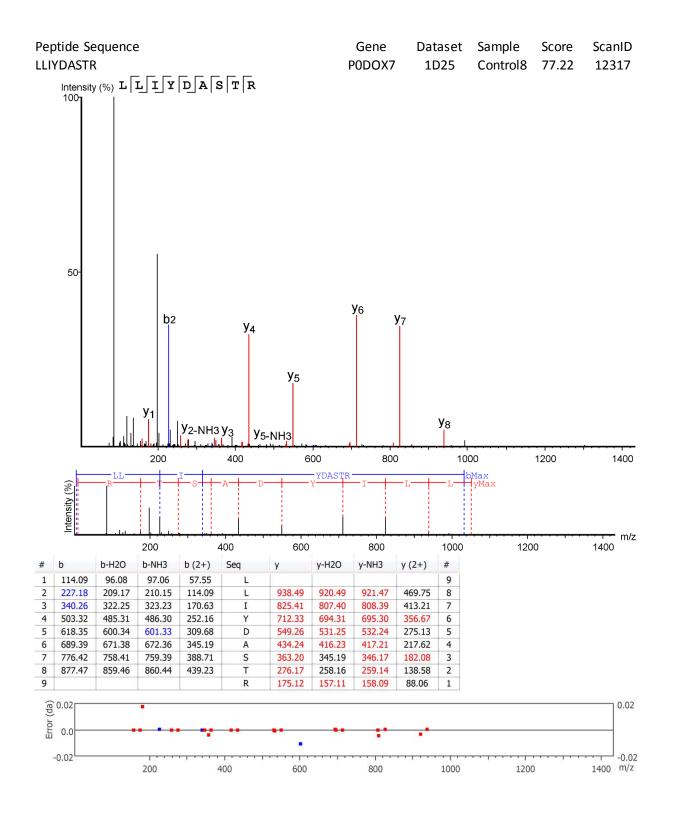


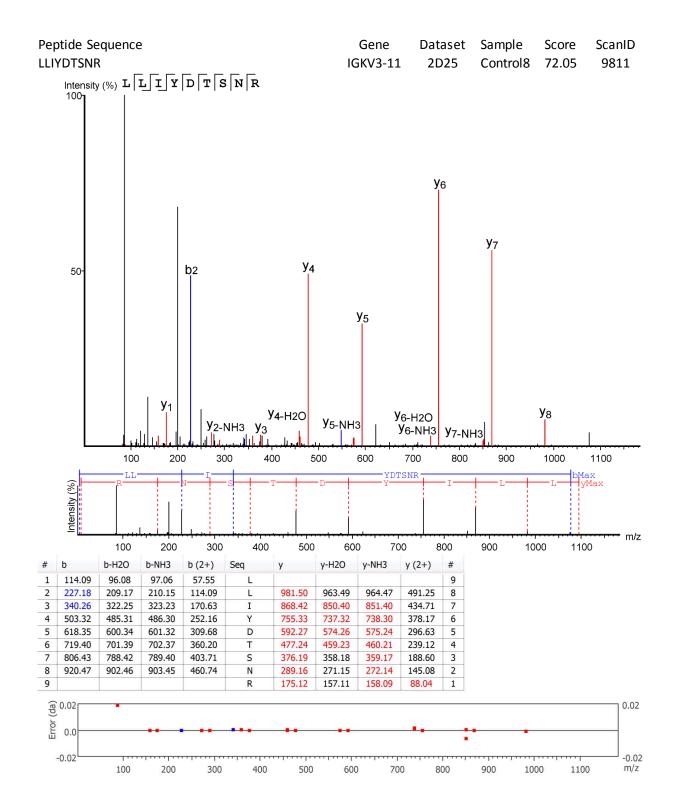


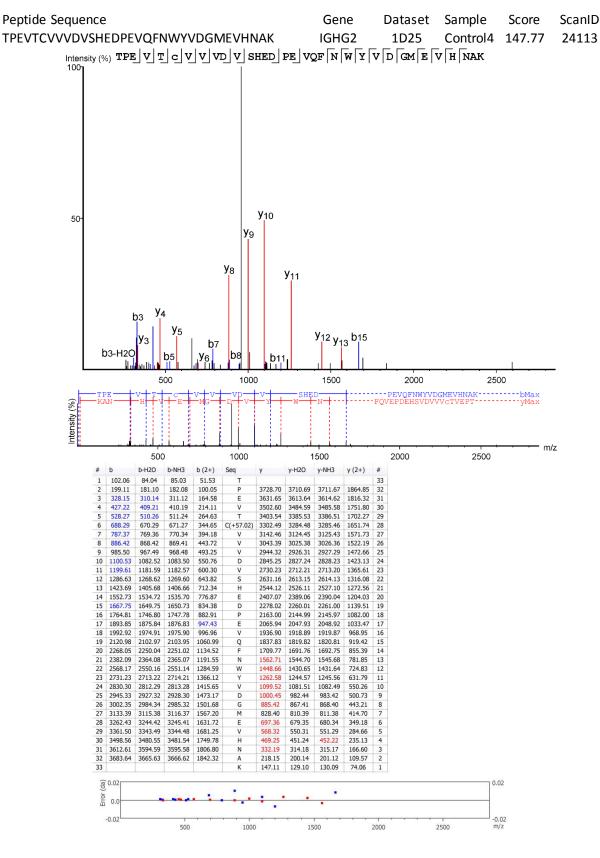


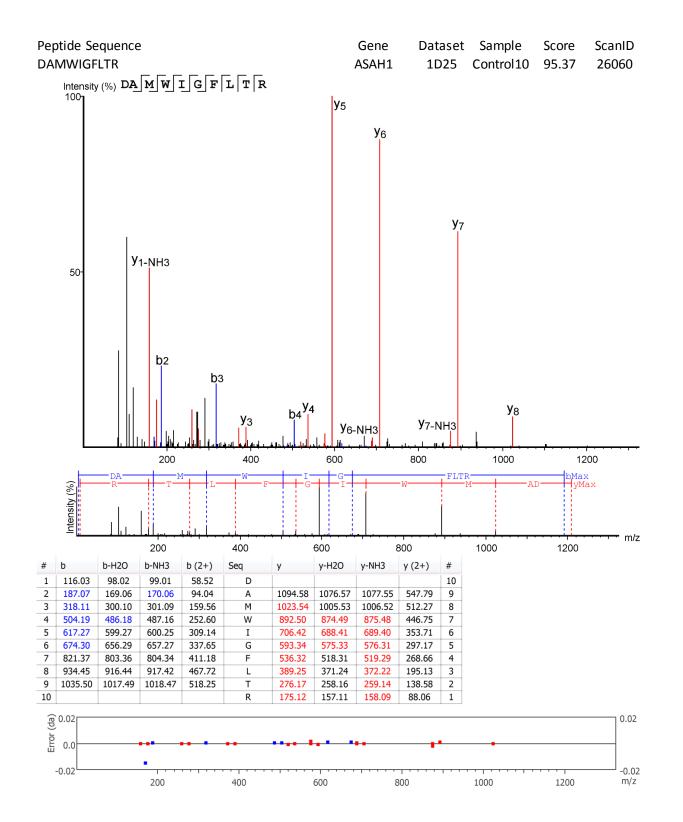


