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Author

Zhang, Xi, Sadowski, Pawel, Punyadeera, Chamindie

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1 Evaluation of sample preparation methods for
2 label-free quantitative profiling of salivary
3 proteome

4 Xi Zhang¹, Pawel Sadowski², Chamindie Punyadeera^{1*}

5 1. Saliva and Liquid Biopsy Translational Research Team, Institute of Health and Biomedical
6 Innovation, School of Biomedical Science, Queensland University of Technology, Australia.

7 2. Central Analytical Research Facility, Queensland University of Technology, Australia

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9 *Correspondence:

10 Chamindie Punyadeera

11 Saliva and Liquid Biopsy Translational Research Team, The School of Biomedical Sciences, Institute of
12 Health and Biomedical Innovations, Queensland University of Technology, 60 Musk Avenue, GPO
13 Box 2434, Brisbane QLD 4001, Australia.

14

15

16 **Abstract**

17 Saliva has become one of the more attractive body fluids for protein biomarker discovery studies
18 because of its ease of collection, non-invasiveness and multiple samples can be collected from an
19 individual at a single time point. With the development of modern data acquisition strategies, such as
20 Sequential Window Acquisition of all Theoretical Mass Spectrometry (SWATH-MS), the quality of saliva
21 sample preparation has become a crucial factor for a successful protein identification and
22 quantification. Several sample preparation methods have been proposed, but there has been no
23 systematic evaluation conducted to date that compared each of these methods. We have therefore,
24 performed an extensive assessment using technical and biological repeats to evaluate the number of
25 protein IDs and repeatability of three most commonly used techniques, in-solution digestion, filter-
26 aided sample preparation (FASP) and in stage-tip (iST) digestion. We discovered that in the case of
27 human saliva sample, FASP provided the highest number of proteins (human and microbial)
28 identifiable from a pool saliva sample, and there were no significant differences in terms of
29 repeatability among the three methods investigated.

30

31 **Introduction**

32 Human saliva contains a wealth of proteins/peptides and as such is gaining attention as an alternative
33 diagnostics medium compared to more traditional methods of sampling, blood. Collecting saliva is
34 easy, non-invasive and safe for the collector [1-4]. The evidence for protein transport across salivary
35 acini cells and blood vessel endothelium cells, suggests that saliva can be used for monitoring systemic
36 events [1].

37 Data-independent acquisition (DIA) mass spectrometry (MS) approaches, such as Sequential Window
38 Acquisition of all Theoretical Mass Spectra (SWATH-MS) available on Sciex instruments, enable
39 unbiased biomarker discovery [5] and have been previously successfully integrated into basic and
40 clinical proteomic research [6-8]. However, being label-free, sample preparation is the key to a
41 successful quantitation. Various sample preparation methods have been utilized for salivary
42 proteomics studies in the past. These include a) in-solution digestion which utilises chemical and

43 thermal denaturation and trypsin digestion using organic or inorganic solvents [9], b) filter-aided
44 sample preparation (FASP) [10], that relies on filtration of detergent that would interfere with trypsin
45 digestion and c) in-StageTip digestion (iST), [11] in which all sample preparation steps are carried out
46 in a single, enclosed volume to minimize sample losses. However, no systematic evaluation of the
47 efficacy of each of these methods for saliva sample preparation has been made. The aim of this study
48 was to evaluate the performance of three most common sample processing techniques (FASP [10],
49 iST [11] and in-solution digestion [9]) prior to protein quantitation using label-free quantitative
50 proteomics profiling of human saliva.

