Label-free quantitative proteomics of oral microbial communities

Marwan Mansoor Ali Mohammed[1*], Jack-Ansgar Bruun[2], Veronika Kuchařová Pettersen[3, 4, 5]

[1]Department of Oral and Craniofacial Health Sciences, College of Dental Medicine, University of Sharjah, United Arab Emirates
[2]Proteomics and Metabolomics Core Facility (PRiME), Department of Medical Biology, UiT The Arctic University of Norway, Tromsø, Norway
[3]Research Group for Host-Microbe Interactions, Department of Medical Biology, UiT The Arctic University of Norway, Tromsø, Norway
[4]Pediatric Research Group, Department of Clinical Medicine, UiT The Arctic University of Norway, Tromsø, Norway
[5]Centre for New Antibacterial Strategies, UiT The Arctic University of Norway, Tromsø, Norway

*Corresponding author: veronika.k.pettersen@uit.no

Abstract

The oral cavity is a habitat for different microorganisms, of which bacteria are best described. Studying different bacterial taxa and their proteins is crucial to understanding their interactions with the host and other microbes. Also, for bacteria with virulence potential, identifying novel antigenic proteins is essential to finding candidates for the development of vaccines.

Here we describe a workflow for gel-free and label-free protein analysis of oral bacterial species grown *in vitro* as a biofilm and a planktonic culture. Details on cultivation, protein extraction and digestion, peptide clean-up, LC-MS/MS run parameters, and subsequent bioinformatics analysis are included. We also discuss challenging steps in the workflow, such as dealing with non-standard protein identifiers and selecting a suitable protein database. This protocol provides a valuable guide for proteomic experiments using multi-species models of oral bacteria.

Keywords: label-free quantitative proteomics, oral pathogenic bacteria, oral microbiology, multi-species model, oral biofilm, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*

29 **1. Introduction**

30 The oral cavity harbours a variety of microorganisms, including fungi, protozoa, archaea, and
31 bacteria, as well as viruses (1). Oral bacteria are responsible for the two most common dental
32 diseases of man: tooth decay (dental caries) and gum disease (periodontal disease) (2). A
33 major etiological factor in initiating these diseases are microbial biofilms that grow on teeth
34 surfaces and are composed of many bacterial species (3). Historically, oral microbiologists
35 often used a reductionist approach that attempts to study the oral microbial community by
36 analysing individual species (4). Nowadays, researchers explore oral microorganisms as an
37 interconnected community with multiple interspecies interactions that contribute to the
38 complexity of biofilm formations and poly-microbial diseases. A limited number of *in vivo*
39 studies on oral biofilms showed variable results and highlighted practical issues with this
40 approach (5). Therefore, multi-species *in vitro* models of oral biofilms are becoming popular
41 (6, 7). Functional omics techniques, such as mass spectrometry (MS)-based proteomics that
42 identify and quantify most proteins in a sample, are well suited for characterising microbial
43 communities' dynamics.

44 Still, the increased complexity of microbial models brings challenges in standardising
45 cultivation conditions and subsequent downstream analyses. Microbial biofilms are dynamic
46 communities affected by environmental factors like pH, oxygen and nutrient gradients, and
47 media composition, making the cultivation of oral microbial communities technically
48 demanding (8). Other challenges include obtaining adequate sample volume and, in turn,
49 sufficient sampling depth that allows taxonomic resolution between related organisms and
50 description of the community interactions and metabolic activity on a species and preferably
51 strain level (9).

52 Proteomics methods have advanced enormously over the last two decades, shifting from
53 dependence on gel-based protein analysis and single protein purification to gel-free
54 proteomics approaches (10). In the gel-based approach, the proteins are first separated using
55 one-dimensional or two-dimensional polyacrylamide gel electrophoresis (2-D PAGE), the
56 target band or spot is excised, and proteins within the band digested into a peptide mixture
57 using proteases such as trypsin. However, the main limitation of gel-based methods is low
58 number of detected proteins. Accordingly, only a small part of the proteome can be analysed
59 using the 2-D approach.

