Supplemental Materials

Supplemental Methods

Informatic Intercomparison Methodologies

Lab 109: The raw files were searched against the metagenomic database employing a 2 round search using PEAKS Studio X. The initial database search was performed to focus the metagenomic database for protein sequences with peptide sequence matches at 5% FDR. The focused database was further used for a second round search, which allowed a parent mass error tolerance of 10.0 ppm and a fragment mass error tolerance of 0.6 Da. The search considered up to 3 missed cleavages, carbamidomethylation as fixed and methionine oxidation and N-terminal acetylation as variable modifications. The cRAP protein sequences (http://ftp.thegpm.org/fasta/cRAP./) were included as contaminant database. Finally, PSMs were filtered for 1% FDR and annotated with taxonomic lineages (obtained from the metagenomic experiments). Non-unique peptide matches were annotated with the LCA of the respective lineages.

Lab 321: SearchGUI (Galaxy Version 3.3.10.1) was used to search using multiple search algorithms (X!Tandem, MS-GF+ and Comet). For each search algorithm, Precursor Tolerance of 10.0 ppm, Fragment Ion Tolerance of 0.6 Da and trypsin was used as an enzyme for proteolytic cleavage. Searches were performed allowing for two missed cleavages fixed modification of Carbamidomethylation at cysteine and Variable Modifications of Acetylation of protein N-term and Oxidation of Methionine. PeptideShaker (Version: 1.16.36) was used to filter peptides with the length of 8-50 aas and a precursor m/z tolerance of 10.0 ppm. Detected Peptide-spectral matches, peptides and proteins were reported at 1% global FDR. All of the analysis was performed within Galaxy platform.

Lab 321: MaxQuant (Galaxy version 1.6.17.0+galaxy3) was used to search the datasets. A fixed modification of Carbamidomethylation at cysteine and Variable Modifications of Acetylation of protein N-term and Oxidation of Methionine was applied along with allowing for two missed cleavages. The detection peptides and proteins were reported at 1% FDR.

Lab 362: The raw files were converted using ThermoRawFileParserGUI (version 1.4.1) to peak lists (.mgf files) using “native Thermo library peak picking” as the peak picking option and “Ignore missing instrument properties” as the error option. The peak lists (.mgf files) obtained from MS/MS spectra were identified using X! Tandem version X! Tandem (Vengeance version 2015.12.1) using SearchGUI version 4.1.0. Here, the parameters provided and suggested by the study were used: tolerances of 10 ppm for MS1 and 0.6 Dalton for MS/MS; dynamic modifications: oxidation of M, and Acetyl on N-terminus; static modifications: Carbamidomethyl of C. Identification was conducted against a concatenated target/decoy database of the provided database (Intercal_ORFs_prodigal_metagenemark_Abbreviated-IDs.fasta).

The X!Tandem files were used as input in MS²ReScore (https://github.com/compomics/ms2rescore), a machine learning-based post-processing tool that improves upon Percolator rescoring of peptide-to-spectrum matches (PSMs). Here, the search engine-dependent features of Percolator were appended with MS2 peak intensity features by comparing the PSM with the corresponding MS²PIP-predicted spectrum. All reported MS²ReScore PSM identifications have a q-value < 0.01. No protein grouping algorithm was applied, and all identified taxa and functions are extracted from the provided database.

Lab 458: The Proteome Discoverer 2.5 platform was used (SequestHT + Percolator (MPS)). Fully tryptic peptides with a minimum length of 6 peptides and a maximum of 2 missed cleavages were required. Precursor Tolerance of 10.0 ppm, Fragment Ion Tolerance of 0.6 Da. carbamidomethylation as fixed and methionine oxidation was set as a variable modification. Filtering

was performed at a 1% PSM- and peptide-level FDR. The MaxQuant contaminant list was used as a contaminant database.

