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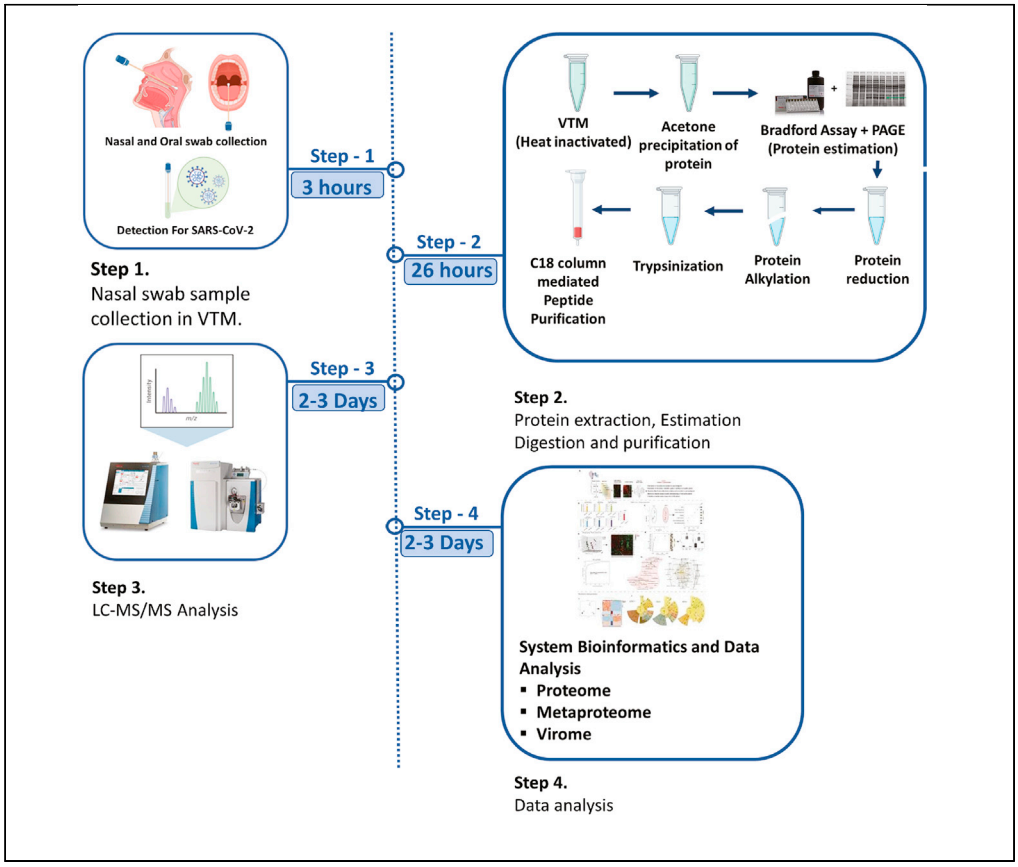
Protocol for global proteome, virome, and metaproteome profiling of respiratory specimen (VTM) in COVID-19 patient by LC-MS/MS-based analysis



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Highlights

HRMS-based Proteome, Virome, and Metaproteome analysis of COVID 19 patients

Protocol for Sample preparation and Database curation for HRMS-based analysis

Downstream bioinformatics data analysis for Proteome, Virome, and Metaproteome dataset

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Protocol for global proteome, virome, and metaproteome profiling of respiratory specimen (VTM) in COVID-19 patient by LC-MS/MS-based analysis

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SUMMARY

In this protocol, we describe global proteome profiling for the respiratory specimen of COVID-19 patients, patients suspected with COVID-19, and H1N1 patients. In this protocol, details for identifying host, viral, or bacterial proteome (Meta-proteome) are provided. Major steps of the protocol include virus inactivation, protein quantification and digestion, desalting of peptides, high-resolution mass spectrometry (HRMS)-based analysis, and downstream bioinformatics analysis.

For complete details on the use and execution of this profile, please refer to Maras et al. (2021).

BEFORE YOU BEGIN

This protocol describes specific steps to execute proteome, metaproteome, and virome study in a COVID VTM sample (Maras et al., 2021). However, this protocol can be used for executing proteome, metaproteome, and virome from any sample. In the case of Plasma samples where some proteins are very high in concentration, e.g., albumin and immunoglobulins, you have to specifically deplete them first and then follow the same protocol {Chutipongtanate, 2017 #57}

Nasopharyngeal specimen (NP) collection (performed by a trained healthcare provider, only): (Pondaven-Letourmy et al., 2020)

Tilt head of patient backward around 70 degrees. Then Cautiously insert a mini tip swab through the nostril, parallel to the palate (not upwards) until you feel resistance or the distance is equivalent to that from the ear to the patient's nostril, indicating contact with the nasopharynx. Cautiously rub and roll the mini tip swab and leave the swab in place for several seconds to absorb secretions. Now gently remove the swab while rotating it. Finally, Place the mini tip swab into the transport tube (VTM media) provided.

> *Note:* If a deviated septum or blockage creates difficulty obtaining the specimen from one nostril, use another nostril.

Oropharyngeal (OP) (throat) specimen collection

Insert the mini tip swab into the pharynx and tonsillar areas. Then roll swab over both tonsillar pillars and posterior oropharynx.

Note: Avoid touching the tongue, teeth, and gums.

Finally, Place the swab, tip first, into the viral transport media provided.

KEY RESOURCE TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Ammonium bicarbonate	Sigma-Aldrich	A6141
Sodium dodecyl sulfate	Sigma-Aldrich	L3771
Dithiothreitol	Sigma-Aldrich	D0632
Iodoacetamide	Acros Organics	122270050
Trypsin protease, MS grade	Thermo Fisher Scientific	90305
Trifluoroacetic acid	Sigma-Aldrich	T6508
Acetonitrile	JT Baker	14650359
Formic acid	Sigma-Aldrich	F0507
Urea	Sigma-Aldrich	U0631
LC-MS grade water	Sigma-Aldrich	39253
Bovine serum albumin	Thermo Fisher Scientific	23208
Acetone	JT Baker	9006–03
Acrylamide	Thermo Fisher Scientific	AM9022
SDS	Thermo Fisher Scientific	15525017
APS	Thermo Fisher Scientific	HC2005
TEMED	Thermo Fisher Scientific	T9281
Tris-Base	Thermo Fisher Scientific	17926
HCL	Thermo Fisher Scientific	24308
Glycine	Thermo Fisher Scientific	A37730IN
Glacial acetic acid	Sigma-Aldrich	A0808
Coomassie Brilliant Blue	Thermo Fisher Scientific	20278
ß-Mercaptoethanol	Sigma-Aldrich	M6250
Bradford reagent	Thermo Fisher Scientific	22663
Software and algorithms		
Proteome Discoverer 2.2 using Sequest HT	Thermo Fisher Scientific	OPTON-30945
Xcalibur	Thermo Fisher Scientific	OPTON-30965
Uniprot	(https://www.uniprot.org):	
Others		
Benchtop centrifuge	Eppendorf	5427R
Sonicator	Helix Biosciences	HBSNII-92
Vortex	Sigma-Aldrich	Z258423
Incubator	Memmert	INB200
Vacuum evaporator	Genevac	DNA-12060-C00
Microplate reader	Thermo Fisher Scientific	VL0000D0
Nano Reverse-phased capillary HPLC system	Thermo Fisher Scientific	UltiMate 3000 HPLC
Q-exactive + orbitrap	Thermo Fisher Scientific	IQLAAEGAAPFADBMBCX
Heated electrospray ionization (H-ESI) ion source	Thermo Fisher Scientific	H-ESI probe
SDS PAGE Apparatus	Bio-Rad Laboratories	165-8000
Viral Transfer media	RMBio	VTM-CHT
Eppendorf tubes	Eppendorf	30125150
ELISA plates	Thermo Fisher Scientific	44-2404-21
Pierce C18 spin columns	Thermo Fisher Scientific	89870
Water bath	LabcOM	76308-830
Nano LC Column	Thermo Fisher Scientific	164570
Calibration solution	Thermo Fisher Scientific	88323/88324

MATERIALS AND EQUIPMENT

Tris's buffer		
Reagent	Final concentration	Volume
Tris's buffer	0.1 M Tris, pH 7.8	500 mL
Dissolve 6.05 g tris in LC-MS grade water and makeup to a total volume of 500 mL. Adjust pH to 7.8 with HCl.		

Note: Tris buffer solutions can be stored at 25°C or at +4°C for two weeks.

Ammonium bicarbonate buffer		
Reagent	Final concentration	Volume
Ammonium bicarbonate buffer	50 mM ammonium bicarbonate in 0.1 M Tris buffer, pH 7.8	250 mL
Dissolve 0.97 g of ammonium bicarbonate in tris buffer and make up a final volume of 250 mL.		

Note: Tris's buffer solutions can be stored at 25°C or at +4°C for two weeks.

Urea buffer		
Reagent	Final concentration	Volume
Urea buffer	8 M urea in 50 mM ammonium bicarbonate buffer	125 mL
Dissolve 110.06 g of urea in 50 mM ammonium bicarbonate buffer and make up a final volume of 125 mL.		

Note: Urea solutions should always be freshly prepared and should be stored below 30°C.

Iodoacetamide solution		
Reagent	Final concentration	Volume
Iodoacetamide solution	50 mM iodoacetamide in urea buffer	10 mL
Dissolve 92 mg of iodoacetamide in urea buffer and makeup to a total volume of 10 mL with 8 M urea buffer.		

⚠ CRITICAL: Iodoacetamide acts as an acutely toxic, irritant, and health hazard, according to MSDS information. To prevent exposure, wear suitable protective gloves, eye protection, face masks, etc.

Note: Iodoacetamide is unstable and light-sensitive. Prepare solutions immediately before use and perform alkylation in the dark.

Dithiothreitol solution		
Reagent	Final concentration	Volume
Sample buffer	1 M DTT in LC-MS grade water	10 mL
Dissolve 1.55 gm of DTT in *LC-MS grade water and makeup to a total volume of 10* mL.		

Note: Dithiothreitol solution is stored at −20°C and should use within 3 months.

LC /MS-MS – Solvent A		
Reagent	Final concentration	Volume
LC - Solvent A	0.1% (v/v) formic acid in LC-MS grade water	1,000 mL
Gently mix 1 mL formic acid with LC-MS grade water and makeup to a total volume of 1,000 mL with LC-MS grade water.		

Note: Always prepare fresh.

LC/MS-MS – Solvent B		
Reagent	Final concentration	Volume
LC - Solvent B	95% (v/v) acetonitrile, 0.1% (v/v) formic acid in LC-MS grade water	1,000 mL
Gently mix 950 mL of acetonitrile with 0.1 mL of formic acid and makeup to a total volume of 1,000 mL with LC-MS grade water.		