51 **Materials and Methods**

52 The study design is summarised in Fig 1. Human saliva samples were collected from 12 healthy
53 volunteers using signed consent form approved by The University Human Research Ethics Committee
54 (approval number: 1400000617). Participants were asked to refrain from eating and drinking (except
55 for water) for two hours before saliva collection. Resting whole mouth saliva was collected as
56 described previously [2, 12-14]. In brief, participants were asked to sit in a comfortable position and
57 to rinse their mouths with water. They were asked to tilt their heads down, pool saliva in the mouth
58 for 1 minute and expectorate into a 50 mL Falcon tubes kept on ice. Saliva samples were transported
59 on ice, aliquoted into 1.5 mL micro centrifuge tubes and stored at -80 °C within 30 minutes until
60 analysis. Upon analysis, 12 saliva samples were randomly divided into three groups and combined
61 together to form three pooled saliva biological replicates. Total protein concentrations of pooled saliva
62 samples were quantified by Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific Inc, Waltham, MA,
63 USA) as per the manufacturer's protocol. In brief, 2 µL of the samples were added into 23 µL of 1x
64 phosphate buffered saline (PBS), mixed with 200 µL of working BCA reagent in a 96 wells microplate,
65 and incubated for 30 minutes at 37°C. The absorbance was read at 562nm on a plate reader and the
66 concentration was calculated against a standard curve ranging from 20- 2,000 µg/mL.

67 The three pooled saliva samples each containing 12.5 µg of total protein were then processed using
68 the methods listed below:

69 **1. Filter-Aided Sample Preparation (FASP) method**

70 Samples were mixed with 15 µL of SDS-Tris buffer (4% sodium dodecyl sulphate (SDS), 100mM Tris-
71 HCl pH 8.5, 100 mM Dithiothreitol (DTT)) and 200 µL of DTT-Urea buffer (25 mM DTT, 8M urea in 100
72 mM Tris-HCl pH 8.5) within a 30 kDa Microcon YM-30 centrifugal filter device (Millipore) and incubated
73 at RT for 60 minutes on agitator. The buffers were then washed away by centrifuging the filters at
74 14,000 g at 21°C for 15 minutes. The filters were washed with 200 µL Urea buffer (8M Urea in 100
75 mM Tris-HCl pH 8.5) by centrifuging the filters at 14,000 g at 21°C for 15 minutes. For cysteine
76 alkylation, 100 µL of 50 mM iodoacetamide (IAM) in Urea buffer was added to each sample and
77 incubated in the dark for 20 minutes. The filters were then washed with 200 µL Urea buffer (8M Urea
78 in 100 mM Tris-HCl pH 8.5) by centrifuging the filters at 14,000 g at 21°C for 15 minutes. Two additional
79 wash steps with 100 µL of 100 mM ammonium bicarbonate were performed before adding proteomics
80 grade trypsin (Sigma-Aldrich, St. Louis, MI, USA) at 1:50 enzyme to protein ratio and digesting at 37 °C
81 overnight. The digested peptides were collected into a clean 1.5 ml microcentrifuge tubes by
82 centrifugation at 14,000 g at 21 °C for 15 minutes. The samples were dried using a Speed Vac vacuum
83 concentrator and reconstituted in 10 µL of 2% ACN/0.1% trifluoroacetic acid (TFA) and transferred
84 into STAGE tips for sample clean up.

85 **2. In-StageTip (iST) method**

86 Samples were mixed with 5x their volume of buffer A consisting of 6M Guanidinium chloride, 10 mM
87 TCEP, 40 mM 2-Chloroacetamide, 10% ACN in 100 mM Tris pH 8.5 and incubated at 21°C for 20
88 minutes. To guanidinium chloride concentration down, the sample was diluted 10 x using 10% ACN in
89 100 mM Tris-HCl pH 8.5. Proteomics grade trypsin (Sigma-Aldrich) at 1:50 enzyme to protein ratio was
90 added and digested at 37 °C. After digestion was complete, 330 µL of 4% TFA was added to the sample

91 for acidification. Insoluble debris was pelleted by centrifuging at 21 °C at 16,000 g for 10 minutes and
92 the supernatant was transferred into STAGE tips for sample clean up.