Lab 501: We first appended the database with a set of common contaminants (Global Proteome Machine Organization common Repository of Adventitious Proteins). Then, we used MSGF+ (Kim and Pevzner, 2014) to match mass spectra with peptide sequences, with cysteine carbamidomethylation as a fixed modification, and methionine oxidation, glutamine modified to pyro-glutamic acid, deamidated asparagine, and deamidated glutamine, as variable modifications. Peptides were searched for with a Target-Decoy approach, with a 1% false discovery rate at the peptide spectrum match level. For spectral counts, we summed MS2 spectra that identified a peptide, and normalized all spectral counts to the total spectral counts per sample.

Proteins were quantified using the median spectral count for all proteotypic peptides (those peptides which uniquely correspond to a protein), specifically using the OpenMS tool ProteinQuantifier. This approach requires at least one proteotypic peptide, but if more are identified, those peptides are also used for quantification.

Lab 828: The raw files were analyzed using Thermal proteome discover. MS/MS spectrums were searched against provided database using SEQUEST-HT engine. MS/MS spectra searches were performed as follows: precursor ion tolerance of 10.0 ppm; fragment ion tolerance of 0.6 Da; carbamidomethyl cysteine was specified as fixed modification, whereas oxidation (M), deamidation (N/Q), and N-terminal protein acetylation were set as variable modifications. Trypsin was specified as the proteolytic enzyme, allowing for two missed cleavages. Percolator-based scoring was chosen to improve the discrimination between correct and incorrect spectrum identifications, learning from the results of a decoy and target database; settings were as follows: maximum delta Cn, 0.05; strict false-discovery rate of 0.01 and validation based on q values.

Lab 902: SEQUEST-HT was used within Proteome Discoverer 2.2 using the following settings: maximum missed cleavage 2, minimum peptide length 6, maximum peptide length 122, precursor mass tolerance 10ppm, fragment mass tolerance 0.6 Dalton; dynamic modifications: M oxidation, acetyl on N-terminus; static modifications: C carbamidomethyl. Percolator PSM validator (within Proteome Discoverer) with following settings: maximum Delta Cn 0.05, target FDR strict 0.01, target FDR relaxed 0.05, validation based on PEP. Scaffold 5.0 used to analyze Proteome Discoverer generated files with following settings: scoring system: prefiltered mode; protein grouping: standard experiment wide protein grouping; protein threshold 1.0% FDR; peptide threshold 0.1% FDR; minimum number of peptides 1.

Lab 932: Mass spectrometry data were transformed from Thermo RAW format (version 66) to mzML and Mascot Generic (MGF) formats using ThermoRawFileParser (version 1.2.0, Hulstaert et al., 2020). Experimental metadata were extracted from mass spectrometry data using the MARMoSET program (Kiweler et al. 2019). Mascot Server (version 2.6.2, Matrix Science, LTD) software performed peptide-spectrum matching between experimental data and a reference sequence database. Reference sequences included a total of 197,824 predicted protein-coding ORFs from a metagenome assembly. Peptides matching an in-house curated inventory of contaminant protein sequences, mass standards, and proteolytic enzyme sequences were removed from the results. Mascot search parameters included the following settings: +10.0 ppm monoisotopic precursor mass tolerance; +0.6 Da monoisotopic fragment ion tolerance; one fixed modification (+57 to C residues); two variable modifications (+16 to M residues, +42 to peptide amino-termini); digestion enzyme trypsin; two missed cleavages; peptide charges +2-+7; and instrument type: electrospray ionization coupled to fourier-transform ion cyclotron resonance (ESI-FTICR). Mascot search results containing peptide-spectrum matches (PSMs) were exported for downstream data analysis. Scaffold Q+S (version 4.8.9) was used to validate MS/MS-based peptide- and protein-level peptide-spectrum matches (PSM) with the Peptide Prophet algorithm. Mascot PSM data were imported into Scaffold Q+S with the

following settings specified: quantitative metric: spectrum counting; scoring system: use legacy Peptide Prophet scoring (high mass accuracy); protein grouping: use standard experiment-wide grouping; optional loading steps: pre-compute false discovery rate (FDR) thresholds; and, use local gene ontology (GO) annotations (UniProt GO annotation data retrieved 25 JUN 2020). Scaffold Q+S identification criteria were set at greater/equals >99.9% probability by the Peptide Prophet algorithm (Keller et al. Anal. Chem. 2002.) and >99.9% probability by the Protein Prophet algorithm (Nesvizhskii et al., Anal. Chem. 2003) with >2 peptides at the protein level.