Note: Always prepare fresh.

Solution A for peptide purification (Wash buffer)		
Reagent	Final concentration	Volume
Wash buffer	50% (v/v) acetonitrile in LC-MS grade water	5 mL
In 25 mL of acetonitrile, add 25 mL of LC-MS grade water to make the final volume 50 mL.		

Note: Always prepare fresh.

Solution B for peptide purification (Equilibration buffer)		
Reagent	Final concentration	Volume
Equilibration buffer	5% (v/v) acetonitrile in LC-MS grade water	50 mL
In 2.5 mL of acetonitrile, add 47.5 mL of LC-MS grade water to make the final volume 50 mL.		

Note: Always prepare fresh.

Solution C for peptide purification (Elution buffer)		
Reagent	Final concentration	Volume
Elution buffer	90% (v/v) acetonitrile in LC-MS grade water	5 mL
In 45 mL of acetonitrile, add 5 mL of LC-MS grade water to make the final volume 50 mL.		

Note: Always prepare fresh.

Tris's HCL buffer (PH-8.8)		
Reagent	Final concentration	Volume
Tris's base	1.5 M Tris's base in deionized water.	100 mL
Dissolve 18.15 gm of Tris base in 80 mL of deionized water, now adjust the pH to 8.8 using 6 N HCL and then adjust final volume to 100 mL.		

Note: Tris buffer solutions can be stored at 25°C or at +4°C for two weeks.

Tris's buffer (pH-6.8)		
Reagent	Final concentration	Volume
Tris's base	0.5 M Tris's base in deionized water.	100 mL
Dissolve 6 gm of Tris base in 60 mL of deionized water, now adjust the pH to 6.8 using 6 N HCL and then adjust final volume to 100 mL.		

Note: Tris's buffer solutions can be stored at 25°C or at +4°C for two weeks.

10% (W/V) SDS		
Reagent	Final concentration	Volume
SDS	10% SDS in deionized water.	100 mL
Dissolve 10 gm of SDS in 90 mL of deionized water, then adjust the final volume to 100 mL.		

Note: Store 10% SDS stock solution at 20°C. Do not store in the fridge, as the SDS will reprecipitate.

10% (W/V) APS		
Reagent	Final concentration	Volume
APS	10% APS in deionized water.	1 mL
Dissolve 0.1 gm of SDS in 1 mL of deionized water.		

Note: Always prepare fresh.

Tris-Glycine buffer (Running buffer)		
Reagent	Final concentration	Volume
Tris's base	250 mM	
Glycine	1.92 M	
SDS	1%	
water	to 1 L	1 L
Dissolve 30.30 gm tris, 144.10 gm Glycine, and SDS 10 gm in 900 mL of water and make final volume 1 L.		

Note: don't adjust it will be reached automatically pH 8.3.

Note: Tris buffer solutions can be stored at 25°C or at +4°C for two weeks.

1% Bromophenol Blue		
Reagent	Final concentration	Volume
Bromophenol blue	1% bromophenol in water.	1 mL
Dissolve 100 mg bromophenol Blue in 10 mL of water.		

Note: 1% Bromophenol Blue can be stored at 25°C or at +4°C for two years.

Staining solution		
Reagent	Final concentration	Volume
Coomassie blue R250	0.1% (w/v),	
methanol	20% (v/v)	
Acetic acid	10% (v/v)	
Dissolve 0.4 gm Coomassie blue in 200 mL of 40% (v/v) HPLC grade methanol in water with stirring as required. Filter the solution to remove any insoluble material. Add 200 mL of 20% (v/v) acetic acid in water.		

Note: Always prepare fresh.

Destaining solution		
Reagent	Final concentration	Volume
methanol	50% (v/v)	
Acetic acid	10% (v/v)	
water	to 1 L	1 L

Add 500 mL of HPLC- grade methanol to 300 mL of HPLC-grade water, Add 100 mL of reagent grade acetic acid mix and adjust final volume to 1000 mL with water. The final concentrations will be 50% (v/v) methanol in water with 10% (v/v) acetic acid.

Note: Always prepare fresh.

STEP-BY-STEP METHOD DETAILS

Heat inactivation of virus (Batejat et al., 2021)

Timing: 30 min

Note: By the end of this step, the virus gets inactivated, and now all the remaining sample processing steps can be performed in the BSL-2 facility.

CAUTION: SARS-CoV-2 Samples should be handled in a Biosafety Level 3 (BSL-3) laboratory using BSL-3 practices till virus inactivation is performed and post inactivation, all other steps can be performed on BSL-2 facility.

1. Nasopharyngeal samples (NPS) and oropharyngeal samples together were collected into 1 mL of viral transport media.
2. Around 500 uL of the sample was incubated with a pre-warmed dry heating block maintained at 92°C for 15 min.

Homogenization of respiratory specimen (Dowling et al., 2020)

Timing: 5–7 min per sample

Note: By the end of this step, all the cell's macromolecules like proteins, metabolites, lipids, etc., will be released in solution.

3. Respiratory specimens may contain host cells, mucus, bacterial and viral components. Thus, it is essential to homogenize the samples.

CAUTION: The high-frequency sound emitted by the Sonicator can damage hearing; therefore, place the sample in a noise isolating chamber and always close the door while operating. Do not grasp an activated horn or touch the tip of a vibrating probe. It can cause severe tissue damage and burns.

4. Take 500 µL of respiratory specimen in a new microcentrifuge tube (MCT) and keep it on the ice during homogenization.
5. Place the sample in a noise isolating chamber and submerge the Sonicator probe into the sample.

Note: The probe should not touch the walls of MCT as it will break the tube and destroy the sample.

6. Close the door of the chamber after properly placing the sample tube.
7. Run the program at Power 20%, Run time 5 min (Cycle – 10 s ON, 10 s OFF) and temperature 22°C.
8. Remove and wipe the probe with ethanol.

Note: Keep the sample on the ice during all the homogenization steps step 2–6.

Pause Point: Respiratory specimen can be stored at −80°C for up to 4 weeks.

Organic solvent-mediated protein precipitation (Bradford, 1976; Simpson and Beynon, 2010)

Timing: 90 min

Note: By the end of this step, all proteins will be precipitated.

9. 200 uL of VTM was placed in acetone compatible tube.
10. Add the 6-fold volume of cold acetone to VTM.
11. Vortex and incubate for 1–4 h at −80°C.
12. Centrifuge for 10 min at 18,000 g.
13. Carefully decant the supernatant without disturbing the protein pellet.
14. Allow pellet to air dry for 10–30 min in an uncapped tube. (Do not over dry otherwise, the pellet may not resuspend properly)
15. Resuspend the pellet in 50 uL of Ammonium Bicarbonate Buffer (ABC buffer).

Protein estimation using Bradford assay (Bradford, 1976)

Timing: 30 min

Note: By the end of this step, the protein concentration of all samples can be quantified.

16. To prepare BSA standards and associated calibration curve, make a stock solution of 2 mg/mL of BSA in PBS.
17. Now label vials A to I, and add the stock or the master solution and Buffer as directed in the table below to achieve the desired results concentration of standard.

Vial	Volume of diluent (µL)	Volume and source vial of BSA (µL)	Final concentration (µg/µL)
A	0	300 of stock	2000
B	125	375 of stock	1500
C	325	325 of stock	1000
D	175	175 of vial B dilution	750
E	325	325 of vial C dilution	500
F	325	325 of vial E dilution	250
G	325	325 of vial F dilution	125
H	400	100 of vial G dilution	25
I	400	0	0

18. In a 96 well plate, 5 uL of pre-prepared BSA standards along with blank were added.
19. 5 uL of unknown samples was also loaded.
20. Now to each well, 245 uL of Bradford reagent was added.
21. Keep it on a shaker for 15–30 s.
22. Now incubate at 25°C for 10 min in the dark.

23. Take absorbance at 595 nm within 60 min post-incubation.
24. Plot a standard absorbance curve at 595 nm on the "Y" axis versus the protein concentration on the "X" axis.
25. Record the value "x" of unknown samples from the graph corresponding to absorbance reading and determine the protein concentration.

SDS PAGE mediated intra-sample variability detection (Nowakowski et al., 2014)

Timing: 5 h

Note: By the end of this step, the intrasample variability among samples and in their associated group can be detected.

26. In-gel casting apparatus assemble glass plates and spacers.
27. Mix the component of resolving gel (12%).
28. Pour the resolving gel mixture between the gel plates till a level 2 cm below the top of the shorter plate.
29. Pace a layer of Distilled H2O over the top of the resolving gel to prevent meniscus formation in the resolving gel.
30. Incubate resolving gel for 30 min at 25°C
31. Drain the water from the top of the resolving gel and wick any remaining water away with a Kimwipe.
32. Mix stacking gel components and pour into gel plates (on top of resolving gel) to fill the gel plates.
33. Insert comb at the top of the spacers.
34. Remove the comb and assemble cast gel into the SDS apparatus.
35. Add 1× bromophenol blue to each sample.
36. Add freshly prepared 1× running buffer to both chambers of the apparatus
37. Now load DNA ladder in first well, 5 ug BSA in another well, and 30 ug equivalent unknown sample protein in other wells.
38. Run the gel at 100 V till the dye front migrates into the running gel (~15 min) and then increase to 120 V until the dye front reaches the bottom of the gel (~1.5 h.).
39. Now Remove the gel from the apparatus and by removing the spacers and glass plates.
40. Place the gel into a small tray.

Note: Never use a metal spatula to separate the glass plates.

41. Add ~25 mL staining solution and incubate for > 30 min with gentle shaking.
42. Add ~5 mL destaining solution and destain for ~1 min with gentle shaking, then Pour off and discard the destain solution.
43. Further, add ~ 30 mL of destain solution and destain with gentle shaking until the gel is visibly distained (> 2 h).
44. Pour off and discard the destain solution and rewash the gel with distilled water.
45. Visualize on GelDoc.

In-solution protein digestion (Gundry et al., 2009)

Timing: 26 h

Note: By the end of this step, all the proteins will be digested, and tryptic peptides will be generated.

46. 50 ug equivalent proteins are diluted using the buffer till the final volume becomes 100 uL.

47. Add 20 uL of 10 mM Dithiothreitol (DTT) and incubate at 60°C for 1 h in a water bath to reduce proteins.
48. Post-reduction, add 15 uL of 10 mM Iodoacetamide and incubate for 30 min in the dark for alkylation of proteins,
49. Add 5 uL of modified sequencing grade trypsin (1 ug/5 uL) reconstituted in 100 uL ABC buffer.
50. Incubate at 37°C for 20–24 h in a water bath.
51. After incubation, add 5 uL of 0.1% (v/v) Formic acid to the reaction mixture to inhibit trypsin activity.