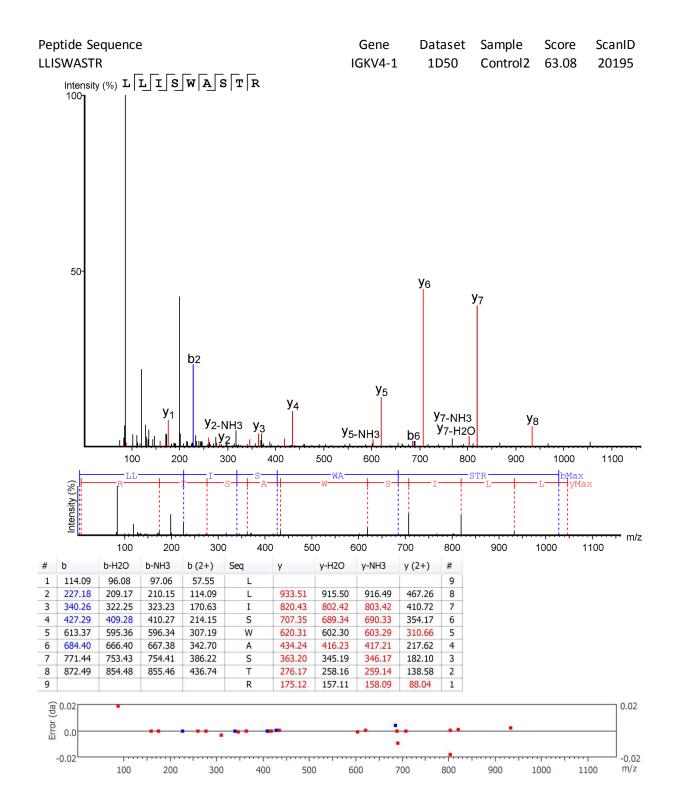


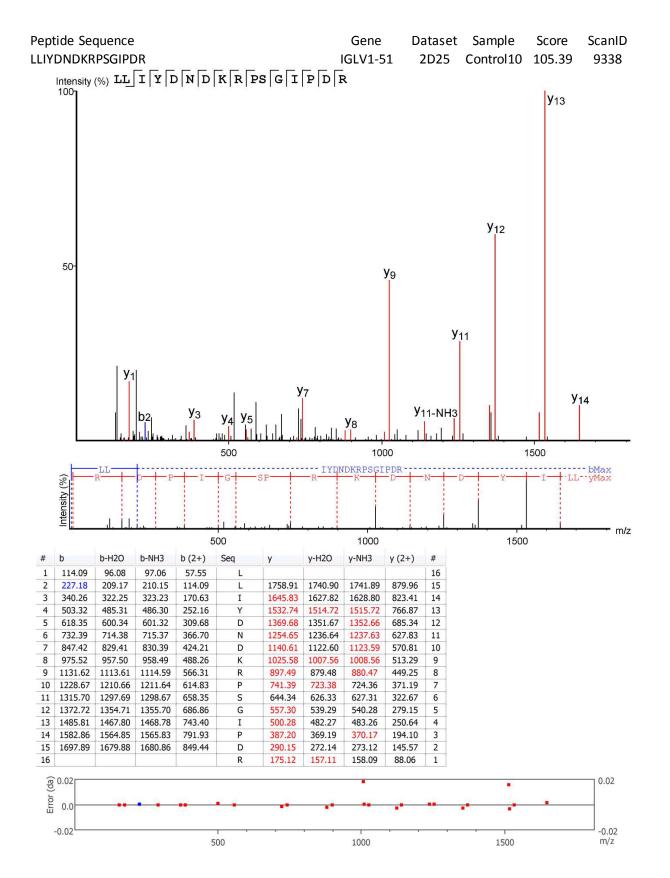


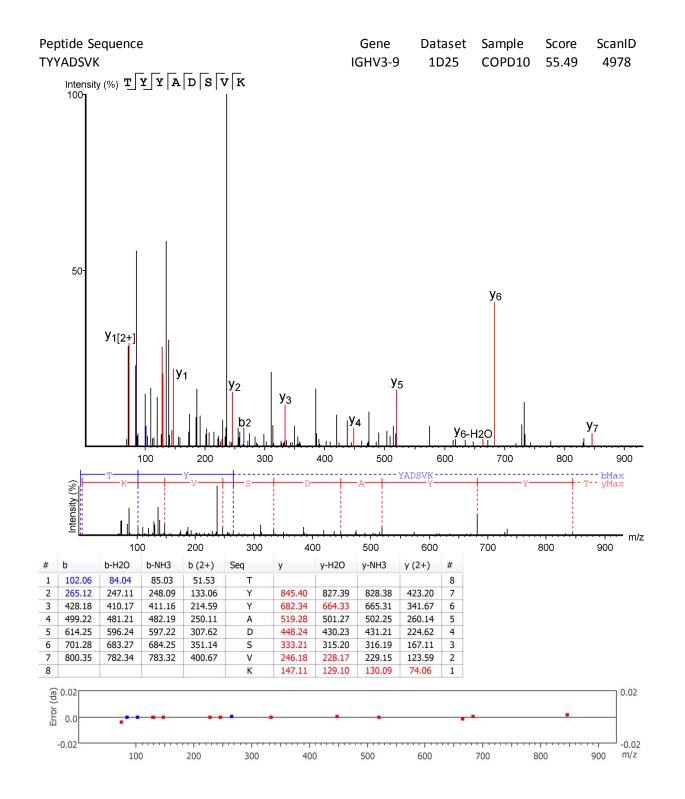


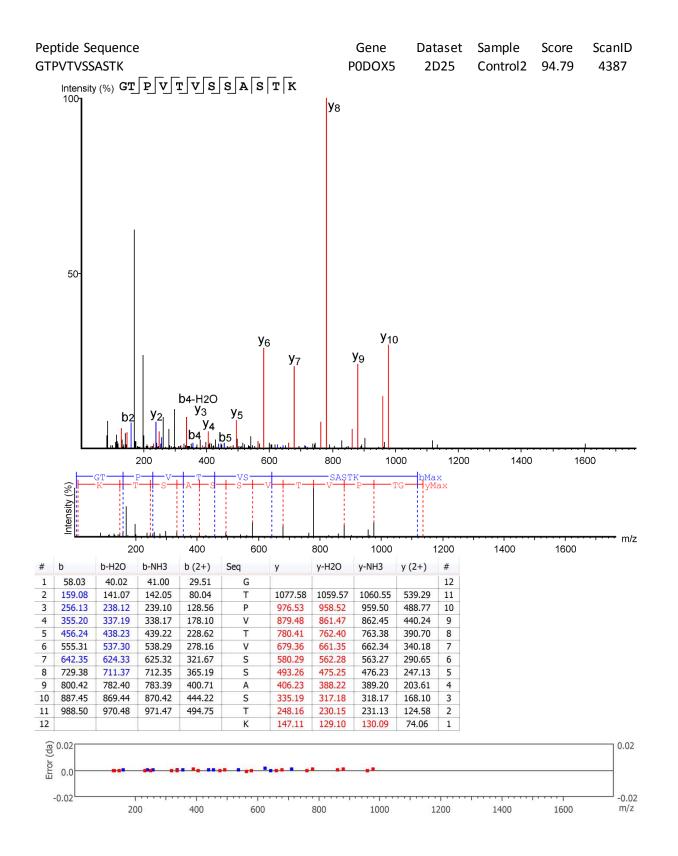


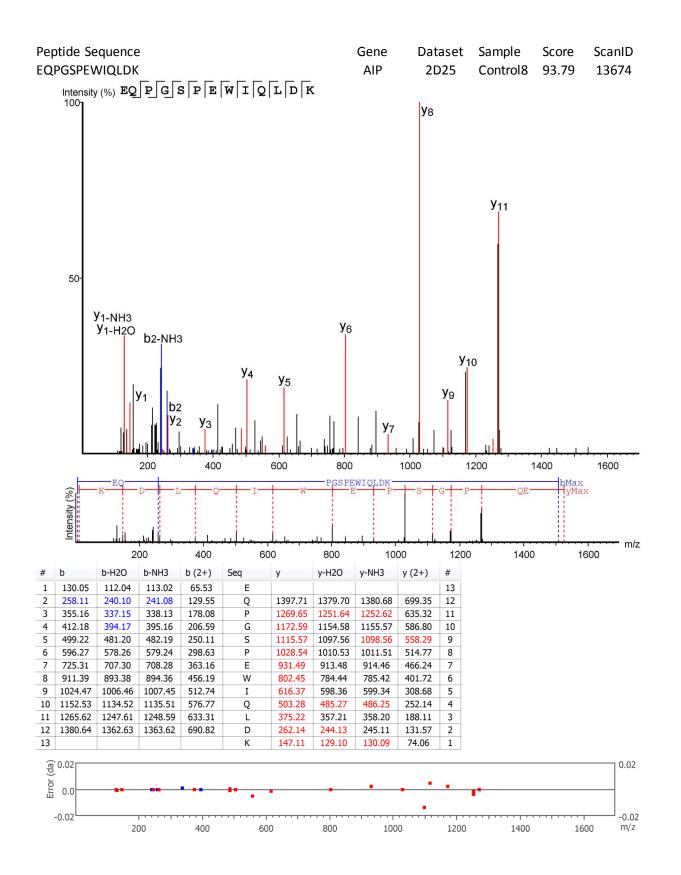