Lab 957: MSFragger 3.3 searches were performed with FragPipe 16.0 and Philosopher 4.0.0. A concatenated target/reverse database was searched with a 50 PPM precursor and 0.4 Da fragment mass tolerance. Automatic mass calibration and parameter optimization was enabled and precursor mass errors for up to +2 neutrons were considered. Peptide candidates were generated from database protein sequences assuming tryptic digestion, allowing for up to one missed cleavage. Peptides were required to have between 8-50 amino acids and range from 500 to 5000 m/z. Cysteines were assumed to be fully carbamidomethylated, and peptides were searched considering variable n-terminal pyroglutamic acid formation and methionine oxidation. PeptideProphet was used for FDR validation with the following default options: “--decoy probs”, “--ppm”, “--accmass”, “--nonparam”, and “--expectscore”, which allow for additional high-mass accuracy analysis and non-parametric distribution fitting. ProteinProphet was used for protein-level FDR validation with the following default option: “--maxppmdiff 2000000”. Filtering was performed using a 1% peptide-level and a 1% protein-level FDR threshold.

Supplementary References

Hulstaert, N., Shofstahl, J., Sachsenberg, T., Walzer, M., Barsnes, H., Martens, L. and Perez-Riverol, Y., 2019. ThermoRawFileParser: modular, scalable, and cross-platform RAW file conversion. *Journal of Proteome Research*, *19*(1), 537-542.

Keller, A., Nesvizhskii, A.I., Kolker, E. and Aebersold, R., 2002. An explanation of the Peptide Prophet algorithm developed. *Anal. Chem*, *74*(2002), 5383-5392.

Kim, S. and Pevzner, P.A., 2014. MS-GF+ makes progress towards a universal database search tool for proteomics. *Nature Communications*, *5*(1), 5277.

Kiweler, M., Looso, M. and Graumann, J., 2019. MARMoSET–extracting publication-ready mass spectrometry metadata from RAW files. *Molecular & Cellular Proteomics*, *18*(8), 1700-1702.

Nesvizhskii, A.I., Keller, A., Kolker, E. and Aebersold, R., 2003. A statistical model for identifying proteins by tandem mass spectrometry. *Analytical Chemistry*, *75*(17), 4646-4658.

Table S1. Participants in wet-lab (W) and informatic (I) components of the 2020-2021 OCB ocean intercomparison study.

Institution(s)	Participants	Role
College of Charleston & NIST	Mike Janech, Ben Neely	W
Dalhousie University	Erin Bertrand, Scott McCain, Elden Rowland	W/I
Ghent University	Tim Van Den Bossche, Lennart Martens	I
Naval Research Laboratory	Judson Hervey, Dasha Leary, Jaimee Compton, Sophie Colston, Gary Vora	I
Rowan University and Rutgers University	Eli Moore, Haiyan Zheng	W
Oak Ridge National Laboratory	Bob Hettich, Samantha Peters, Richard Giannone	W/I
Ohio State University	Brian Searle	I
TU Delft	Martin Pabst and Hugo Kleikamp	I
University of Chicago	Jake Waldbauer	W
University of Minnesota	Pratik Jagtap, Tim Griffin, Subina Mehta	I
University of Vienna	Gerhard J. Herndl and Zihao Zhao	W/I
University of Washington Genome Sciences	Brook Nunn	W
University of Washington Oceanography	Rick Keil, Jacqui Neibauer, Megan Duffy	W
Woods Hole Oceanographic Institution	Mak Saito, Matthew McIlvin, Dawn Moran	W/I

Table S2. Metadata for laboratory intercomparison samples. Volumes filtered through 142 mm pump heads and corresponding volume per slice.