C18 column mediated desaltation of peptides (Dowling et al., 2020)

Timing: 90 min

Note: By the end of this step, all the contaminants, e.g., salt, will be removed.

52. Please open a new Pierce C18 column, place it in an empty 1.5 mL Eppendorf tube,
53. Incubate it with 250 uL of Buffer C for 5 min (Column Washing).
54. Centrifuge for 2 min on a tabletop centrifuge and discard the flowthrough.
55. Add 200 uL of Buffer A in the column, then incubate for 2 min (Column Washing).
56. Centrifuge for 2 min on a tabletop centrifuge and discard the flowthrough.
57. Add 200 uL of Buffer B in the column,
58. Incubate for 2 min (Column equilibration).
59. Discard the flowthrough.
60. Now load the digested sample.
61. Incubate it for 5 min.
62. Centrifuge for 2 min on a tabletop centrifuge and then reload the flowthrough (peptide-binding).
63. Incubate again for 5 min and repeat step 49.
64. Repeat steps 49 and 50 two more times.
65. Centrifuge for 2 min on a tabletop centrifuge and discard the flowthrough.
66. Add 200 uL of Buffer B in the column, then incubate for 2 min (Washing).
67. Centrifuge for 2 min on a tabletop centrifuge and discard the flowthrough.
68. Add 30 uL of Buffer C and Centrifuge for 2 min on a tabletop centrifuge.
69. Repeat step 55.
70. Finally, add 40 uL Buffer C and Centrifuge for 2 min on a tabletop centrifuge.
71. Take the flowthrough and discard the used column.
72. Lyophilize the samples at 4°C and 60 millibar pressure till the sample is dry (do not over dry sample; otherwise, they will be tough to reconstitute).
73. Reconstitute in 40 uL of 0.1% (v/v) Formic acid.
74. Centrifuge at 15,000 g for and load the supernatant in an HPLC vial.

LC-MS/MS analysis (Bhat et al., 2020; Das et al., 2017, 2019; Dowling et al., 2020)

Timing: 180 min per sample

Note: By the end of this step, the MS.raw files would be curated.

75. The peptides were ionized by nano-electrospray and subsequent tandem mass spectrometry (MS/MS) on a Q-ExactiveTM Plus (Thermo Fisher Scientific, San Jose, CA, United States).
76. The peptides were eluted by a 3–95% gradient of buffer B (aqueous 80% acetonitrile in 0.1% formic acid) with a continuous flow rate of 300 nL/min for about 60 min on a 25-cm analytical C18 column (C18, 3 mm, 100 A).

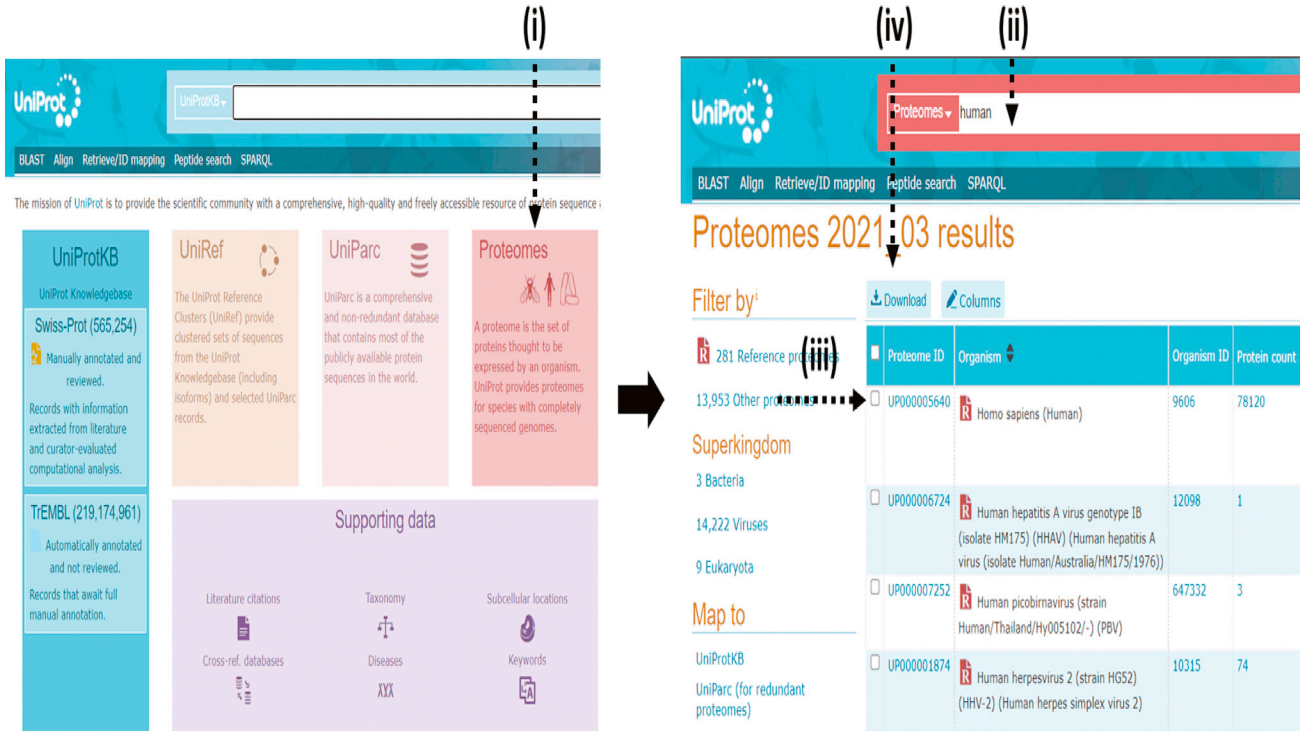


Figure 1. Overview of data curation process at UNIPROT website

77. The peptides were analyzed using a mass spectrometer with the collision-induced dissociation mode with the electrospray voltage 2.3 kV.
78. Analysis on the orbitrap was performed with full scan MS spectra with a resolution of 70,000 from m/z 350 to 1800. Mascot algorithm (Mascot 2.4, Matrix Science).
79. Significant proteins were identified at ($p < 0.05$) and q values ($p < 0.05$). The threshold of false discovery rate was kept at 0.01.

Database curation for host proteome, metaproteome, and virome (Maras et al., 2021)

Note: By the end of this step, the database would be curated on which MS.raw files would be mapped.

80. The raw files generated post-LC-MS/MS analysis were analyzed using Proteome discoverer 2.2 for protein identification and quantification.
81. One of the most crucial parts is database curation, against which the mass spectrometric files need to be searched. So, in our case, we created three databases: a Virome (COVID) database, another for humans, and one for metaproteome.
82. To curate the database, visit https://www.uniprot.org/proteomes/.
83. Find proteome for your organism of interest by searching for them from proteome ID, e.g., UP000005640 – Homo sapiens) or taxonomic name or ID.
84. Now click on the **download** button> Select **all protein entries** > **Fasta** (canonical, compressed, and isoform). (Figure 1)

Note: the databases in Uniprot are of three categories reviewed (Uniprot/Swissport), Unreviewed (Uniprot/TREMBL), or both (UniprotKB) options. Always prefer the reviewed databases because reviewed databases are carefully annotated with minimal redundancy and can integrate into other databases efficiently.

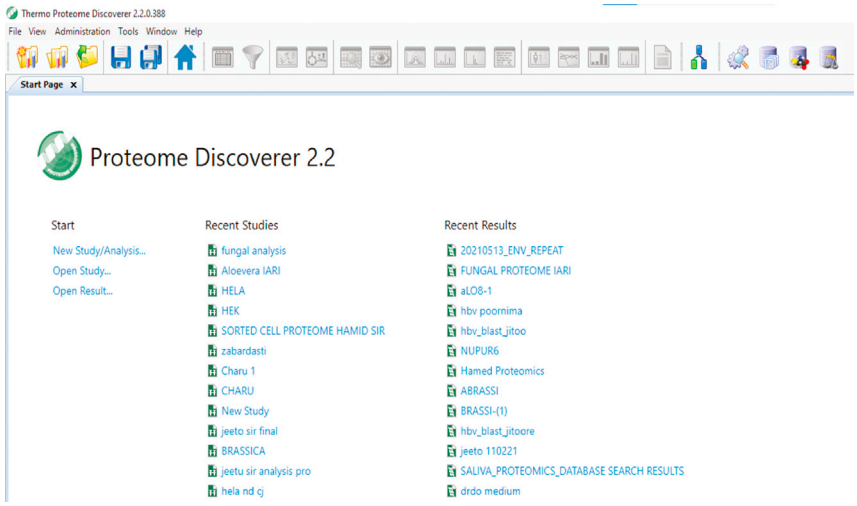


Figure 2. Pictographic representation of step 87 associated to proteome discoverer 2.2 mediated data analysis section

85. We used the following databases

Application	Organism	Source
Host Proteome	Homo sapiens	Uniprot homo sapiens (Human) database (UP000005640; reviewed)
Virome	COVID-19	https://covid-19.uniprot.org/uniprote
Metaproteome	Amalgamation	bacterial/fungal sequence (UniprotSwP_20170609, with sequences 467231 and MG_BG_UPSP with sequences2019194)

Proteome discoverer 2.2 mediated data analysis (Maras et al., 2021)

Note: By the end of this step, the excel data files would be curated.

Note: The steps for data curation from raw files for Proteome, Metaproteome, and virome are the same, and the same raw files can be used for data curation analysis of all three.

The only difference among the three is that each need to be run individually against their respective database, e.g., Host proteome should be mapped against Human Uniprot database)

86. Open Proteome discoverer 2.2.
87. Click New Study. (Figure 2)
88. Name your study > Decide the root directory > Add your raw files > Press ok. (Figure 3)
89. Click on New Analysis. (Figure 4)
90. Click on Input files > Select all files and drag it below the processing workflow window > Click on the icon of the processing step. (Figure 5)
91. Click on Open common > Select your processing workflow and press enter. (Figure 6)
92. Click on Spectrum files and > go to Search settings > Select the Protein database, e.g., for COVID virome, select the already curated COVID database> Select the type of digestion we performed, e.g., tryptic peptides. (Figure 7)

Note: This is the place where you will select the database for proteome, metaproteome, and virome or any other organism associated.