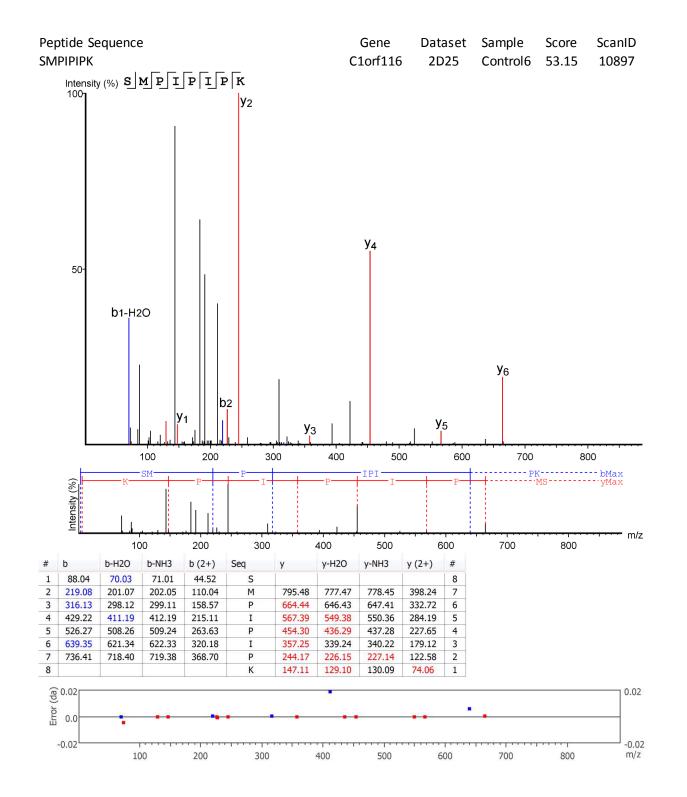


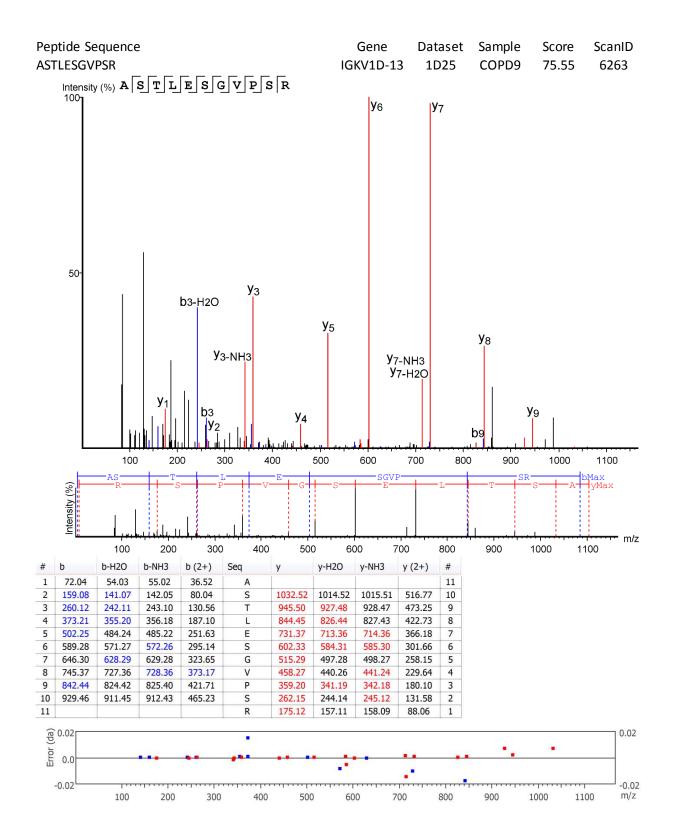


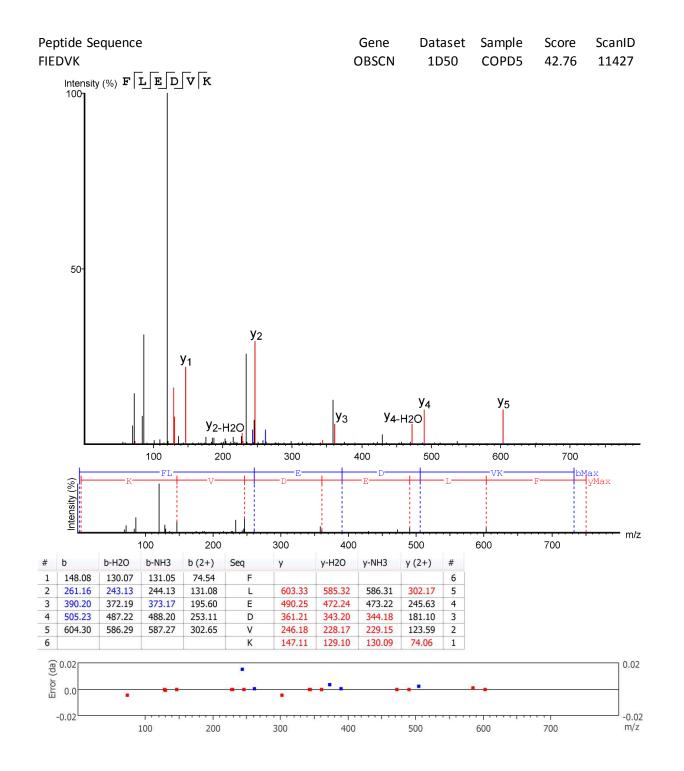


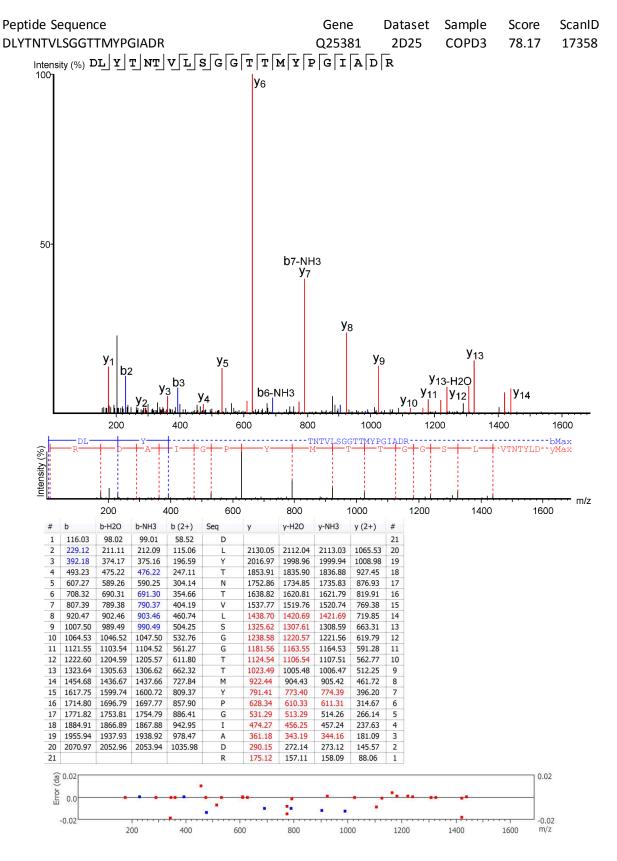