60 A more complete study of proteomes has been possible with gel-free approaches, which have
61 been driven by advances in liquid chromatography (LC)-based separation techniques and the

62 resolution power of MS. These approaches use proteases to digest the whole sample without
63 prior separation on the gel (11). The peptide mixtures are then subjected to strong cation
64 exchange chromatography or microcapillary reverse-phase LC coupled to MS or tandem MS
65 (LC-MS/MS) analysis (11). The most common approach for peptide identification is
66 comparing the experimentally obtained spectra against theoretical spectra of peptides derived
67 from in silico digestion of a protein sequence database. Currently, shared peptides originating
68 from homologous proteins remain a challenge when assigning proteins to specific species,
69 which is greatly enhanced when profiling microbial communities (11).

70 LC-MS/MS-based proteomics has become the conventional approach for functional analysis
71 of microbial communities since the method combines high sensitivity and specificity with
72 high-throughput qualitative and quantitative protein characterisation (11, 12). Label-free
73 methods are currently the most widely used form of quantitative proteomics (13), as they
74 avoid additional expense in sample preparation steps. The approach is also feasible on
75 hundreds of samples. Different labelling techniques have been developed and provide good
76 results for the relative and absolute quantification of proteins (12), and in many studies,
77 multiplexing proteomics can be the best-suited method. However, the label-free methods
78 provide a higher dynamic range of quantification, which means one can measure changes
79 within a complex mixture or across an entire proteome in one experiment (13, 14). Label-free
80 protein quantitation is traditionally done using Data-Dependent Acquisition (DDA) methods.
81 Still, Data-Independent Acquisition (DIA) is also becoming a popular method in label-free
82 quantification (14).

83 Here we describe a workflow for gel-free and label-free protein analysis of oral bacteria
84 grown in biofilm, with a planktonic condition as a control. Details on cultivation, protein
85 extraction and digestion, peptide clean-up, LC-MS/MS run parameters for DDA, and
86 subsequent bioinformatics analysis are included (15, 16). We also discuss challenging steps in
87 the workflow, such as selecting a suitable protein database. The method has been beneficial in
88 identifying virulence factors produced by oral bacteria *Fusobacterium nucleatum* and
89 *Porphyromonas gingivalis* that directly contribute to the development and progression of
90 periodontal diseases. Further understanding of how microbial communities work together and
91 form oral biofilms is of great demand as it will guide strategies for oral disease prevention and
92 therapy.

93 **2. Materials**

94 **2.1 Solutions and Reagents for Cultivation of Bacteria**

- 95 Strains of oral bacterial species (*e.g.*, *Fusobacterium nucleatum* subsp.
96 *nucleatum* type strain ATCC 25586 and *Porphyromonas gingivalis* type strain
97 ATCC 33277)
- 98 Fastidious anaerobic agar (FAA) plates
- 99 Brucella broth (Becton Dickinson) supplemented with 5 µg/ml hemin and 0.25
100 µg/ml Vitamin K
- 101 Phosphate buffered saline (PBS)

102 **2.2 Solutions and Reagents for Filter Aided Sample Preparation (FASP)**

- 103 PBS
- 104 MS grade water
- 105 Protein extraction buffer [10 mM Tris-HCl, 2.5 % sodium dodecyl sulfate
106 (SDS), pH 8.0]
- 107 100 mM ammonium bicarbonate (NH_4HCO_3 - ABC) - 79 mg ABC into 100 ml
108 MS grade water.
- 109 10 mM dithiothreitol (DTT)- 1.54 mg DTT into 1mL 100mM ABC
- 110 Urea buffer (8 M urea, 10 mM HEPES, pH 8.0)
- 111 0.05 M iodoacetamide
- 112 40 mM ABC - dilute from 100 mM ABC
- 113 Proteomics grade Trypsin in 40 mM ABC buffer

114 **2.3 Solutions and Reagents for C18-based Peptide Clean-Up** (note here that all
115 reagents have to be MS grade)

- 116 Methanol
- 117 Acetonitrile (ACN) 2%
- 118 Formic acid (FA) 0.1% and 100%
- 119 elution buffer (80% ACN, 0.1% FA)

120 **2.4 Consumables and Equipment**

- 121 Anaerobic growth jars (see Note 1)
- 122 Polystyrene cell culture flasks 25 cm^2 (area)
- 123 Test tubes with screw caps