93. Now, select each step of the Processing workflow.

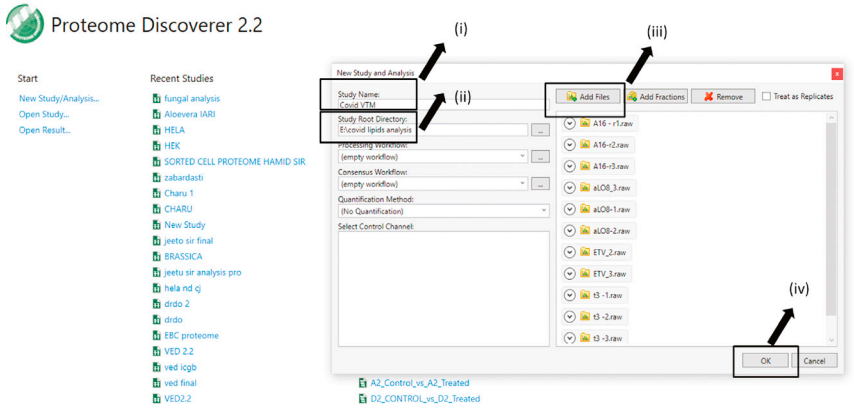


Figure 3. Pictographic representation of step 88 associated to proteome discoverer 2.2 mediated data analysis section

Note: Setting for each parameter group is given in the image below. (Figure 8)

94. Now click on consensus workflow > then Open common > and select your workflow. (Figure 9)
Step 10-Now select each step of the processing workflow.

Note: Setting for each parameter group is given in the image below. (Figure 10)

95. Click on grouping and quantification> Then select Sample type. (Figure 11)
96. Click on Run. (Figure 12)

97. Wait for 24–72 h. for data analysis to happen, and finally, you will get a file like below. (Figure 13)

Note: You have to repeat Proteome discoverer 2.2 mediated data analysis five times.

a. For proteome run all raw files of COVID positive, COVID negative, and H1N1 samples together.
b. For virome, similarly run all raw files of COVID positive, COVID negative, and H1N1 samples together.
c. For the metaproteome, run three times for COVID positive, once for COVID negative, and then finally once for H1N1.)

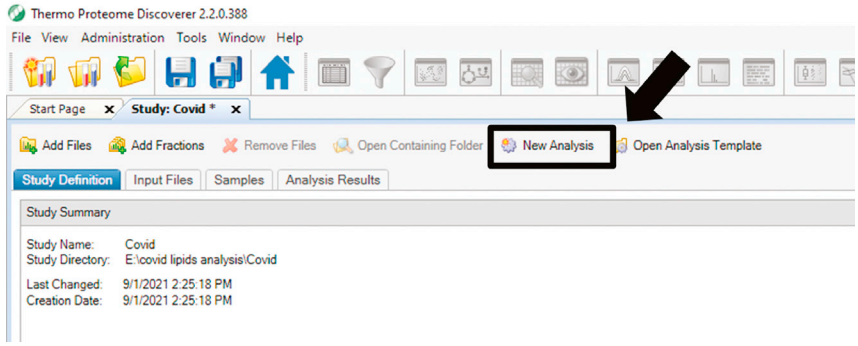


Figure 4. Pictographic representation of step 89 associated to proteome discoverer 2.2 mediated data analysis section

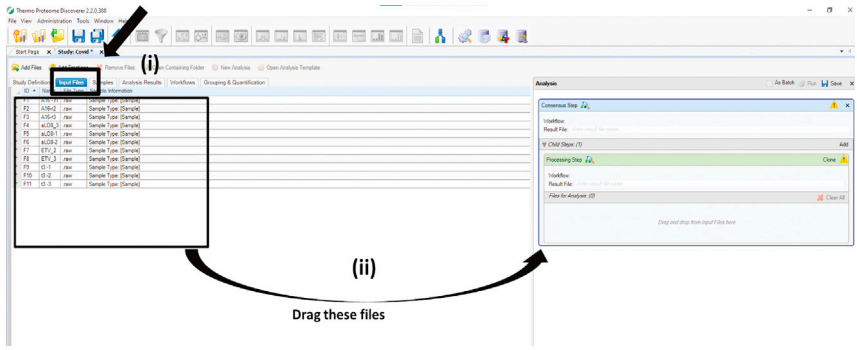


Figure 5. Pictographic representation of step 90 associated to Proteome discoverer 2.2 mediated data analysis section

Note: From here, the further downstream data processing is the same for Host proteome and virome and different for metaproteome.

Downstream data Processing for Metaproteome analysis (Maras et al., 2021)

Note: By the end of this step final metaproteome excel file will be curated.

98. By the end of step 13, you will have three files for the metaproteome, one for each group.
99. Open the excel file of one group. (Figure 14)
100. Copy all the tryptic peptides
101. Visit the **Unipept** website https://unipept.ugent.be/. (Figure 15)
102. Enter the list of tryptic peptides here > Click search (Figure 16).

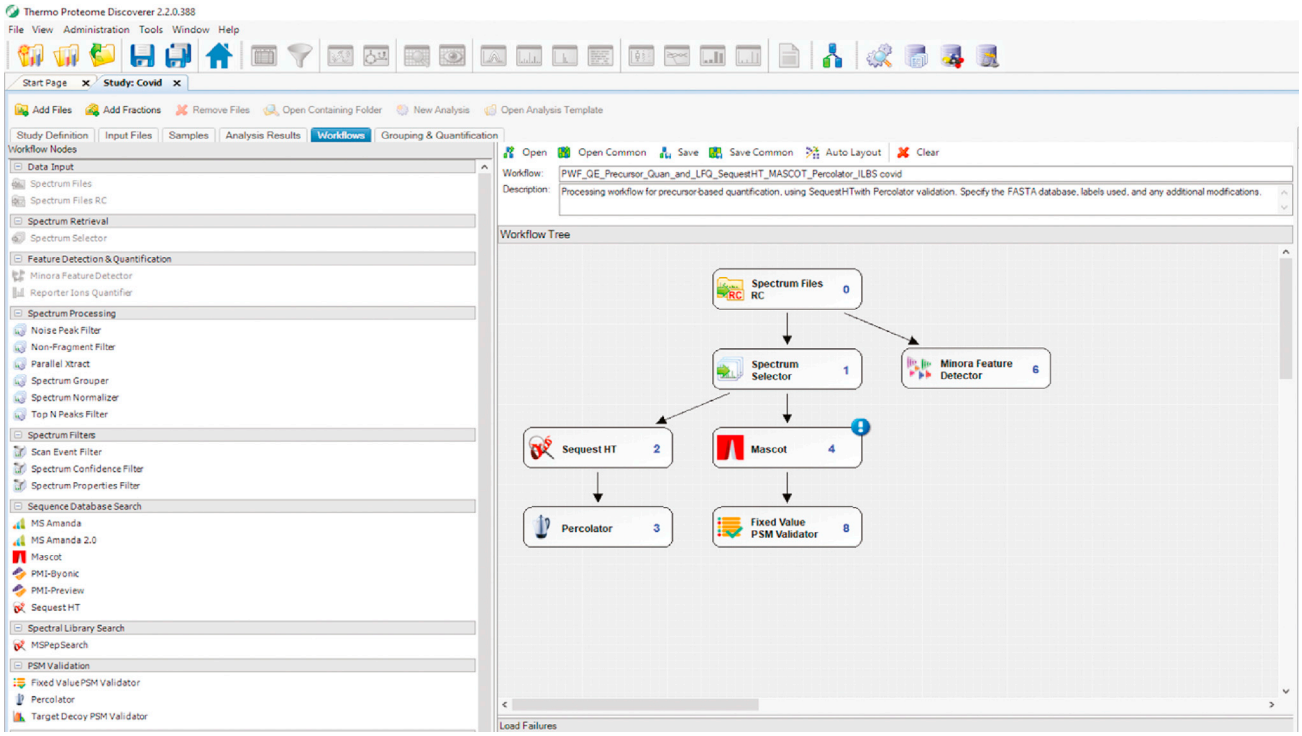


Figure 6. Pictographic representation of step 91 associated to proteome discoverer 2.2 mediated data analysis section

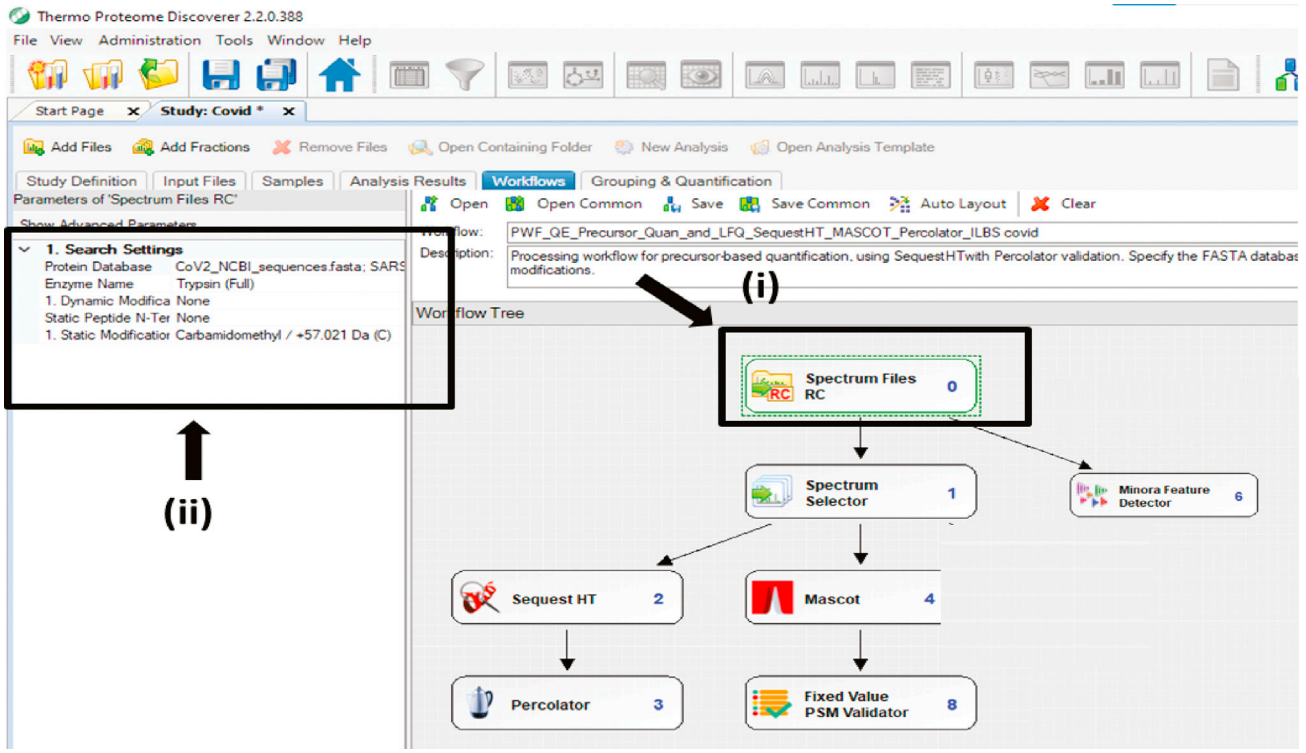


Figure 7. Pictographic representation of step 92 associated to proteome discoverer 2.2 mediated data analysis section

103. You will get a sunburst plot from Unipept (Figure 17).
104. Now download the data from Unipept by clicking on the download tab. (Figure 18).
105. You will get a file like this (Figure 19).
106. Repeat these steps for the other two groups.