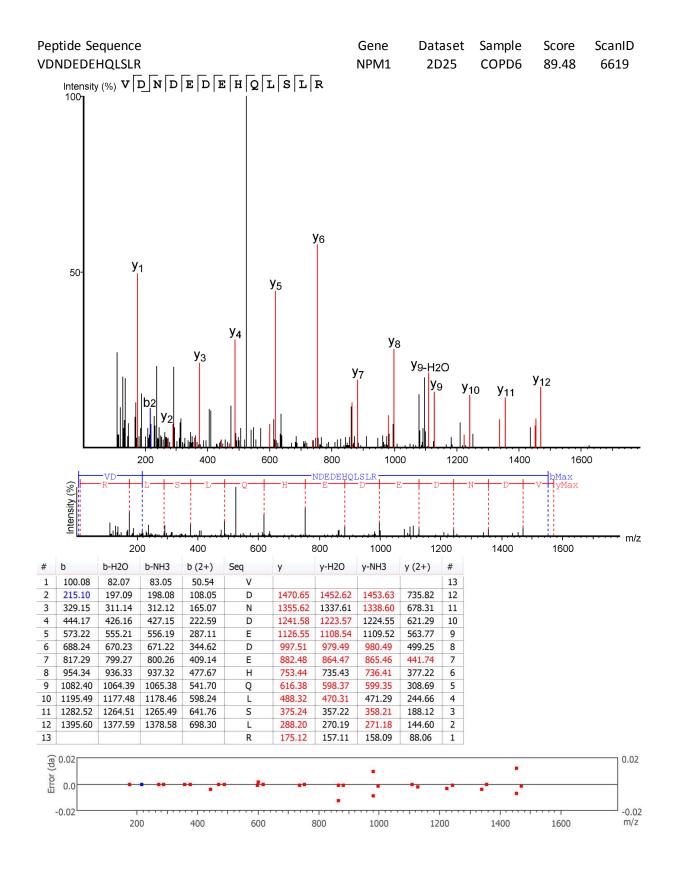


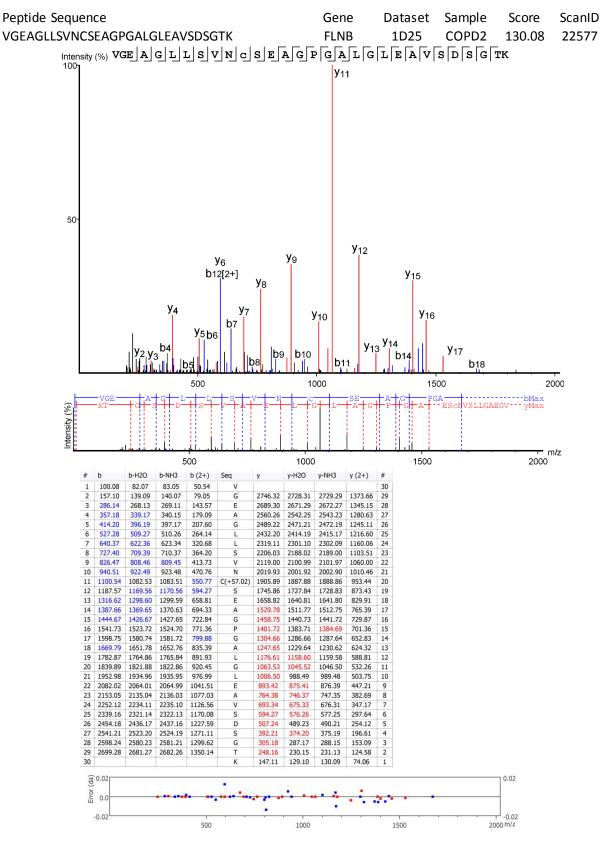




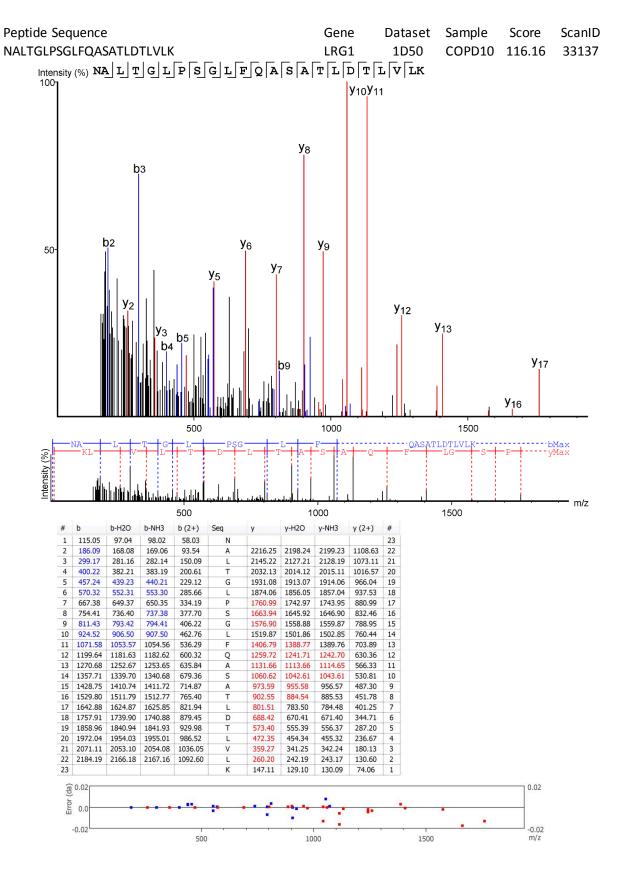


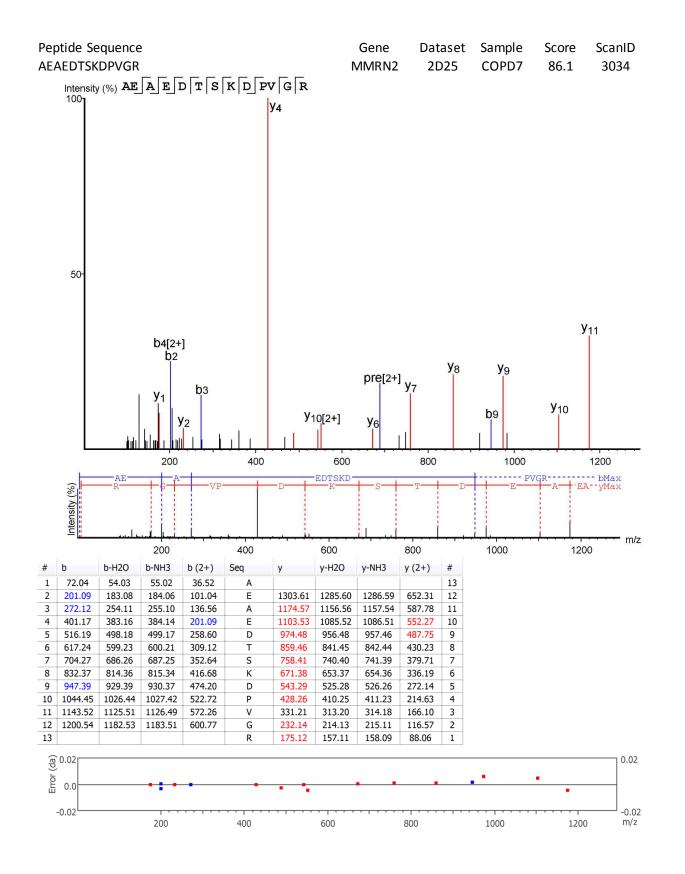


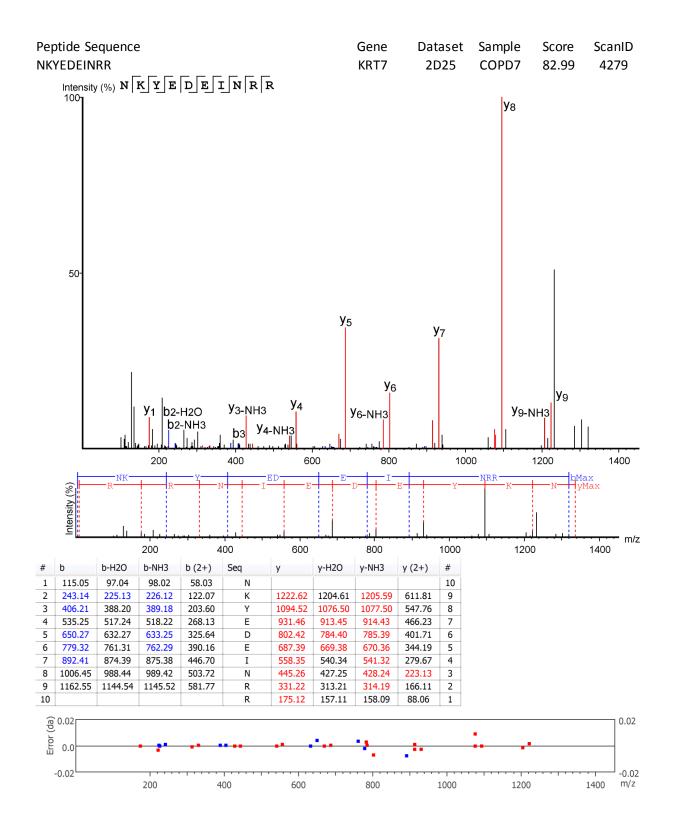