Pump / Pump head / Sample name	Volume filtered (L)	Volume per 1/8th slice (L)
Pump 2L / BATS 1 / pump 1A	221.6*	27.7
Pump 2R / BATS 2 / pump 1B	167.3*	20.9
Pump 1L / BATS 3 / pump 2A	235.1+	29.4
Pump 1R / BATS 4 / pump 2B	211.1+	26.3

* Pump 1 total gauge = 447 L, sum of two pump gauges = 446.2 L

+ Pump 2 total gauge = 478 L, sum of two gauges = 388.9 L, discrepancy of 89 L, gauges on pump head are assumed more accurate, as leaks in system could create the additional flow for the total pump gauge.

Table S3. Sample metadata and accession numbers.

Expedition ID, Sample name	Location (Lat/Long)	Depth (m)	Date (mm-dd-yyyy)	Time (UTC; sampler recovery)	ProteomXchange ID
Laboratory Intercomparison	--	--	--	--	--
BATS 348, Lab 127	31.66 N 64.166 W	80	06-16-2018	08:00:00	PXD043218
BATS 348, Lab 135	31.66 N 64.166 W	80	06-16-2018	08:00:00	PXD043218
BATS 348, Lab 209	31.66 N 64.166 W	80	06-16-2018	08:00:00	PXD043218
BATS 348, Lab 438	31.66 N 64.166 W	80	06-16-2018	08:00:00	PXD043218
BATS 348, Lab 593	31.66 N 64.166 W	80	06-16-2018	08:00:00	PXD043218
BATS 348, Lab 652	31.66 N 64.166 W	80	06-16-2018	08:00:00	PXD043218
BATS 348, Lab 729	31.66 N 64.166 W	80	06-16-2018	08:00:00	PXD043218
BATS 348, Lab 774	31.66 N 64.166 W	80	06-16-2018	08:00:00	PXD043218
BATS 348, Lab 811	31.66 N 64.166 W	80	06-16-2018	08:00:00	PXD043218
BATS 348, paired metagenomic sample	31.66 N 64.166 W	80	06-16-2018	08:00:00	Bioproject Accession: PRJNA932835; SRA submission: SUB12819843
Informatics intercomparison	--	--	--	--	--
AE1913, Ocean 8 Clio020	33.128 N 65.967 W	120	06-19-2019	16:56:57	PXD044234
AE1913, Ocean 11 Clio020	33.128 N 65.967 W	20	06-19-2019	16:56:57	PXD044234

Table S4. Experimental guidelines in ocean metaproteome intercomparison project

Parameter	Guideline(s)
Extraction and digestion	Extraction of participant's choice, trypsin digestion
Chromatography	1-dimension of chromatography, at least 60 minutes of separation time, triplicate analyses
Total protein injected	1 μg suggested. Allowable range 0.25 - 2 μg
Isotope Tagging	No isotope tags
Mass spectrometry	Data Dependent Analyses (DDA), participant's choice of parameters
Informatics pipeline	Participants choice of software tools. Report in Spectral Counts. Protein and peptide results to be <1 % false discovery rate (FDR), 1 peptide per protein

Table S5. Laboratory intercomparison sample extraction method and LC method.