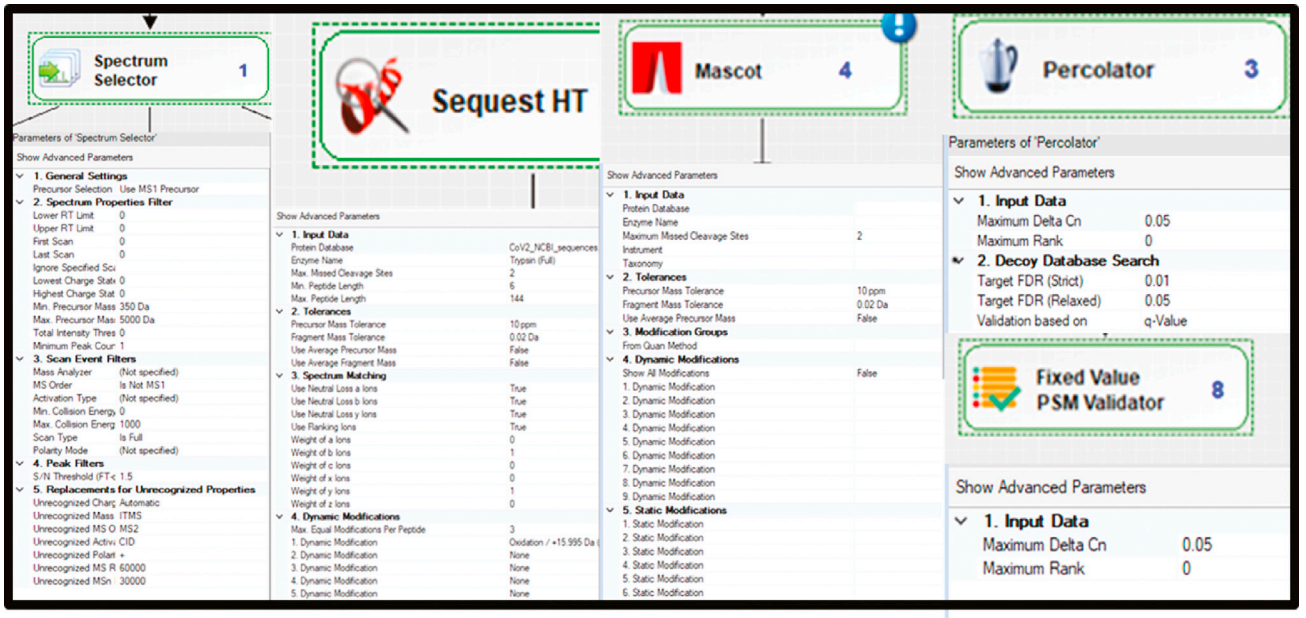


Figure 8. Pictographic representation of step 93 associated to proteome discoverer 2.2 mediated data analysis section

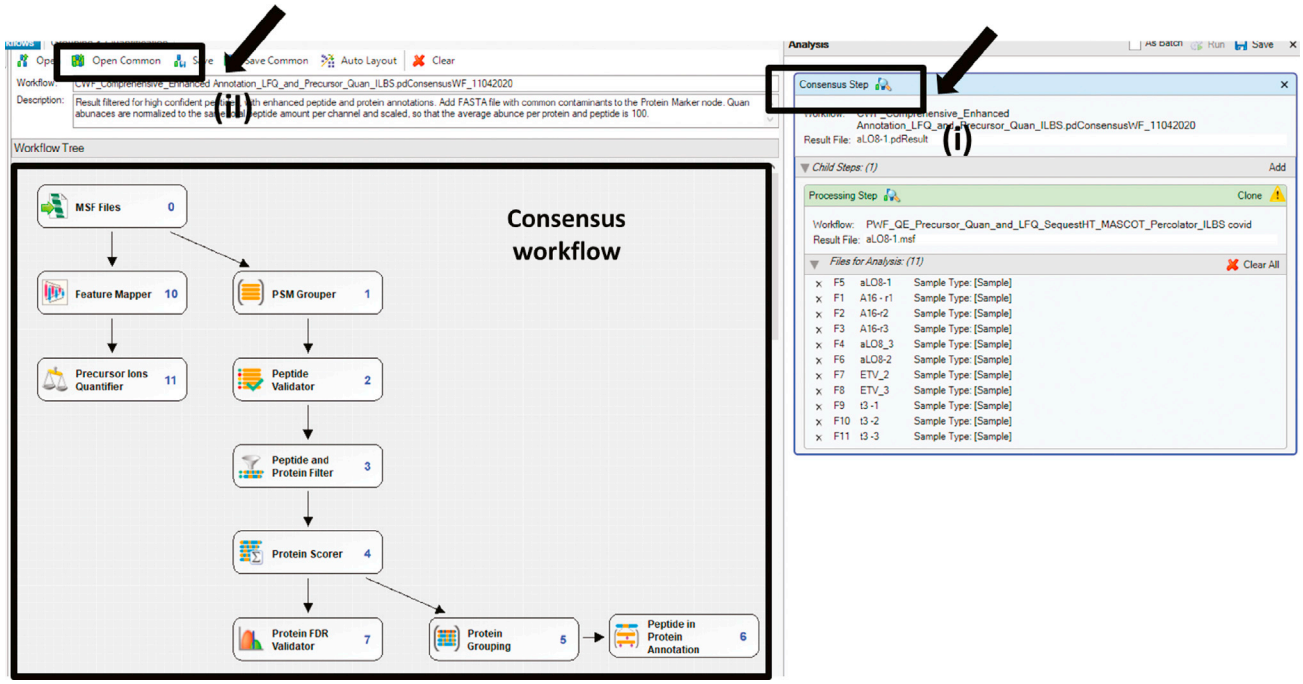


Figure 9. Pictographic representation of step 94 associated to proteome discoverer 2.2 mediated data analysis section

107. Now, you will have this excel file for all groups.
108. Now, compile all three files and master peptide files to get the abundance of each bacterium (Figure 20).
109. During this process, you can identify each disease group-specific metaproteome and compile the relative abundance of metaproteome.
110. Now use this file for statistical analysis

Note: the files generated at step 13 of proteome and virome can also be used for statistical analysis.

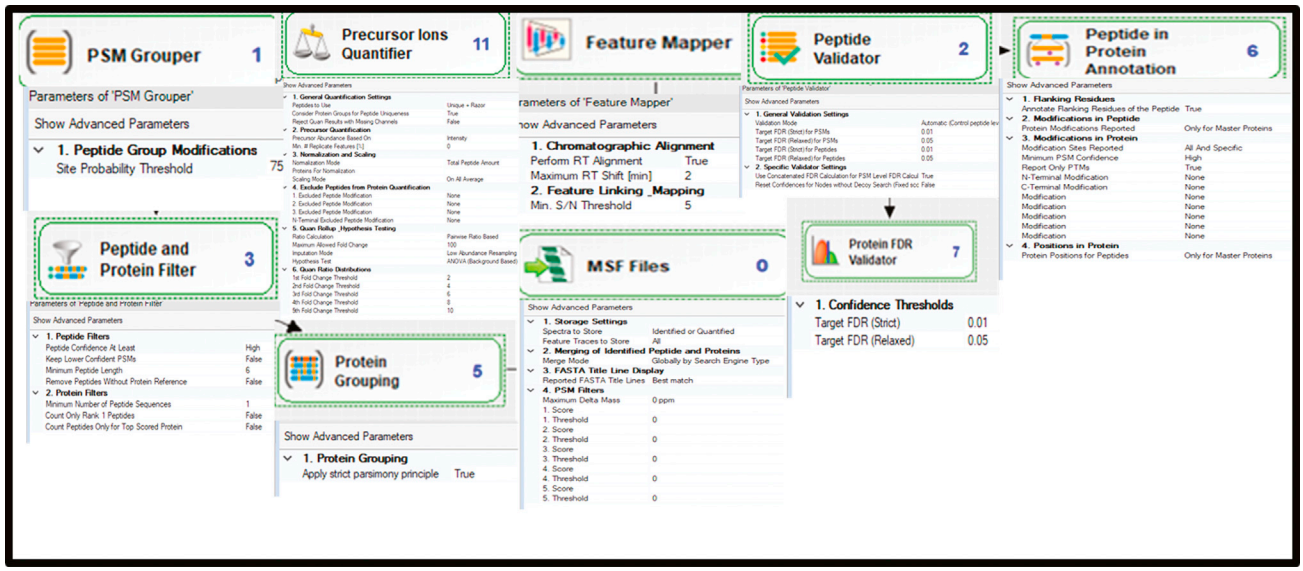


Figure 10. Pictographic representation of step 94 associated to proteome discoverer 2.2 mediated data analysis section