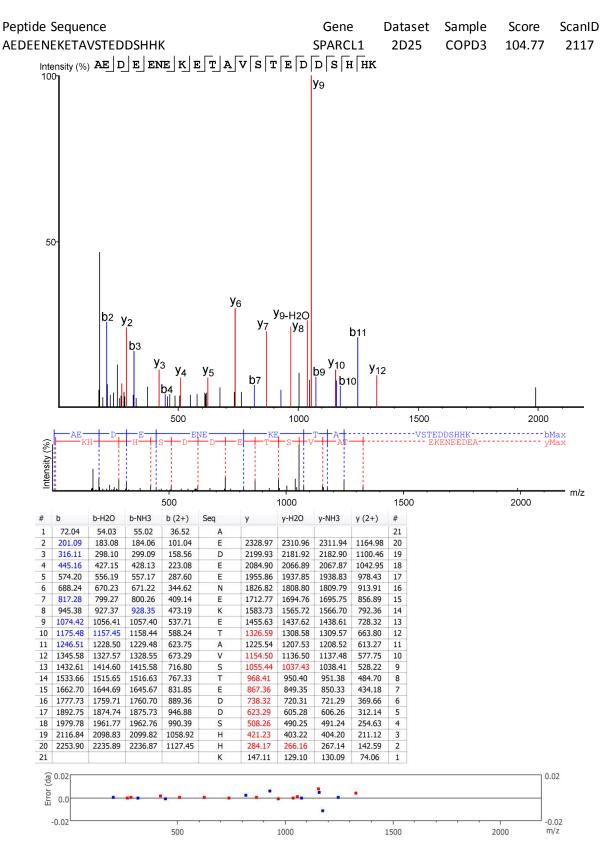


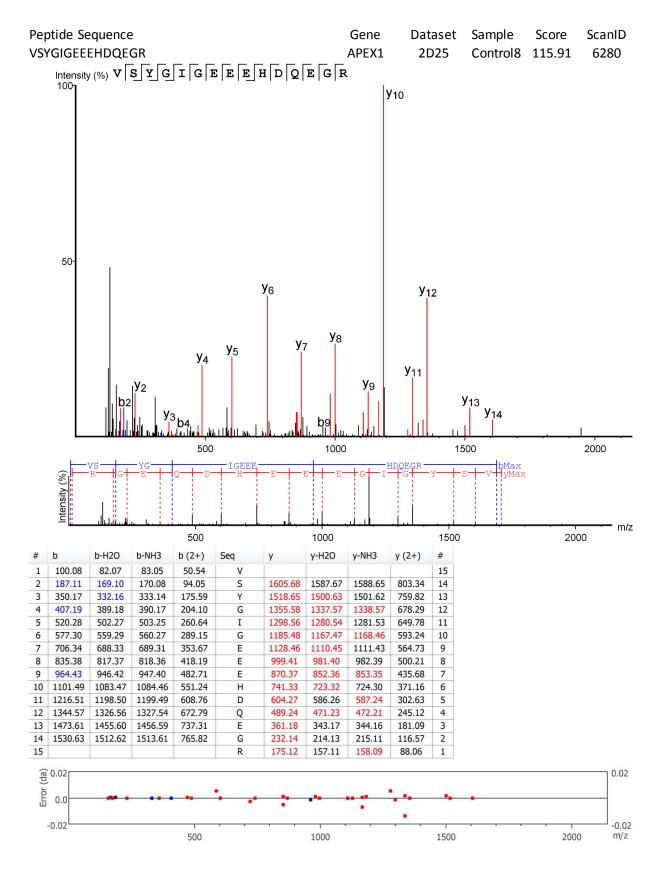


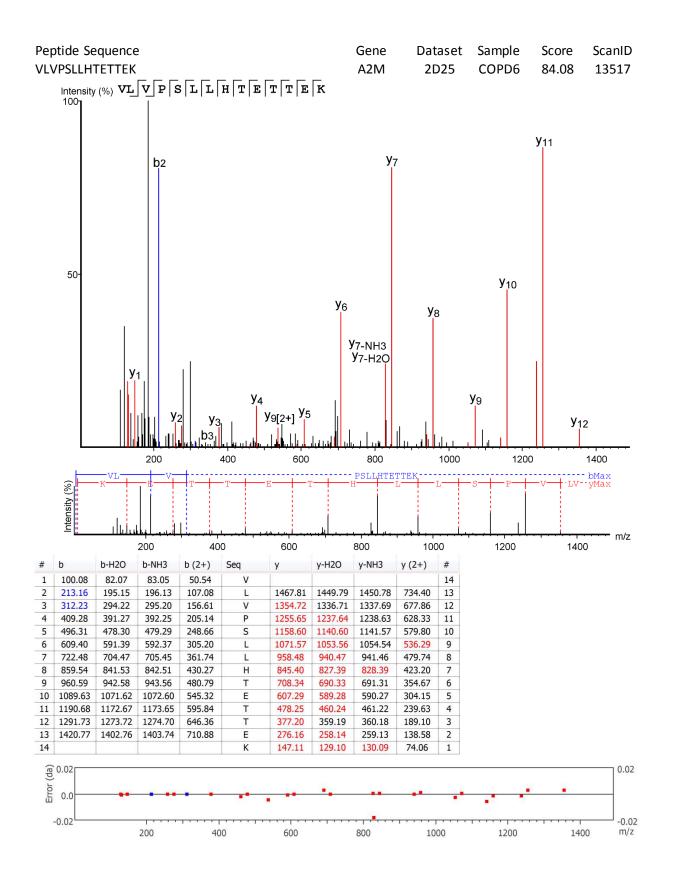


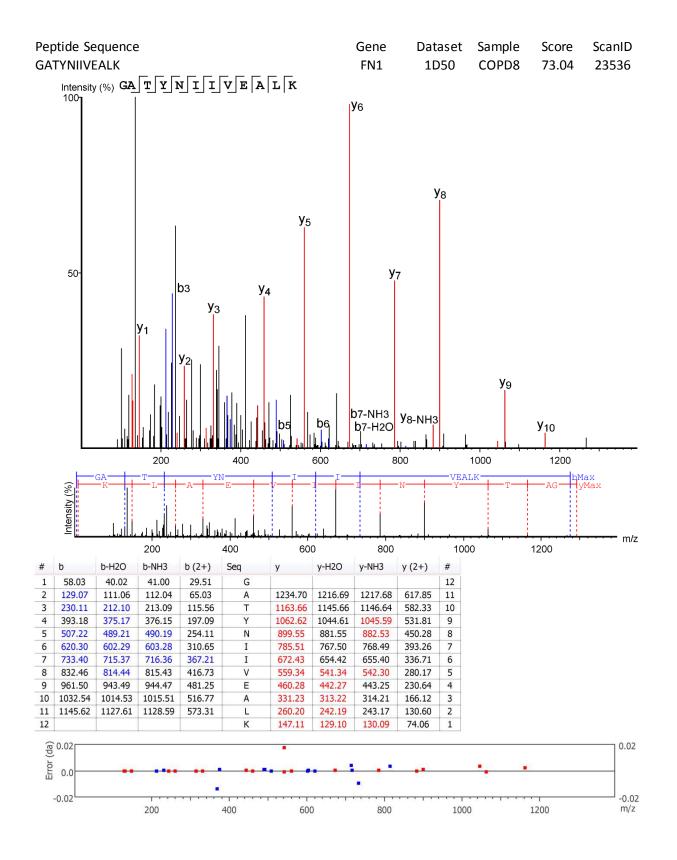


