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Standardization of a protocol for shotgun proteomic analysis of saliva

Abstract

Saliva contains numerous proteins and peptides, each of them carries a number of biological functions that are very important in maintaining the oral cavity health and also yields information about both local and systemic diseases. Currently, proteomic analysis is the basis for large-scale identification of these proteins and discovery of new biomarkers for distinct diseases. Objective: This study compared methodologies to extract salivary proteins for proteomic analysis. Material and Methods: Saliva samples were collected from 10 healthy volunteers. In the first test, the necessity for using an albumin and IgG depletion column was evaluated, employing pooled samples from the 10 volunteers. In the second test, the analysis of the pooled samples was compared with individual analysis of one sample. Salivary proteins were extracted and processed for analysis by LC-ESI-MS/ MS. Results: In the first test, we identified only 35 proteins using the albumin and IgG depletion column, while we identified 248 proteins without using the column. In the second test, the pooled sample identified 212 proteins, such as carbonic anhydrase 6, cystatin isoforms, histatins 1 and 3, lysozyme C, mucin 7, protein S100A8 and S100A9, and statherin, while individual analysis identified 239 proteins, among which are carbonic anhydrase 6, cystatin isoforms, histatin 1 and 3, lactotransferrin, lyzozyme C, mucin 7, protein S100A8 and S100A9, serotransferrin, and statherin. Conclusions: The standardization of protocol for salivary proteomic analysis was satisfactory, since the identification detected typical salivary proteins, among others. The results indicate that using the column for depletion of albumin and IgG is not necessary and that performing individual analysis of saliva samples is possible.

Keywords: Methods. Proteomics. Standardization. Saliva.

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Introduction

Saliva is a biological fluid composed of more than 99% water and less than 1% protein, electrolytes and other low-molecular-weight components. It originates mainly from three pairs of major salivary glands (parotid, submandibular and sublingual glands), as well as from 300 to 400 minor salivary glands present in the oral cavity. Saliva plays a key role in lubrication, chewing, swallowing and digestion. It protects the oral tissues and also provides biomarkers for local and systemic diseases¹⁷. Therefore, saliva contains more than 2000 proteins and peptides that are involved in an infinity of different biological functions in the oral cavity¹⁷. Saliva still plays a large role in the formation of acquired pellicle, which begins only a few seconds after exposure of the enamel to saliva⁵.

Human saliva is a biological fluid with enormous diagnostic potential. Because saliva can be noninvasively collected, it provides an attractive alternative for blood, serum or plasma¹³.

In the human saliva were identified 1166 proteins, and high portions of these proteins were found in serum. Currently, progress in salivary diagnostics has demonstrated that these contents can be very informative for detection of oral and systematic diseases²⁰.

Proteomics, a new field of research centered on identification, quantitation, and characterization of proteins and their interplay, is largely based on the robustness, sensitivity, speed, and throughput of mass spectrometric procedures⁶. Currently, mass spectrometry is the basic technology for large-scale identification of these salivary proteins, and proteomic analysis of saliva has distinct advantages over blood, especially for proteins of low abundance^{17,18}. One of the main challenges in proteomic analysis is the fact that highly abundant proteins can impair the identification of low-abundance proteins, considering the equipment dynamic range. In the case of saliva, albumin and immunoglobulin G (IgG), they are very abundant, and some authors have recommended using columns for depletion of these proteins during the extraction procedure^{7,8}. Saliva functions are not only restricted to process food for digestion, considering that it contains a large number of proteins, which play important roles in the regulation of the immune defense and endocrine system and in the maintenance of mucosal tissue and dental health¹.

Saliva may contain locally expressed proteins and other substances called biomarkers, which can be used as diseases' indicators, be closely related to an individual's health condition and change greatly when diseases occur. In general, most studies view saliva wrongly as a homogeneous body fluid. It is also not stable, but constantly in change, and its composition is affected among other things by sampling methodology, environment, periodicity, oral hygiene, psychological status and general health^{6,13,20}.

Considering the importance of saliva in the oral cavity homeostasis, as well as its great potential as a diagnostic fluid, the aim of this study was to standardize a protocol to extract salivary proteins for further proteomic analysis. In the first test, we evaluated the need for using an albumin and IgG column to deplete these proteins during protein extraction. In the second test, we compared analysis of samples pooled from 10 volunteers with samples from individual analysis.

Material and methods

Ethical aspects and human subjects

The protocol of this study was submitted and approved by the Ethics Committee in Research with Human Beings of the Bauru School of Dentistry -FOB/USP (CAAE No. 61484116.0.0000.5417). Ten participants with good general and oral health took part of this study, which was based on previous *in vivo* studies¹⁸. Inclusion criteria were: nonsmokers with good general and oral health, stimulated salivary flow >1 mL/min and unstimulated salivary flow >0.25 mL/min, salivary pH>6.0.