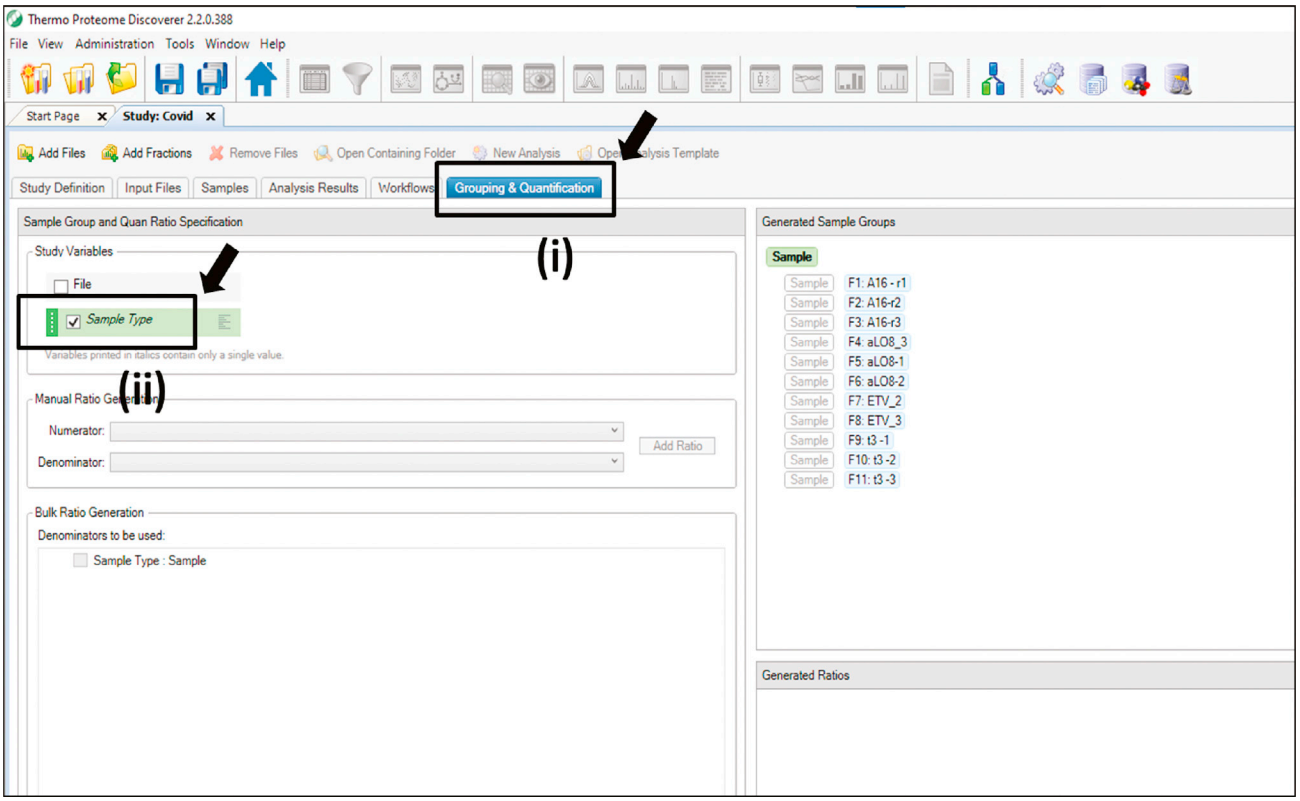


Figure 11. Pictographic representation of step 95 associated to proteome discoverer 2.2 mediated data analysis section

EXPECTED OUTCOMES

The importance of Global Proteomics in SARS-CoV-2 biology can help us understand the virus-host protein interaction and provide the index of its variability depending on the cell type used or sample type studied. Together, this can help us understand the mechanism of virus infection and provide us with potential biomarkers. On the contrary SARS-CoV-2 infection often precedes with bacterial co-infection and is linked with longer duration and more severe infection (van den Brand et al., 2014) thus metaproteome studies can give us insights into how the COVID-19 infection changes the microbiome of the human body and is this change in microflora can be associated to the severity of the disease. This protocol describes how to execute global proteomics to identify host proteome, virus-linked proteome (Virome), and bacteria-linked proteome (metaproteome) in the respiratory specimen of the study group. This protocol describes an approach that combines the Sample collection, virus inactivation, protein isolation, alkylation, digestion, desalination, and peptide sequencing using a Q-exactive + mass spectrometer bioinformatics approaches for data analysis like database curation, using proteome discoverer 2.2 and metaproteome analysis.

The expected outcome of these studies would be a list of a few hundred to several thousands of proteins and their expression pattern. Now via the proteomics approach, you can understand the change in host proteome pattern, virome can reveal the insights of virus proteins in the host, and finally, the metaproteome study can reveal the change in it and intensity of change. For example, in our COVID study (Maras et al., 2021), we identified 6 significantly differentially expressed COVID-19 viral proteins; around 1256 host proteins were identified. We also found an increase in respiratory pathogens (e.g., Burkholderiales, Klebsiella pneumonia) and decreased lactobacillus salivary (FDR<0.05) in the COVID-19 specimen. Finally, an increase in the basal level of WARS (tryptophan-tRNA ligase) and MX1 (MX dynamin-like GTPase 1) was correlated with SARS-CoV-2 infection outcome. Further, these were validated in a separate cohort of 200 patients.

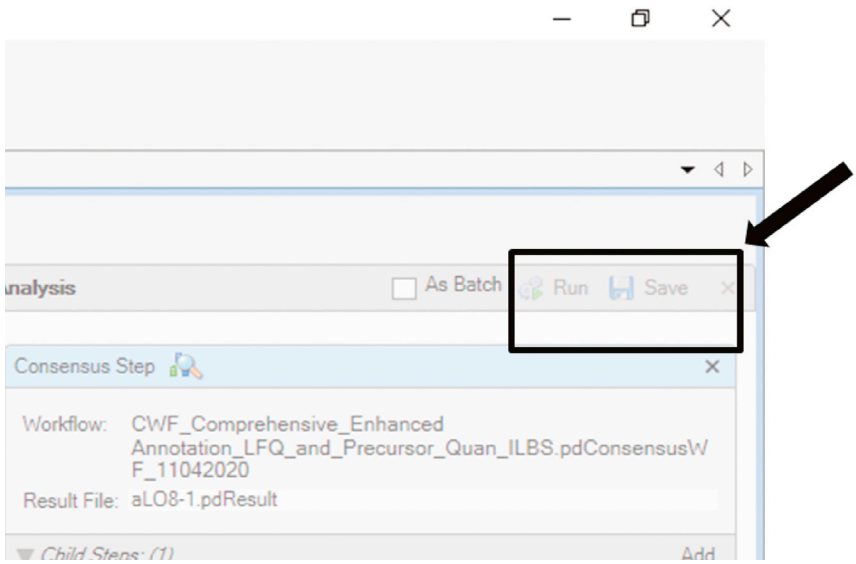


Figure 12. Pictographic representation of step 96 associated to proteome discoverer 2.2 mediated data analysis section

QUANTIFICATION AND STATISTICAL ANALYSIS

Systems bioinformatics

Timing: days to weeks

For further downstream analysis of proteome, virome, and Metaproteome data is performed using the following software's-

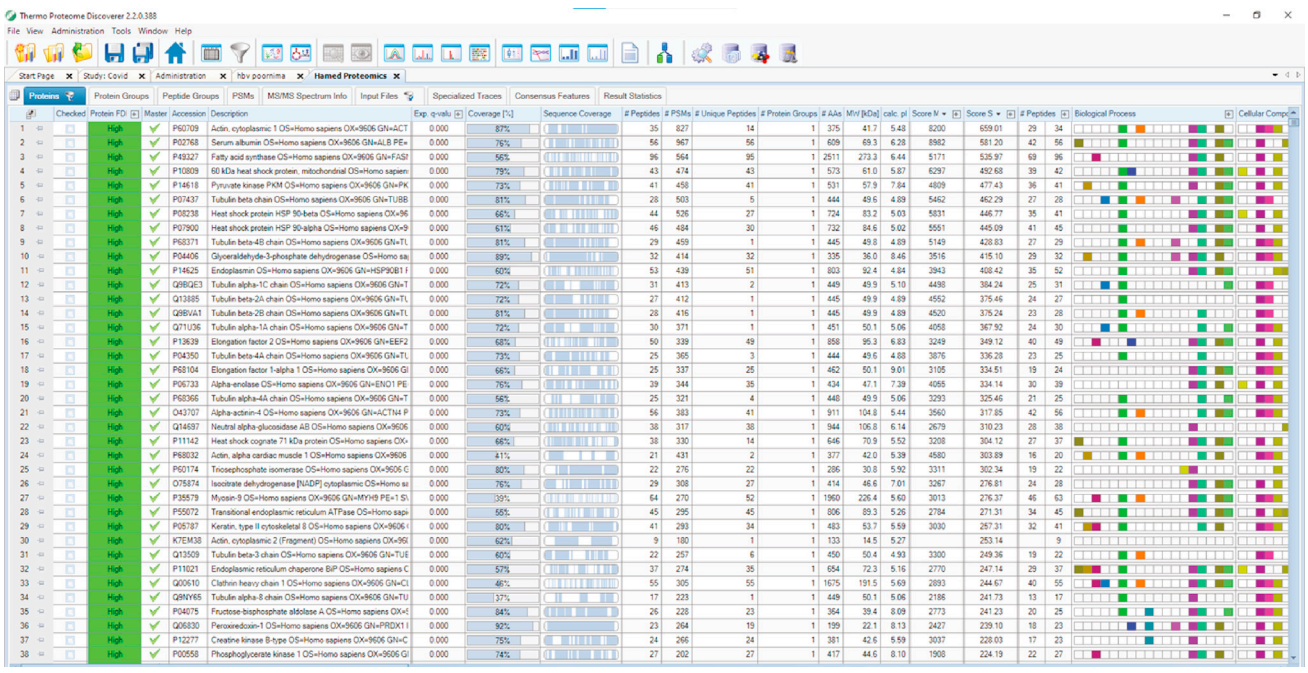


Figure 13. Final output result file from proteome discoverer (step 105)

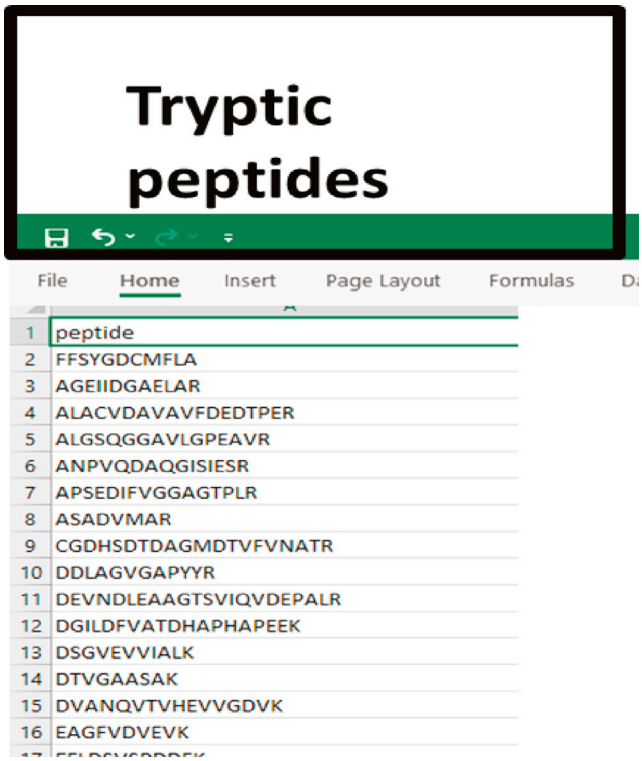


Figure 14. Pictographic representation of step 99 associated to downstream data processing for metaproteome analysis

a. Uniprot (https://www.uniprot.org): A comprehensive database of protein sequences. (The usage is explained in detail in the bioinformatics section, especially database curation).
b. Enricher (https://maayanlab.cloud/Enrichr/): Program for the functional enrichment analysis of proteins.
(Open the site > Copy the list of Gene symbols and paste in the Query section > Press Submit > Open the Reactome database > Download)
c. PANTHER (http://www.pantherdb.org): Program for the functional enrichment analysis of proteins.
(Open the site > Copy the list of Gene symbols and paste in Query section > Select ID type > Select organism > Select statistical overrepresentation test > Choose Reactome annotation > Submit > Select reference genome and run.
d. Metaboanalyst 4.0 (https://www.metaboanalyst.ca/): A web server used for all the statistical analysis associated with the proteome, virome, and Metaproteome data.
(Open site > Click on start > Click on statistical analysis > select data type, file and format > Submit and select preferred normalization > Perform desired statistics)
e. Cytoscape (https://cytoscape.org/): a global cross-correlation map between the virome, metaproteome, and the pathways linked to the proteins and metabolites using Cytoscape.
f. Unipept (https://unipept.ugent.be/): Database that's designed for metaproteomic data analysis with a focus on interactive data visualizations.
(The usage is explained in detail in the bioinformatics section, especially database curation)