124 • Spectrophotometer (for example Direct Detect® Spectrometer from Merck
125 Millipore, Darmstadt, Germany).
126 • Anoxomat System for anaerobic growth conditions
127 • Cell scraper
128 • lysing matrix (e.g., Fastprep lysing matrix type A,MP Biomedicals, California,
129 USA))
130 • Ribolyser (e.g., Fastprep, Thermo Savant)
131 • Microcon device YM-10 filters (Merck Millipore, Darmstadt, Germany)
132 • Vacuum concentrator (Eppendorf, Hamburg, Germany)
133 • 3M Empore C18 extraction disks
134 • 200 µl pipette tips
135 • Blunt-ended needle and a plunger or metal rod that helps to fit the C18 disks in
136 the 200 µl pipet tips
137 • 1 ml syringe – it is used to push the solution through the disk
138 • Eppendorf tubes (0.5, 1 and 2 ml)
139 • Low-bind protein tubes (*e.g.*, 1.5 ml Proteon Low Bind tubes from Eppendorf)

140 **2.5 LC-MS/MS and Data Analysis**

141 • LC-MS/MS: EASY nLC 1200 connected to a Orbitrap Exploris 480 (Thermo
142 scientific) with an EASY-Spray column (ES903, thermo scientific)
143 • Data analysis: Freely available MaxQuant software package (17) with the
144 integrated search engine Andromeda. Protein sequence database matching the
145 studied bacterial strains in FASTA format (*e.g.*, downloaded from UniprotKB).
146 • Spreadsheet editor or the freely available Perseus software package (18).

147 **3. Methods**

148 **3.1 Cultivation of bacteria in biofilm and planktonic culture**

149 1. Inoculate the bacteria from -80°C stock on the FAA plates and allow them to grow
150 anaerobically at 37°C for 48 hours.
151 2. Harvest a few colonies from agar to inoculate 8 ml supplemented Brucella broth in 10
152 ml flasks and allow them to grow at 37°C overnight (16h).
153 3. Next morning, adjust the absorbance of the overnight grown culture to 0.15 at 600 nm
154 (A_{600}) with sterile supplemented Brucella broth.

155 4. For biofilms, transfer 10 ml of the A_{600}-adjusted culture (5 ml from each species in a
156 dual-species biofilm) to polystyrene cell culture flasks and incubate the flasks at 37°C
157 for 4 days (See Note 2)
158 5. For planktonic culture, transfer 10 ml of adjusted culture to glass round bottom test
159 tubes with screw caps and incubate the flasks at 37°C for 4 days (See Note 3)
160 6. Decant the medium and gently wash the biofilm once with 3 ml of PBS without
161 disturbing the formed biofilm. During the washes, one should not press the liquid
162 against the biofilm but rather slide the liquid slowly over the biofilm and slowly
163 decant the liquid.
164 7. Harvest the biofilm with the help of a cell scraper.
165 8. Resuspend the harvested biofilms in 500 µl PBS and store the samples at -20°C until
166 further processing.
167 9. To harvest the planktonic bacteria, centrifuge the cultures at 3,000 x g for 3 min at
168 room temperature and discard the supernatant.
169 10. Wash the bacterial pellet three times by resuspension in 1 ml PBS followed by
170 centrifugation for 10 min at 6,000 × g at +4°C.
171 11. Resuspend the pelleted cells in 500 µl PBS, transfer them to 1.5 ml Eppendorf tube,
172 and store it at -20°C until further processing.

173 **3.2 Protein extraction from the biofilm and planktonic samples**

174 1. Resuspend the bacterial samples prepared in section 3.1 in 1 ml of extraction buffer.
175 2. Transfer the suspended bacteria in the extraction buffer to the lysing matrix and then
176 bead beat in ribolyser (*e.g.*, Fastprep), for example, for 45 sec at 6.5 m/s speed.
177 3. Cool the extract on ice for 5 minutes, followed by centrifugation for 30 minutes at
178 10,000 × g, +4°C.
179 4. Collect the supernatant (protein extract) and keep it on ice.
180 5. Measure the protein concentration by using a spectrophotometer.

181 **3.3 Sample preparation for the proteomic analysis**

182 1. Prepare the samples with protein extracts of different culture conditions in three
183 biological replicates and apply the Filter Aided Sample Preparation (FASP) method
184 developed by Wisniewski and co-workers (19), with minor modifications.