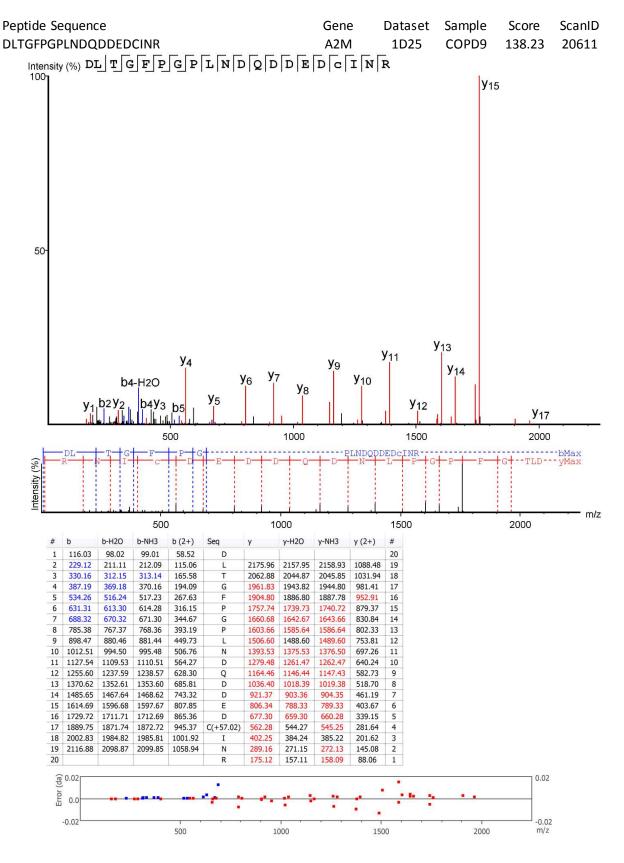




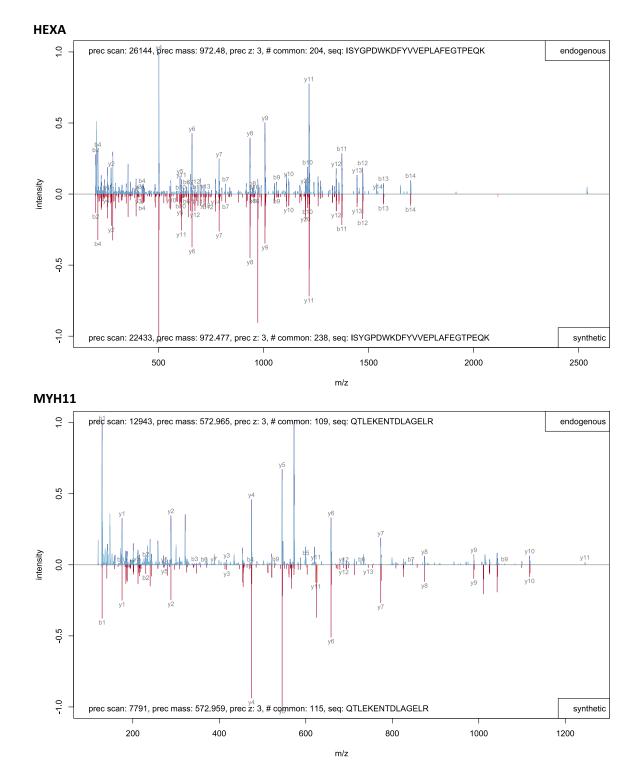


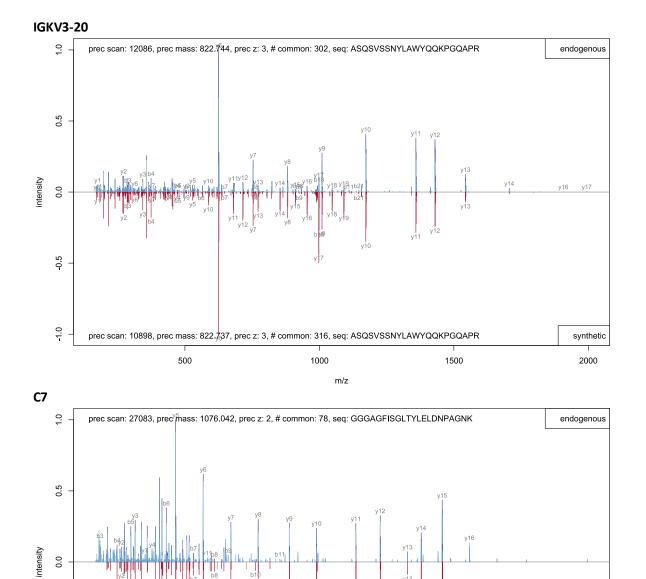


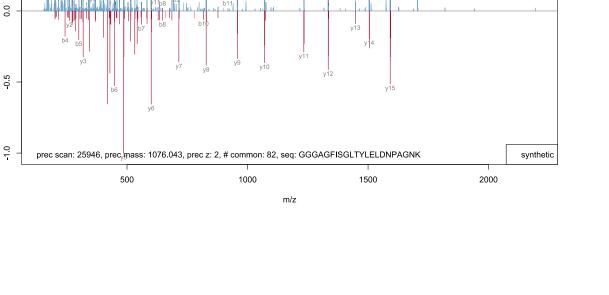