LIMITATIONS

Although mass spectrometry-based proteomics and viromics are non-replaceable techniques in terms of how much they are essential in giving the snapshot of Protein expression of whole-cell,

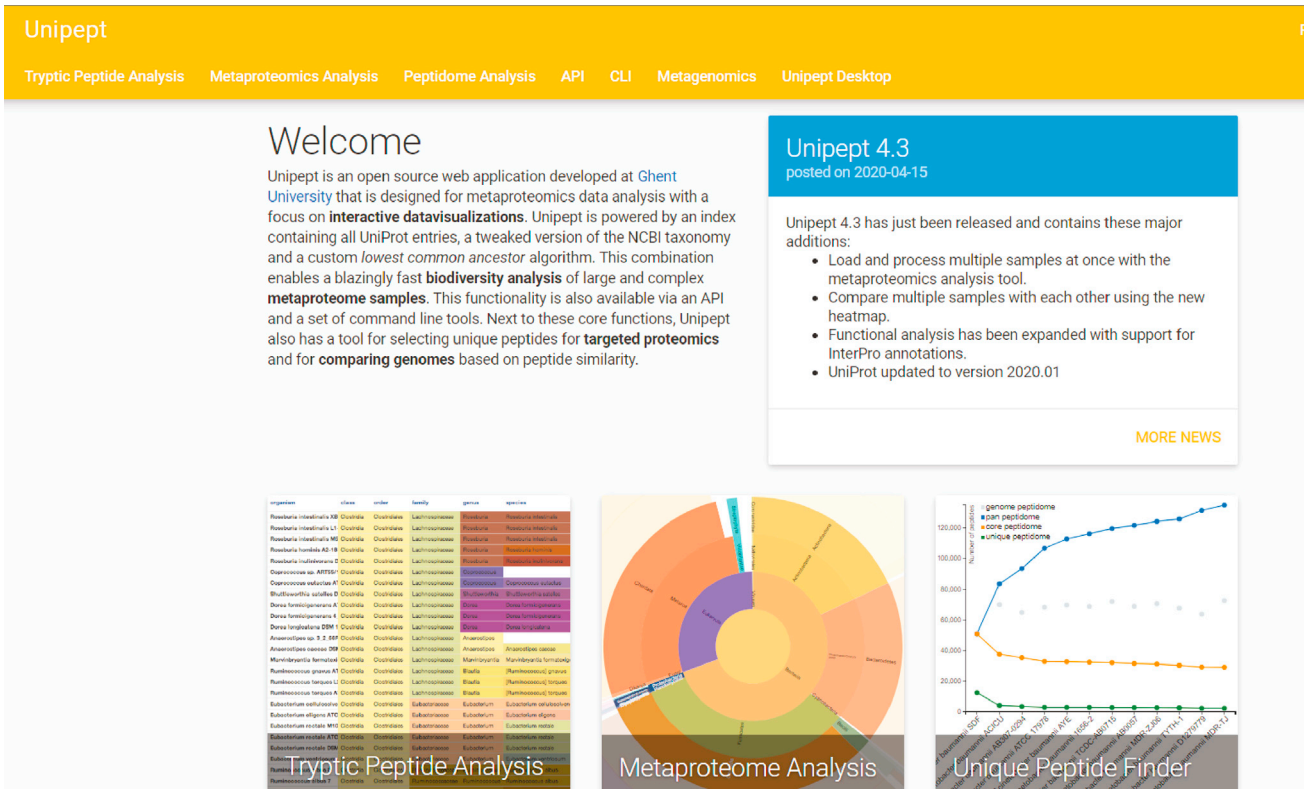


Figure 15. Pictographic representation of step 101 associated to downstream data processing for metaproteome analysis

unlike genome, the proteome of cells is highly dynamic, and thus, multiple samples and time points need to be considered if study demands temporal profiling (Betzen et al., 2015). The starting sample should be fresh as degradation in the proteins would result in lower identification. The extensive protein and peptide cleanup procedure used in the protocol tends to lose some proteins and peptides, resulting in lower identification.

TROUBLESHOOTING

Problem 1

Interference of chemicals such as polymers in LC-MS/MS analysis (steps 83–87).

Potential solution

In such a scenario, the prepared peptides samples need to be reprecipitated. Add 6× acetone to your peptide samples and incubate them at −20°C for 1 h > centrifuge at maximum g possible and discard the supernatant. Dissolve the pellet again and reanalyze in mass-spectrometer.

If still the problem persists, reprepare your samples, and this time before using any plastic ware, soak them in any organic solvent (e.g., ethanol or acetone) for 24–72 h to remove any contaminant polymer.

Problem 2

The trap column of Nano-LC gets blocked (steps 83–87).

Potential solution

In such a scenario, first, you disconnect the column from source > then reverse the direction of flow in the column by reversing the direction of column > then start the isocratic flow and wait till water

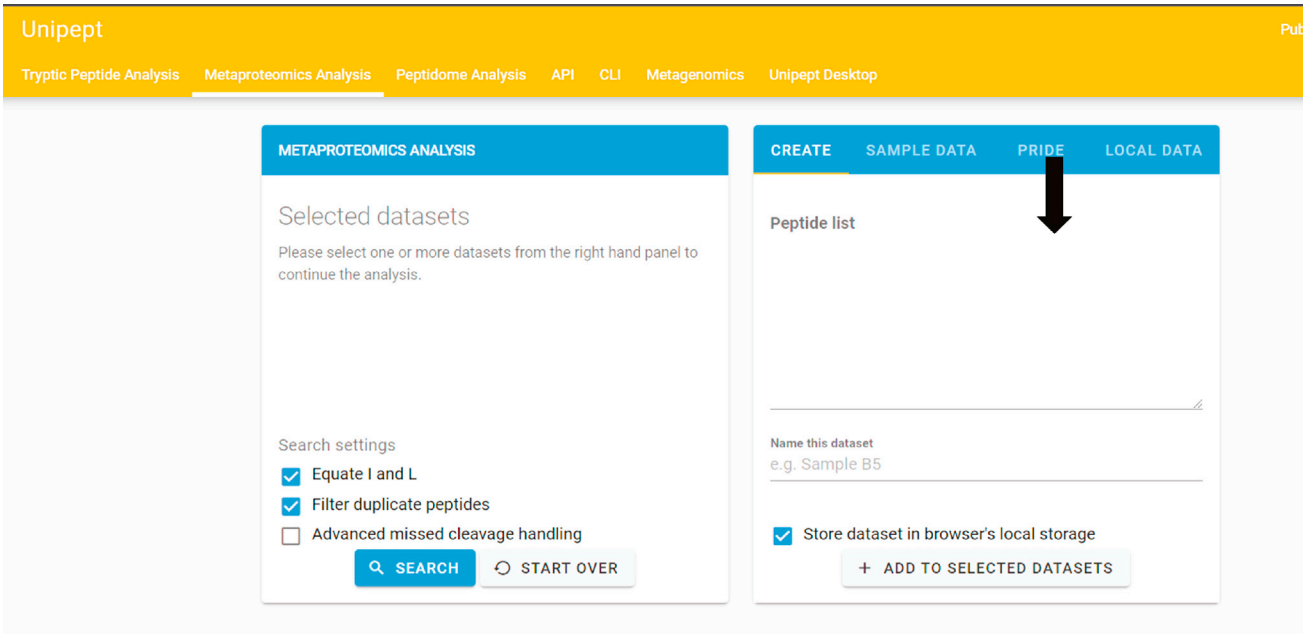


Figure 16. Pictographic representation of step 102 associated to downstream data processing for metaproteome analysis

starts to appear or flow from the open end of the column once it happens. Rearrange the column and start.

Problem 3

The air bubble got trapped in the nano-LC pump (steps 83–87).

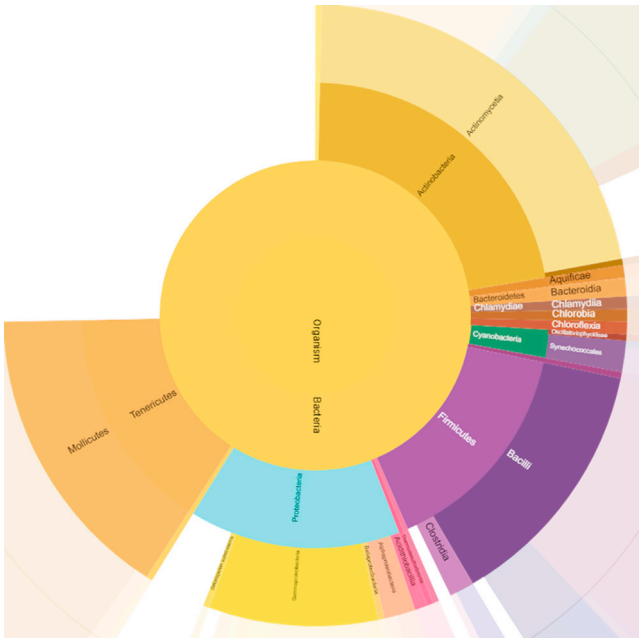


Figure 17. Pictographic representation of step 102 associated to downstream data processing for metaproteome analysis