2. Mix 50 µg of protein extracts with 5 µl of a solution of 10 mM DTT in 100 mM ABC [solution to total protein ratio (v/w) 1:10] and incubate for 45 min at 56°C without shaking.
3. Condition the Microcon device YM-10 filters by adding 100 µl of urea buffer and centrifuge at 14,000 × *g* for 5 min (See Note 4)
4. Mix the denatured protein sample from step 2 with 200 µl urea buffer in the filter unit and centrifuge at 14,000× *g* for 15 min.
5. Wash the sample with 200 µl urea buffer by centrifuging at 14,000× *g* for 15 min.
6. Discard the filtrate and add 100 µl of 0.05 M iodoacetamide to each sample.
7. Mix the samples at 600 rpm for 1 min in a thermo-mixer, then incubate without mixing in the dark for 20 min,
8. Centrifuge at 14,000 x *g* for 10 min, then wash with 100 µl urea buffer three times, followed by another three washes with 100 µl 40 mM ABC.
9. Digest the proteins on the filter with trypsin in 40 mM ABC (enzyme to protein ratio 1:50) at 37°C for 16 h.
10. Collect the released peptides by adding 50 µl of MS grade water followed by centrifugation at 14,000 x *g* for 15 min. Repeat this step twice.
11. Concentrate the samples (to 20-40 µl volume) in a vacuum concentrator (See Note 5).

3.4 ~~Filtration~~ Enrichement and desalting

1. Stamped out the 3M Empore C18 extraction disks using a blunt-ended syringe needle (Note 6). The portion size is determined by the inner diameter of the needle, and can thus be adapted to the size needed
2. Pack up to five of extraction disks in 200 µl pipet tips with a blunt-ended needle and a plunger or metal rod that helps to fit the extracted disks in the pipet tips, according to the protocol developed by Rappsilber and colleagues (20).
3. Wet the disks by passing 20 µl of methanol, followed by 20 µl of elution buffer [80% ACN) 0.1% FA].
4. Condition and equilibrate the disks with 20 µl of 0.1% FA just before the last residue of the previous buffer leaves the tip to avoid drying the disks.
5. Load the sample (volumes 20-40 µl) on top of the disks.
6. Desalt the disks with samples by washing with 20 µl of 0.1% FA.
7. Elute the peptides by adding 20 µl elution buffer and collect the desalted sample in a new clean low-bind protein tube. Repeat the elution step one more time.

218 8. Dry the collected samples in the vacuum concentrator and store at -80°C until further
219 analyses.
220 9. Resuspend the peptide samples by adding 1 µl of 100% FA and 19 µl of 2% ACN
221 prior to LC-MS/MS analysis (See Note 7)

222 **3.5 LC-MS/MS**

223 1. The MS/MS analysis is typically carried out at a dedicated proteomic facility by
224 personnel operating the instruments. An example of a potential LC-MS/MS run setup
225 is the EASY nLC 1200 connected to an Orbitrap Exploris 480 (Thermo Scientific)
226 with an EASY-Spray column (ES903, Thermo Scientific)
227 2. Load 1 µg protein onto a pre-concentration column (Acclaim PepMap 100, 2 cm ×75
228 µm i.d. nanoViper column, packed with 3 µm C18 beads) at a flow rate of 5 µl/min for
229 5 min using an isocratic flow of 0.1% trifluoroacetic acid, vol/vol (TFA).
230 3. Separate the peptides by a biphasic ACN gradient (flow rate of 300 nl/min) on the
231 analytical column (EASY-Spray column (C18, 2µm, 100 Å, 50µm, 50 cm)).
232 4. Apply solvent A [0.1% FA in water (vol/vol)] and solvent B [0.1% FA in 80 %
233 ACN/Water (vol/vol)], during a 140 min LC run with the following gradient
234 composition: 0 min 5% B, 0-5 min 8% B, 5-125 min 8–40% B, 125-130 min 40–90%
235 B, and 130-140 min 90% B.
236 5. The separated peptides are then directly sprayed into the MS instrument by an EASY-
237 Spray Source.
238 6. Operate the mass spectrometer in the DDA mode to automatically switch between MS
239 and MS/MS acquisition.
240 7. Use a label-free quantification method from the instrument associated software
241 Excalibur.
242 8. Full scans are acquired at orbitrap Resolution 60,000 with Scan Range from 350-1,200
243 m/z. The predetermined number of dependent scans is 38. MS2 scans are acquired at
244 15,000 resolution. Target ions already selected for MS/MS are dynamically excluded
245 for 45s.