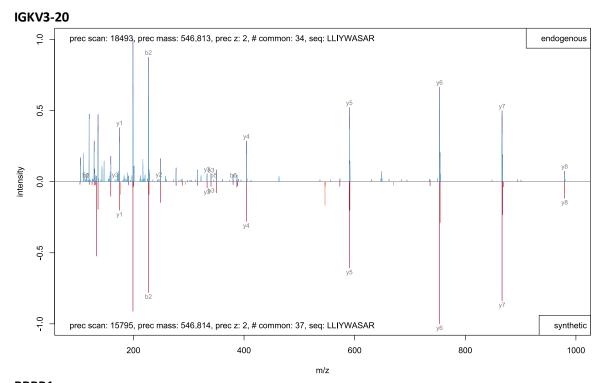


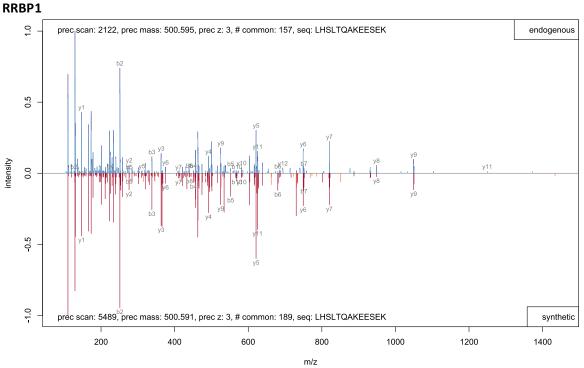
6 MS/MS spectra visualisation of synthetic and endogenous 23 nonreference peptides present only on COPD or control samples