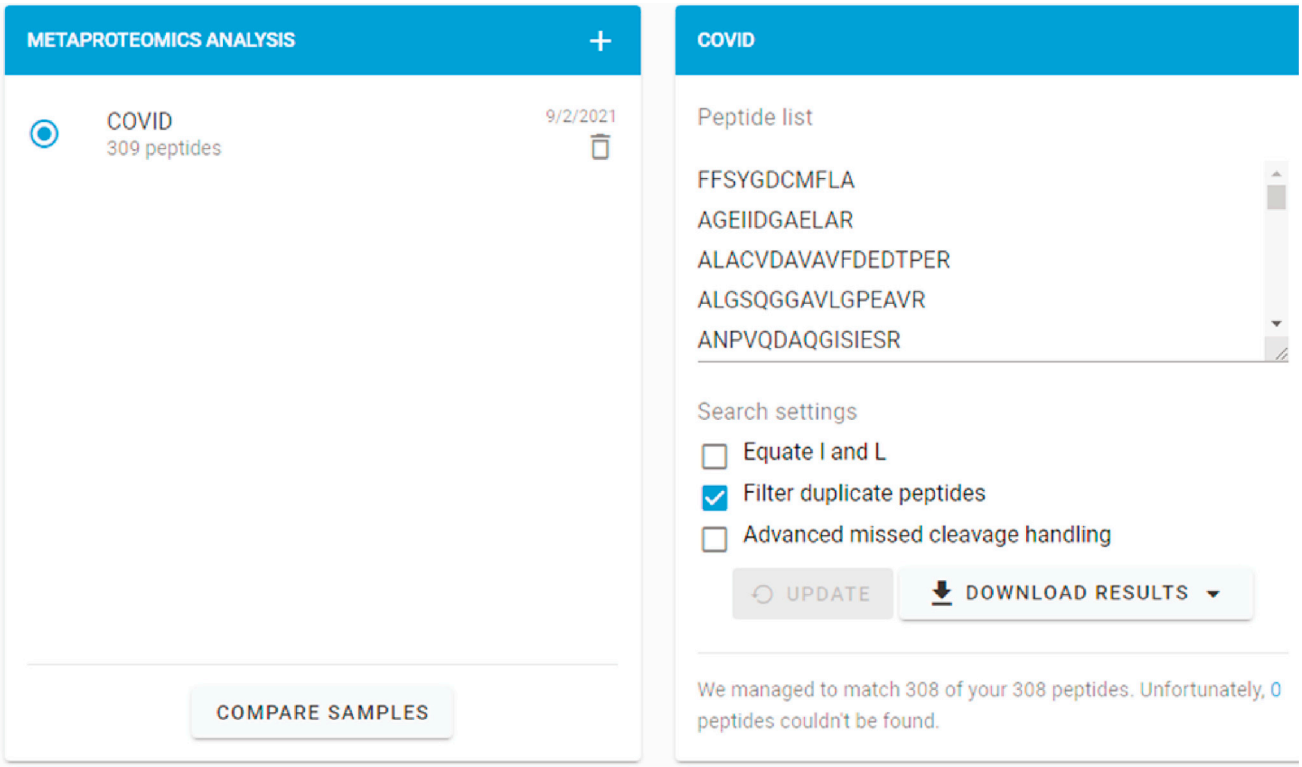


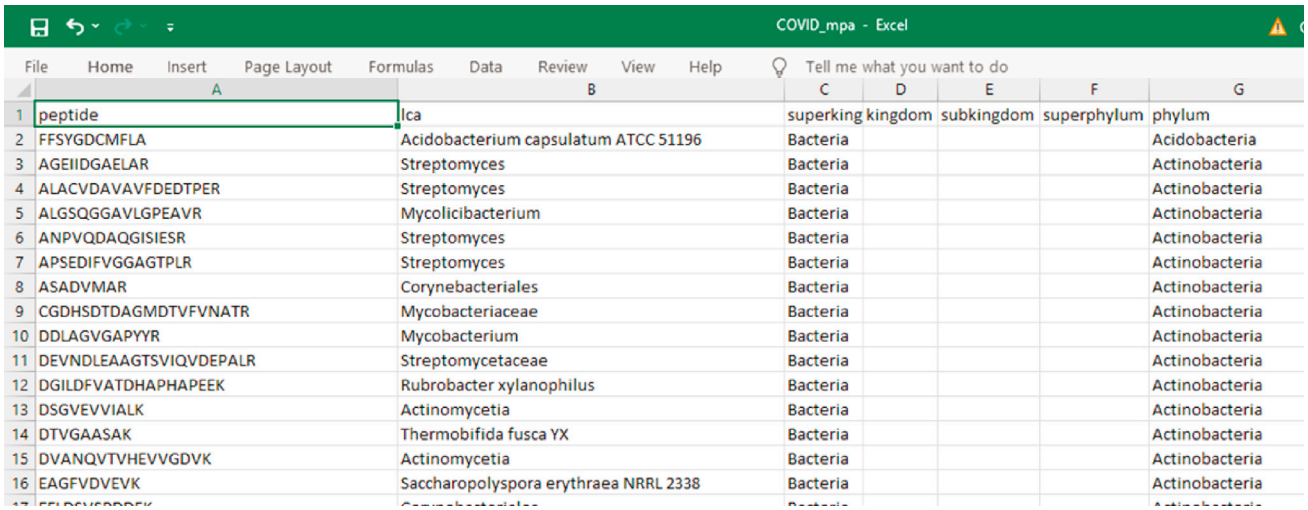
Figure 18. Pictographic representation of step 104 associated to downstream data processing for metaproteome analysis

Potential solution

In such a scenario, first, you disconnect the column from source > then join the column end with waste outlet> then start purge > the flush finally performs a leak test if passed. Rearrange the column and start.

Problem 4

No Peaks/Very Small Peaks (steps 83–87).



	A	B	C	D	E	F	G
1	peptide	lca	superking	kingdom	subkingdom	superphylum	phylum
2	FFSYGDCMFLA	Acidobacterium capsulatum ATCC 51196	Bacteria				Acidobacteria
3	AGEIIDGAELAR	Streptomyces	Bacteria				Actinobacteria
4	ALACVDAVAVFDEDTPER	Streptomyces	Bacteria				Actinobacteria
5	ALGSQGGAVLGPEAVR	Mycolicibacterium	Bacteria				Actinobacteria
6	ANPVQDAQGISIESR	Streptomyces	Bacteria				Actinobacteria
7	APSEDIFVGGAGTPLR	Streptomyces	Bacteria				Actinobacteria
8	ASADVMAR	Corynebacteriales	Bacteria				Actinobacteria
9	CGDHSDTDAGMDTVFVNATR	Mycobacteriaceae	Bacteria				Actinobacteria
10	DDLAGVGAPYYR	Mycobacterium	Bacteria				Actinobacteria
11	DEVNDLEAAGTSVIQVDEPALR	Streptomycetaceae	Bacteria				Actinobacteria
12	DGILDFVATDHAPHAPEEK	Rubrobacter xylanophilus	Bacteria				Actinobacteria
13	DSGVEVVIALK	Actinomycetia	Bacteria				Actinobacteria
14	DTVGAASAK	Thermobifida fusca YX	Bacteria				Actinobacteria
15	DVANQVTVHEVVGDVK	Actinomycetia	Bacteria				Actinobacteria
16	EAGFVDVEVK	Saccharopolyspora erythraea NRRL 2338	Bacteria				Actinobacteria

Figure 19. Pictographic representation of step 105 associated to downstream data processing for metaproteome analysis

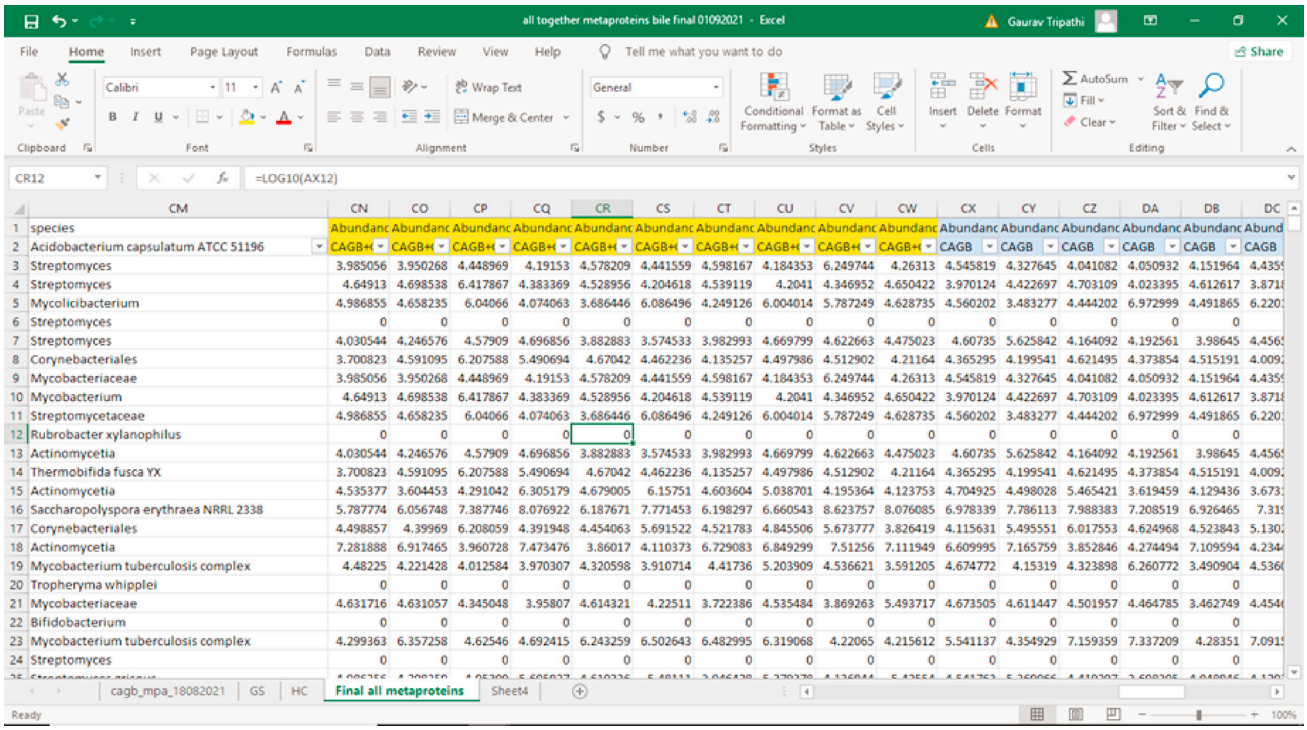


Figure 20. Pictographic representation of step 108 associated to downstream data processing for metaproteome analysis

Potential solution

In such a scenario, check that the lamp is on and the cables are well connected. Next, ensure that the flow is normal and the automatic sampler is working properly, not blocked or jammed.

Problem 5

No or low protein detection (steps 60–82).

Potential solution

In such a scenario take your flowthrough and repurify peptide from it again. Never discard the flowthrough.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Jaswinder Singh Maras (jassi2param@gmail.com).

Materials availability

The study did not generate any materials.

Data and code availability

No data is provided, and the raw data for the manuscript is available on request to the lead contact Dr. Jaswinder Singh Maras (jassi2param@gmail.com).

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AUTHOR CONTRIBUTIONS

J.S.M. conceptualized the work. The manuscript was written by G.T. and J.S.M., with help from M.Y., N.S., B.M., V.B., S.S., and S.K.S. The manuscript was read and approved by all authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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