93 **3. In-solution method**

94 Proteins in the samples were denatured and reduced by the addition of 100 µL of 1% SDS, 100 mM
95 Tris-HCl buffer pH 8.0 and 10 mM DTT, and incubation at 95 °C for 10 min. Cysteine in the samples
96 were then alkylated by addition of 30 mM IAM and incubating at RT for 30 min. Excess IAM was
97 quenched with the addition of DTT to a final concentration of 10 mM. Proteins were then precipitated
98 by addition of 800 µL of 1:1 methanol/acetone, incubation at -20 °C overnight and centrifugation at
99 18,000 × g for 10 min. The protein pellet was resuspended in 50 µL of 50 mM ammonium acetate and
100 digested with 0.5 µg of proteomics grade trypsin (Sigma-Aldrich) at 37 °C for 16 h. The samples were
101 dried using a concentrator and reconstituted in 10 µL of 2% ACN/0.1% TFA.

102 **Clean up**

103 All the samples from the above mentioned methods were subjected to clean up procedure using
104 STAGE tips with double SCX membrane (Empore, part no: 2251, 3M, Maplewood, MI, US) [15]. In short,
105 the STAGE tips were activated by passing through 30 µL of 100% ACN and equilibrated with 30 µL of
106 0.2% TFA. The acidified samples were loaded to the STAGE tips, followed by three subsequent washes
107 with 30 µL of 0.2% TFA. The samples were eluted with 30 µL of 5% Ammonium hydroxide/80% ACN.
108 The eluted peptides were dried using a concentrator and reconstituted with 10 µL 2% ACN/0.1%
109 formic acid and transferred into polypropylene autosampler vial (Thermo Scientific).

110 **DDA data acquisition and data processing**

111 Peptide spectral data from approximately 400 ng – 1 µg of injected tryptic peptides per sample were
112 generated using nanoLC-nanoESI-MS/MS instrument (TripleTOF® 5600+, SCIEX). Peptide spectral data
113 were generated using nanoLC-nanoESI-MS/MS on a TripleTOF® 5600+ instrument (SCIEX). Peptides

114 were separated by performing reversed-phase chromatography using an Eksigent ekspert™ nanoLC
115 400 System directly interfaced to the MS/MS instrument. The LC platform was set up in a trap and
116 elute configuration with a 10 mm × 0.3 mm trap cartridge packed with ChromXP C18CL 5 μm 120 Å
117 material and a 150 mm × 75 μm analytical column packed with ChromXP C18 3 μm 120 Å (Eksigent
118 Technologies, Dublin, CA). The mobile phase solvents were composed of mobile phase A: water/0.1%
119 FA; mobile phase B: ACN/0.1% FA; and mobile phase C: water/2% ACN/0.1% FA. Trapping was
120 performed in mobile phase C for 5 min at 5 uL/min. Separation involved mobile phases A and B
121 running at a conserved flowrate of 300 nL/min. and adjusting the proportions of solvents (98, 60, 35,
122 10, 10, 98 and 98 % of solvent A) at specified time-points (0, 30, 35, 40, 49, 50 and 60 min). To minimise
123 retention time drift, the analytical column was maintained at 40°C.

124 After chromatography, peptides were analysed by data-dependent acquisition (DDA). The DDA mode
125 of the instrument was set to obtain high resolution (30,000) TOF-MS scans over a range of 350-1350
126 m/z, followed by 40 high sensitivity MS/MS scans of the most abundant peptide ions per cycle over
127 the range of 100- 2000 m/z. The selection criteria for the peptide ions included intensity greater than
128 150 cps and charge state of 2-5. The dynamic exclusion duration was set at 9 s. Each survey (TOF-MS)
129 scan lasted 250 ms and the product ion (MS/MS) scan was acquired for 50 ms resulting in a total cycle
130 time of either 2.3 s. The ions were fragmented in the collision cell using rolling collision energy, and
131 collision energy spread (CES) was set to 5. An overlay of Total Ion Chromatograms are shown in Fig 2.