Lab ID	Extraction Method	LC method
127	2% SDS buffer 95oC; S-Trap purification and digestion	180 min run, 5% B (0.1% FA in acetontirile) to 30% B over 135 min, 30% B to 55% B over 12 min. A solvent 0.1% formic acid in water
135	5% SDS + 0.1M TEAB, tip sonication, S-trap digestion, c18 SPE	--
209	2% SDS, 95˚C + sonication; acetone precipitation; FASP cleanup & digestion	270 min run; 98% A (0.1 formic acid in water)/2% B (0.1% formic acid in acetonitrile) to 30% B over 130min, to 70% B over 45min
438	1% SDS buffer 95oC; SP3 bead purification and digestion	200 min run, 95% A (0.1 formic acid in water) to 95% B (0.1% formic acid in acetonitrile) nonlinear over 170 min, with a flow rate of 500nM min-1--
593	7M Urea 2M Thiourea, 1% DTT 2% CHAPS, vortex and sonicate, spin, ultrafiltration 30kD, filter aided sample prep (FASP) in solution digestion, desalt with C18 tips	180min gradient from 98% solution A (0.1% formic acid) and 2% solution B (90% acetonitrile and 0.1% formic acid) at 0 min to 40% solution B at 180 min with a flow rate of 300 nL min−1.
652	5% SDS + 0.1M TEAB applied to filters in ziplock, tip sonication, S-trap digestion, c18 SPE	120 min run, 5% B (0.1% FA in acetontirile) to 30% B over 90 min, 30% B to 55% B over 10 min. A solvent 0.1% formic acid in water
729	4% SDS sonication, protein aggregation capture	a linear organic gradient of 100% solvent A (95% water, 5% acetonitrile, 0.1% formic acid) to 25% solvent B (70% acetonitrile, 30% water, and 0.1% formic acid) for 180 minutes
774	2.1% SDS (2X Laemmeli buffer); SDS gel plug; 8M Urea; sonication	Sample was loaded on to a fused silica trap column (Acclaim PepMap 100, 75umx2cm, ThermoFisher). After washing for 5 min at 5 µl/min with 0.1% TFA, the trap column was brought in-line with an analytical column (Nanoease MZ peptide BEH C18, 130A, 1.7um, 75umx250mm, Waters) for LC-MS/MS. Peptides were fractionated at 300 nL/min using a segmented linear gradient 4-15% B in 30min (where A: 0.2% formic acid, and B: 0.16% formic acid, 80% acetonitrile), 15-25%B in 40min, 25-50%B in 44min, and 50-90%B in 11min. Solution B then returns at 4% for 5 minutes for the next run.
811	Bead beating and 3 freeze thaw cycles with Ammonium bicarbonate (50mM) and EDTA (5mM), centrifugation, then TCEP, iodoacetaminde, DTT, trypsin, desalted with C18 spin columns	Solvents of 100% LC/MS grade water with 0.1% formic acid (A) and 100% LC/MS grade acetonitrile with 0.1% formic acid (B) were used to elute peptides over a 90-minute gradient from 5-35% solvent B

Table S6. Chromatographic parameters and mass spectrometer and resolution employed. See Table S5 for LC method and Table S7 for mass spectrometer parameters.

Lab ID	Column Length (cm)	Column Width (µm)	LC Resin	LC flow rate (nl/min)	LC gradient time (min)	Trap Column or Direct Injection	LC system	Mass Spectrometer	MS1 resolution
127	50	100	C18 Jupiter	250	147	direct	Dionex LC	QExactive	35,000 or 140,000
135	25	75	C18 Acclaim PepMap RSLC 2um	300	65	trap	Dionex LC 3000	Lumos Tribrid	60,000
209	200	100	C18 monolith (GL Sciences)	360	188	trap	Dionex 3000	Orbitrap Elite	120,000
438	25	100	3 µm C18 beads (Dr. Maisch)	500	200	trap	Dionex 3000	Fusion Tribrid	240,000
593	50	75	2µm C18 beads	300	270	--	Dionex UltiMate 3000	QExactive	120,000
652	30	75	3 µm C18 beads (Dr. Maisch)	250	90	trap	Thermo Easy-LC UPLC	QExactive	70,000
729	15	75	1.7µm Kinetex C-18 (Phenomenex)	150	180	trap	Vanquish Ultra-HPLC	QExactive Plus	70,000
774	25	75	Peptide BEH	300	120	trap	Dionex RSLC	Thermo Eclipse	120,000
811	37	75	C18 particles (Magic C18AQ, 100˚A, 5µm; Michrom)	300	90	precolumn	Easy-nLC 1200	Thermo Q Exactive Plus HRMS	70,000

Table S7. Chromatographic parameters and mass spectrometer and resolution employed. See Table S5 for LC method and Table S6 for chromatographic parameters and mass spectrometer and resolution employed.