246 **3.6 Data analysis**

247 1. Process the acquired MS raw data by using the MaxQuant software (17) with default
248 settings and the following additional options (see Note 8): Label-Free Quantification
249 (LFQ), match between runs, and 0.01 false discovery rate (FDR) at both peptide and

250 protein level. By using the LFQ option, the software will derive normalised spectral
251 protein intensities by the MaxLFQ algorithm that applies protein-specific correction
252 coefficients during the normalisation process (21).
253 2. Upload into the MaxQuant user interface matching protein databases for the strains
254 used in the experiment (*e.g.*, *F. nucleatum* strain ATCC 25586 and *P. gingivalis* strain
255 ATCC 33277), which can be downloaded from the Universal Protein Knowledgebase
256 (Note 9).
257 3. Perform the MS searches by analysing each species separately, that is, searching raw
258 files of single-species cultures/bifilms together with raw files from the dual-species
259 model against the protein database of the specific species.
260 4. Analyse the MaxQuant output data ('proteingroups.txt') with the Perseus module (18).
261 5. Filter the generated 'proteingroups.txt' table for contaminants, only identified by site,
262 and reverse hits in Perseus software (see Note 8)
263 6. Consider each protein identified in at least 2 of the 3 replicates as valid.
264 7. Proteins with significant differential levels can be identified by statistical analysis
265 based on two-sided t-test performed on proteins $\log_2$ transformed LFQ values.
266 8. Consider protein levels as significantly different between conditions if it is marked as
267 significant in the t-test and showed more than 2 $\log_2$ difference from the mean LFQ
268 intensity.
269 9. Perform the functional protein classification using The Database for Annotation,
270 Visualization and Integrated Discovery (DAVID) website (22) and QuickGO
271 annotation database (23). Analyse the potentially interesting clusters identified by
272 DAVID individually.
273 10. Use the web-based application SOSUI-GramN (24) to predict the subcellular
274 localisation of the identified proteins.
275 11. Deposit the mass spectrometry proteomics data to a preferred repository database (e.g
276 ProteomeXchange).

277 **4. Notes**

278 1. The anaerobic growth condition is required if one works with strict anaerobic bacteria.
279 The available anaerobic culture system like Anoxomat Mart, AnaeroPack or an
280 anaerobic chamber can be used.
281 2. The time for bacterial biofilm to mature varies between species, usually anaerobic oral
282 bacteria need longer cultivation time of 4-5 days compared to aerobic bacteria.

283 Therefore, a pilot study may be required to find the optimal growth time to have a
284 mature biofilm.

285 3. The glass round bottom test tubes with screw caps can be used to grow the planktonic
286 bacteria, but other cultivation tubes might be prefered as long as minimal biofilm
287 formation is seen on the inner surfaces. Both *P. gingivalis* and *F. nucleatum* can form
288 biofilm on glass and polystyrene surfaces. For practical reasons, polystyrene flasks are
289 often recommended to grow the biofilms, while planktonic cultures are easier to
290 cultivate in glass flasks. However, whether and how the different surfaces affect the
291 bacteria protein expression is currently unknown.

292 4. There are several types of microcon devices based on molecular weight cut-offs. For
293 example, one can also use 30kD - YM-30.

294 5. This step for concentrating the samples using a vacuum concentrator requires several
295 hours, so using disposable ultrafiltration centrifugal devices can be an alternative.
296 However, a portion of the protein sample is lost during the process.

297 6. Readymade C18 for enrichement and desalting of peptides can be purchased from
298 several suppliers

299 7. The expected yield should not be less than 20 ug of peptides, and it can be checked
300 with different methods, such as PIERCE colorimetric peptide assay for concentration
301 determination.

302 8. MaxQuant is a quantitative proteomics software package designed for analysing large
303 mass-spectrometric data sets (to download https://www.maxquant.org/) and the user
304 gide is here http://coxdocs.org/doku.php?id=maxquant:start. Perseus is a companion
305 software and it's user guide is here (software documentation available here
306 http://coxdocs.org/doku.php?id=perseus:start)

307 9. Using strain-specific databases allows for accurate assignment of the proteins and
308 avoids cross-species identification between *F. nucleatum* and *P. gingivalis* proteins.

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