132 The collected peptide ion fragmentation spectra were stored in .wiff and .wiff.scan formats (SCIEX).
133 Peptide identification was performed by the Protein Pilot 5.0 software (SCIEX) using the Uni-
134 Prot/Swiss-Prot database with the following setting: Sample Type, identification; cysteine alkylation,
135 iodoacetamide or Iodoacetic acid; instrument, TripleTof 5600; Species, human; ID focus, Biological
136 modification; enzyme, trypsin; Search effort, thorough ID. We downloaded the LudwigNR database
137 (downloaded from <http://apcf.edu.au> as at 27 January 2012; 16 818 973 sequences; 5 891 363 821
138 residues) for human protein identification and the Human Oral Microbiome Database [16] for oral

139 microbial protein identification. Protein Pilot was used to perform false discovery rate (FDR) analysis
140 for all searches. The search result with a confidence interval of 80% -99%, including False Discovery
141 Rate (FDR) analysis were shown in Table 1. The lower limit of peptide identification confidence and
142 cut off of global FDR was set at 99% and 1% respectively for further analysis.

143 **SWATH-MS data acquisition and label-free quantitation**

144 The LC conditions were the same as for DDA acquisition. Mass spectrometry acquisition followed the
145 strategy described in Gillet *et al.* [17] with the following modifications. High-resolution TOF-MS scans
146 were collected over a range of m/z of 350–1500 for 0.08 s followed by high-sensitivity TOF-MS/MS
147 scans collected over a range of m/z 100-1800 on all peptide ions whose mass fell within a particular
148 SWATH window and for 0.08 s per window. There were 36 overlapping windows, each 26 m/z units
149 wide, that covered a range of m/z 350–1500 which resulted in a cycle time of 3s. Collision energy was
150 automatically assigned by the Analyst software (SCIEX) based on m/z window ranges Protein library
151 was generated with the DDA data as described above for the targeted data extraction and peptide
152 quantification.. The abundance of peptides was measured using PeakView Software with standard
153 settings essentially as described [18], summing the integrated areas of up to six fragment ions per
154 peptide. Protein abundance was measured using the sum of the abundances of two to six peptides per
155 protein. The accuracy of peak selection by PeakView was manually confirmed for proteins of interest
156 in each sample.

157 **Results**

158 **Proteome coverage**

159 A Venn diagram depicts the unique proteins identified per sample preparation method as well as the
160 impact of each sample preparation method on the human proteome coverage (Fig 3A). Grassi *et al*
161 reported significant number of microbial proteins in saliva [19]. Therefore, on a separate Venn

162 diagram we are reporting the number of oral microbial proteins after grouping proteins together from
163 each species for the three sample preparations used (Fig 3B).

164 **Label-free quantitation**

165 Next, we evaluated the reproducibility of label-free quantitation resulting from these three methods
166 by calculating the variability of the protein intensity across three pooled saliva samples. Comparing
167 the reproducibility of protein intensities generated by the three methods, there was no statistically
168 significant differences (as evaluated using repeated measures ANOVA, Fig 4)

169 **Discussion**

170 The total number of human proteins detected by FASP, iST and in-solution methods with 99%
171 confidence were 488, 230 and 130 respectively. The proteins covered by FASP, iST method and in
172 solution methods were 94%, 92% and 92% respectively. In addition, we determined FASP, iST and in
173 solution methods identified 206, 153 and 81 oral microbial protein with 99% confidence respectively.
174 With respect to protein identification, the results demonstrated that the FASP saliva sample
175 preparation method yielded a higher number of both human and microbial proteins. The
176 reproducibility of FASP protocol was also comparable to other methods, making it an ideal sample
177 processing method for protein biomarker discovery research using saliva samples. Strong detergent
178 like SDS is a powerful solution to solubilise proteins, but can negatively influence protein digestion
179 and mass-spectrometric measurement even in very small concentration. With the filter device used in
180 this method, SDS is efficiently removed and poses no threat to the downstream procedure and
181 analysis. FASP protocol also allows uniform representation of the samples' proteome, without the
182 need to separate it into insoluble and supernatant fractions. This potentially contributes to the larger
183 number of proteins detected in our samples. Even though the filter device used in the FASP method
184 could increase the total cost of the proteomics studies, the reliability and higher performance of the
185 FASP method still makes it an ideal method for processing saliva samples. Recently, plate-based FASP