Lab ID	MS1 AGC target	Max Injection Time (ms)	MS1 Scan Range	MS2 Detector	Resolution or Scan rate	Minimum AGC target	Max Injection Time (ms)	Loop count (N) or cycle time (s)	Isolation Window	Activation Type	Collision Energy	Charge States Included	Dynamic Exclusion (s)
127	3.00E+06	100	400-2000	orbitrap	17,500	5.00E+03	60	Top N 12, Top N 8	2	HCD	27	2,3,4	30
135	4.00E+05	50	375-1500	orbitrap	15,000	2.00E+05	30	TopN, 3sec	1.3	HCD	32	2,3,4,5,6	60
209	1.00E+06	100	300-1800	ion trap	rapid	1.00E+04	100	TopN 15	2	CID	35	>1	30
438	4.00E+05	50	380-1280	ion trap	normal rate	2.00E+04	150	2 s cycle	1.6	HCD	30	2,3,4,5,6,7,8	15
593	--	--	350-1800	orbitrap	--	--	--	20	--	CID	--	>1	30
652	1.00E+06	100	400-1400	orbitrap	35,000	5.00E+04	50	20	1.2	HCD	30	2,3,4,5	10
729	1.00E+06	25	300-1500	orbitrap	17,500	1.00E+05	50	20	1.8	HCD	27	2,3,4,5	30
774	8.00E+05	auto	375-1500	orbitrap	15,000	1.00E+05	50	3	1.2	HCD	30	2-7	60
811	5.00E+04	50	375-1575	orbitrap	17,500	5.00E+04	50	20	1.2	HCD	25	2,3,4,5	30

Table S8. Participant laboratory results: User provided results from diverse informatic pipelines. NA – not available. Multiple values reported if protein groupings were used, based on the output formats and protein inference methods of the various informatic pipelines used.

Lab	Total Unique Peptides	Protein IDs
127	22382	3520
135	9797	NA
209	2363	4359 / 1049
438	15903	5771
593	131	89
652	11979	2089
729	11204	4907
774	18859	5946
811	3515	NA

Table S9. Participant laboratory results using the single pipeline re-analysis. Raw data files were processed SEQUEST HT and Scaffold resulted in these sums of total unique peptides, total proteins, and protein groups.

Lab	Total Unique Peptides	Total Protein IDs	Protein Groups
135	9600	3919	3533
209	3354	1586	1461
438	15646	6221	5621
593	0	0	0
652	9106	3518	3189
729	6626	3522	3202
774	16500	5676	5111
811	14	12	12
127	12615	5080	4595

Table S10. Participant laboratory results passed through the single pipeline re-analysis, using alternate chromatographic techniques. Raw data files were processed SEQUEST HT and Scaffold resulted in these sums of total unique peptides, total proteins, and protein groups.

Lab	Total Unique Peptides	Total Protein IDs
Alt-1 (12h run)	7060	2832
Alt-2 (2D)	18477	7765
Alt-3 (2D)	5852	2746

Table S11. Informatic intercomparison study: anonymous laboratory identification numbers, software used, and results. NA – not available.

ID	Software	Unique Peptides Oceans 8	Unique Peptides Oceans 11
109	Peaks Studio X	4522	4898
321	SearchGUI / Peptide Shaker	2768	7389
321	MaxQuant	3342	4751
362	X!Tandem / SearchGUI	4890	8079
458	SEQUEST-HT / Percolator	6369	8288
501	MSGF+ OpenMS	4025	7463
828	SEQUEST-HT PD	NA	NA
902	SEQUEST-HT / Percolator	4653	7649
932	MASCOT	1724	3019
957	MsFragger / PeptideProphet / ProteinProphet	3687	6144

Figure S1. Results from user submitted data reports for laboratory intercomparison. a) Total number of unique peptide identifications by laboratory. A total of 35715 unique peptides were detected across all six laboratories. Note any peptides with PTMs were removed and not counted. b) Total number of protein identifications, note that some laboratory groups did not provide protein results (135 and 811). c) Pairwise comparisons of shared peptides between six laboratories ranged from 3844 to 10877 and averaged 7142 +/- 2074 identified peptides, demonstrating reproducibility of peptides identifications between laboratories. Note that PTMs were not taken into account for the uniqueness of peptides.