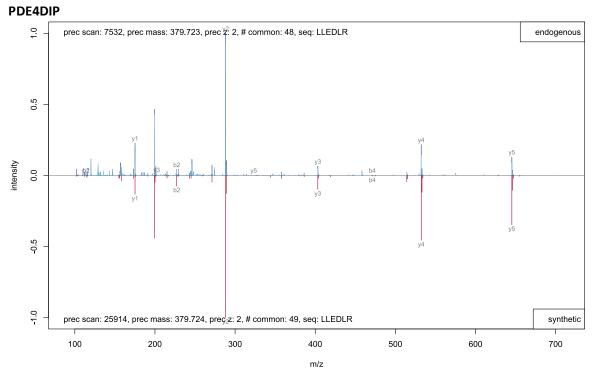




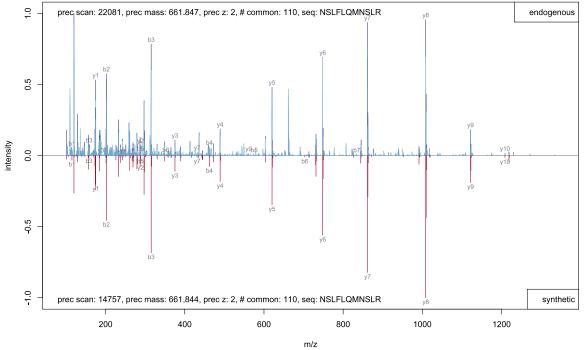


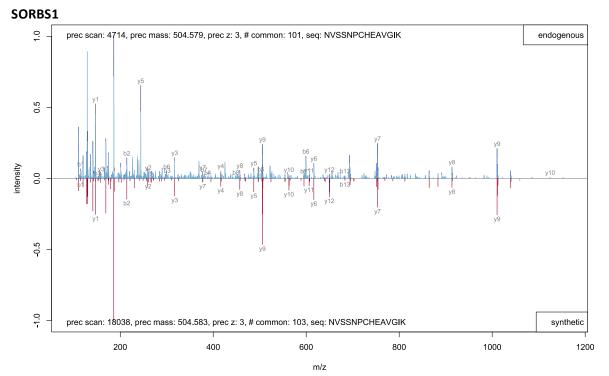




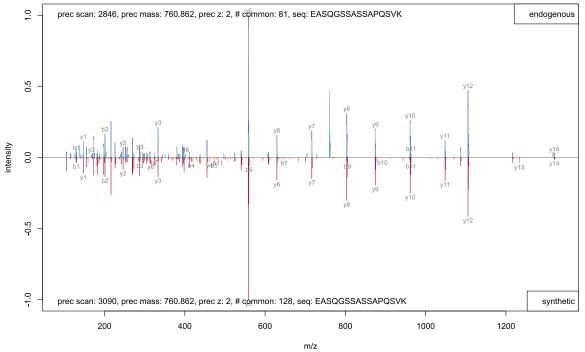


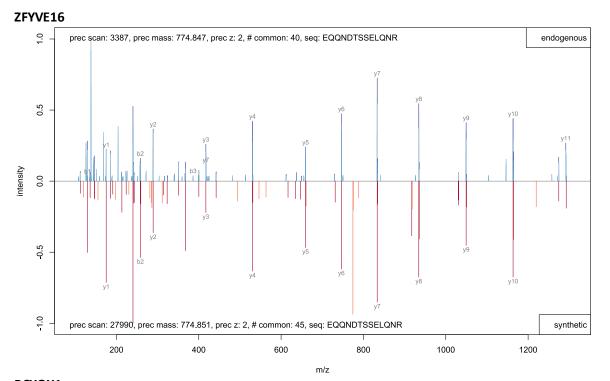


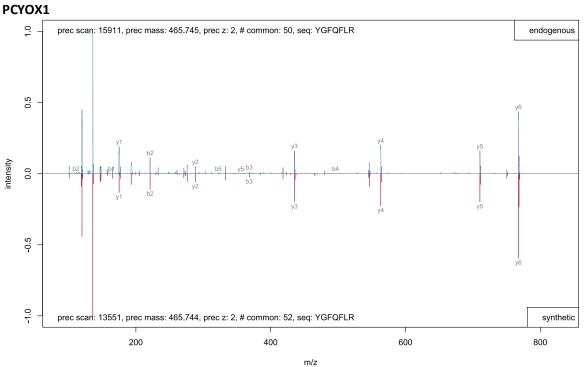


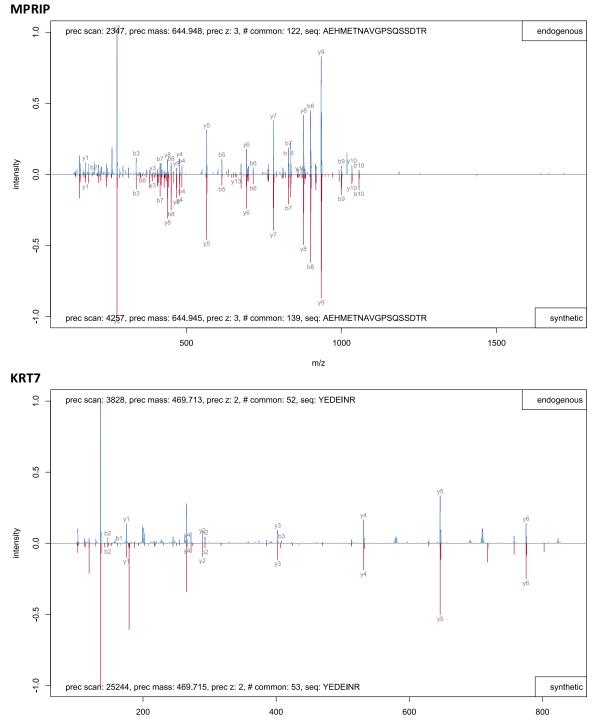












m/z