186 protocol has been introduced [20] which has a potential to significantly reduce the cost of FASP
187 digestion. In conclusion, saliva samples processed using the FASP method provides a much higher
188 number of identifiable proteins compared to both iST method and in solution method. Both iST and
189 in solution methods only provide a small number of unique identifiable proteins compared to FASP
190 method. Our results suggest that FASP method of saliva sample processing is superior label-free
191 quantitative technique to profile the saliva proteome. Recently, new sample processing techniques
192 have been developed with the focus of efficient SDS removal for label free quantitative mass
193 spectrometry. Both Suspension trapping (S-trap) [21] and Single-Pot Solid -Phase-enhanced Sample
194 Preparation (SP3) [22] were developed and evaluated against FASP for their efficiency of sample
195 processing respectively [23, 24]. It was found that both of these techniques provide better proteomics
196 coverage and quantification consistency in comparison to FASP. However currently these techniques
197 have not been applied to saliva sample processing. Sequential window acquisition of all theoretical
198 fragment ion spectra mass spectrometry (SWATH-MS) is recently developed and a popular label-free
199 quantitative strategy that provides fast and reliable quantitative information of sample's proteome.
200 Due to its advantage over the traditional methods, SWATH-MS has attracted interest from the saliva
201 research community. The finding in this study are directly translatable to SWATH-based quantitation.

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205

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270

271 Figure legends

272 Figure 1 Schematic workflow for the experiment.

273 Figure 2 Overlay of Total Ion Current chromatograms generated for samples processed by (A) FASP,
274 (B) iST and (C) in solution method

275

276 Figure 3 Venn Diagram showing the overlap of proteins identified by FASP, iST and in solution
277 method in (A) human and (B) oral microbiome