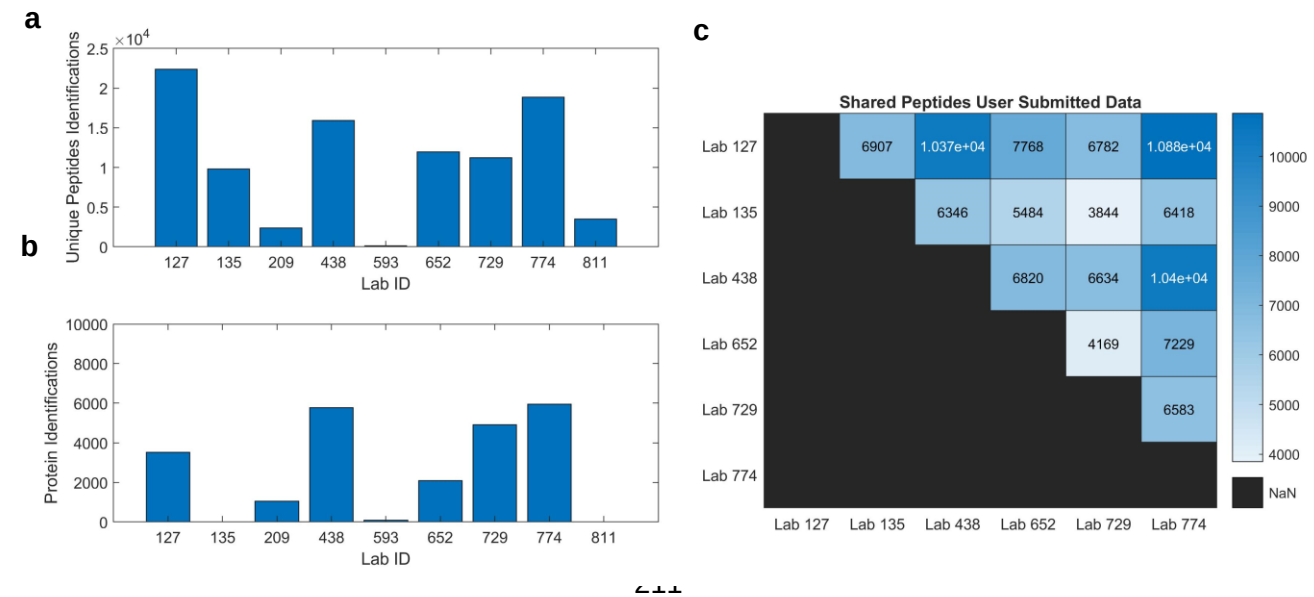

Figure S2. Results of pair-wise two-way linear regression analyses for re-analysis of submitted raw data from laboratory intercomparison, corresponding to Figure 4.

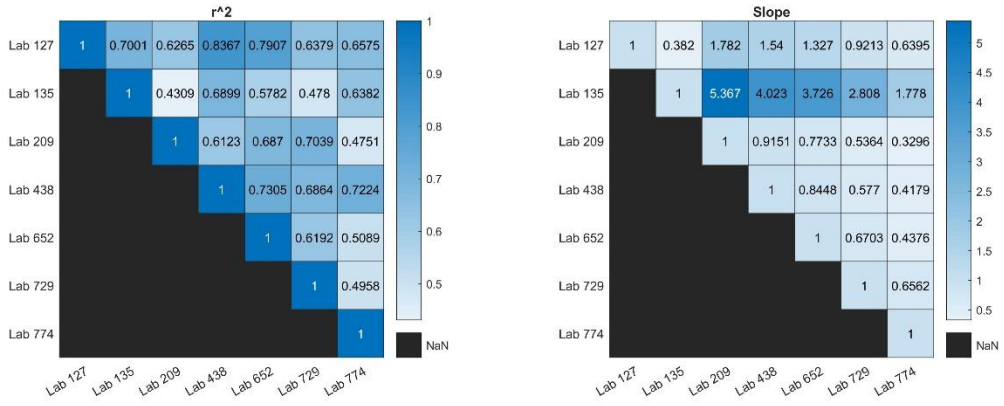

Figure S3. Quantitative Sørensen similarity analysis. Sørenson similarity analysis on full protein dataset. See Fig. 6 for analysis of top 1000 proteins.