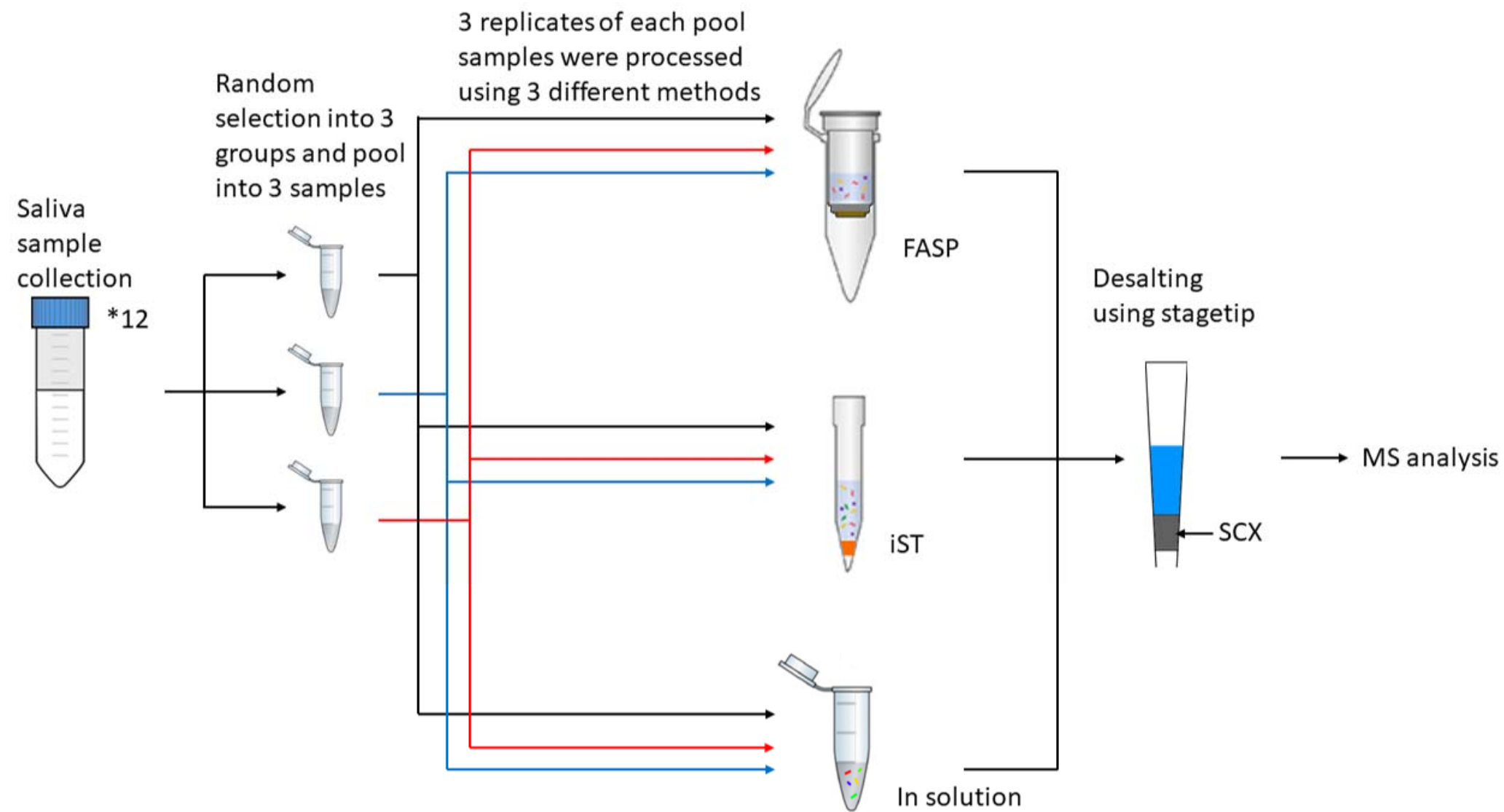
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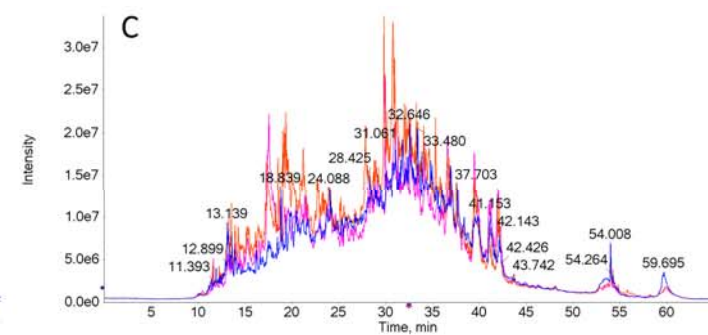
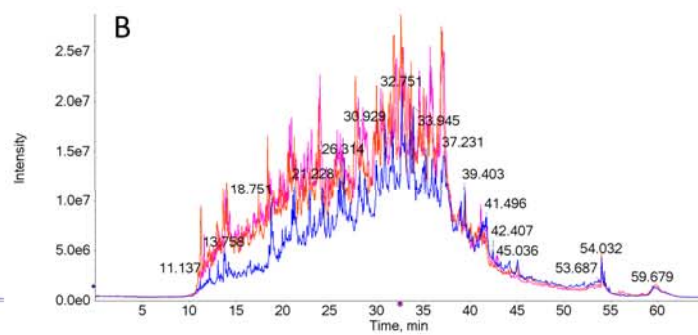
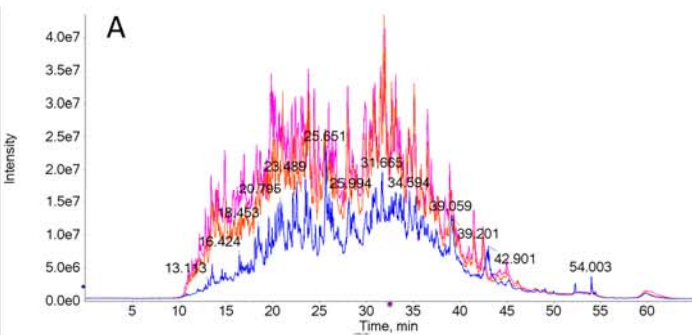
279 Figure 4 Percentage coefficient of variations of shared protein intensities between the three
280 different processing methods

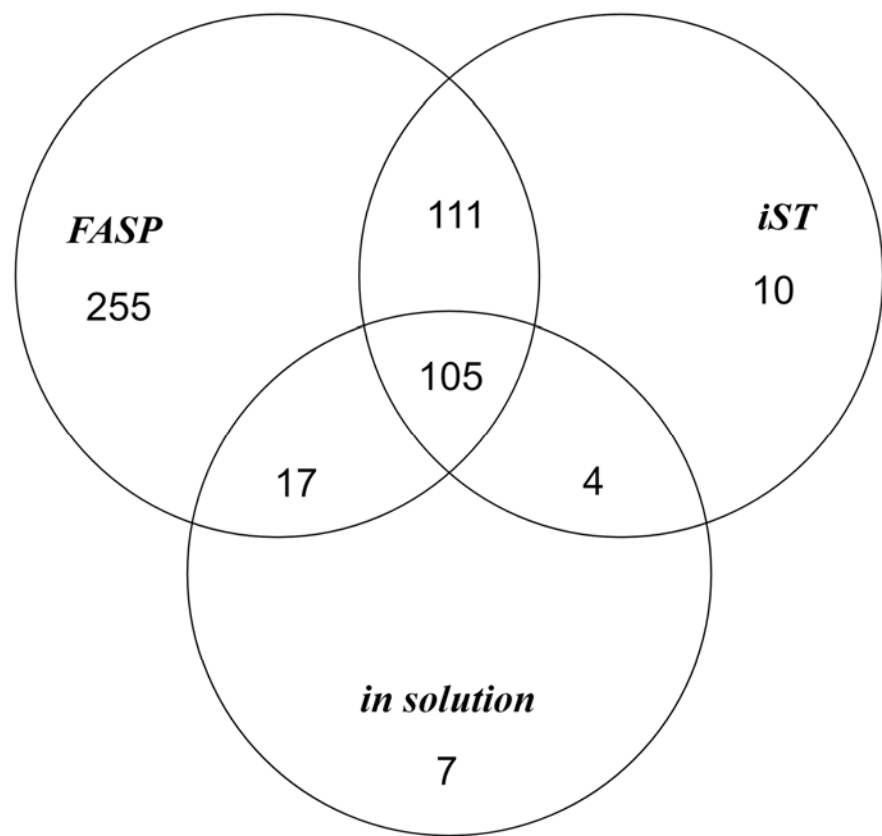
281

Table 1 Summary of the number of human Protein Identified using three methods

	FASP		iST		in solution	
Confidence	Protein ID		Protein ID		Protein ID	
> 99%	488		230		133	
> 95%	583		285		167	
> 90%	598		305		173	
> 80%	615		326		187	
FDR analysis	Protein	Peptide	Protein	Peptide	Protein	Peptide
1%	488	7538	219	4747	120	3253
5%	488	9312	230	5900	133	4032
10%	488	10560	230	6666	133	4572





A**B**