Saliva collection

The volunteers were asked to rest for 15 min before collecting saliva, sitting upright. They were asked not to speak or eat before beginning to collect saliva. First, they rinsed their mouths with 5 mL of drinking deionized water, then they were asked to swallow saliva for 5 min. After this period, the volunteers spit out all the saliva accumulated in the mouth in a plastic tube immersed in ice for 10 min (unstimulated flow). The saliva samples were immediately centrifuged at 14,000 g for 15 min at 4°C to remove all debris, such as insoluble material, cell debris and food debris. The supernatant from each sample was collected and

frozen at -80°C until analysis. These procedures were based on previous studies 6,18 .

Preparation of the saliva samples

The experiments were performed into two phases. The first test was done to evaluate whether or not the albumin & IgG Depletion SpinTrap column (GE Healthcare[®], Buckinghamshire, UK) should be used. The second test was performed after the results of the first to compare analysis of salivary samples pooled from all the 10 volunteers with analysis of an individual sample from one selected volunteer.

For the first test, 100 μ l of saliva from each volunteer was taken and transferred to 10 new tubes. For the second test, 100 μ l of each saliva sample was also taken and transferred to 10 new tubes to constitute the pool, while 1 ml of saliva was taken from only one of the volunteers (randomly selected) for individual analysis.

Proteins from the saliva samples were extracted using an equal volume of a solution containing 6 M urea, 2 thiourea in 50 mM NH₄HCO₂ pH 7.8. The samples were vortexed at 4°C for 10 min, sonicated for 5 min and centrifuged at 14,000 g at 4°C for 10 min. This step was repeated once more. For the first test (with or without the use of the albumin and IgG depletion column), we added 100 µl of the extraction solution to each Eppendorf tube. For the second test (pool X individual analysis), we added 100 µl of the extraction solution in each Eppendorf tube (for the samples that will be pooled later on), while for the individual sample, we added 1 ml of the extraction solution. In all the cases, an equal volume of saliva sample and extraction solution was used. For the pooled samples, we placed the content of the 10 tubes in one tube after the extraction procedure, constituting the pool for further analysis.

After extraction, for the first test, the pooled sample was loaded into the albumin & IgG depletion columns, according to the manufacturer's instructions Albumin & IgG Depletion SpinTrap column (GE Healthcare[®], Buckinghamshire, UK). We did not use this column in the second test.

The samples were then concentrated to 150 µl in Falcon Amicon tubes (Merck Millipore®, Tullagreen, County Cork, Ireland). After concentration, the samples were reduced with 5 mM dithiothreitol (DTT) for 40 min at 37°C, alkylated with 10 mM iodoacetamide (IAA) for 30 min in the dark. After this procedure, we added 100

µl of 50 mM NH₄HCO₃, and the samples were digested with 2% (w/w) trypsin (Promega[®], Madison, USA) for 14 hours at 37°C. After this period, we added 10 µl of 5% formic acid to stop the trypsin reaction, then the samples were purified and desalted using the C18 Spin columns (Thermo Scientific[®], Rockford, Illinois, USA) and we withdrew a 1 ul aliquot of each sample from the tests for protein quantification by the Bradford method (Bio-Rad[®], Hercules, Califórnia, USA)¹⁶. We resuspended the samples in the solution containing 3% acetonitrile and 0.1% formic acid to be submitted to Nano Liquid Chromatography Electron Spray Ionization Tandem Mass Spectrometry - LC-ESI-MS/MS (Waters, Manchester, New Hampshire, UK).

Shotgun label-free quantitative proteomic analysis

Peptides identification was performed on a nanoACQUITY UPLC-Xevo QTof MS system (Waters, Manchester, New Hampshire, UK). The nanoACQUITY UPLC was equipped with nanoACQUITY HSS T3, analytical reverse phase column (75 µm X 150 mm, 1.8 µm particle size (Waters, Manchester, New Hampshire, UK). The column was equilibrated with mobile phase A (0.1% formic acid in water). Then, the peptides were separated with a linear gradient of 7-85% mobile phase B (0.1% formic acid in ACN) for 70 min at a flow rate of 0.35 μ L/min. The column temperature was maintained at 55°C. The Xevo G2 Q-TOF mass spectrometer was operated in positive nano-electrospray ion mode, and data were collected using the MSE method in elevated energy (19-45 V), which allows data acquisition of both precursor and fragment ions, in one injection. Source conditions used included capillary voltage, 2.5 kV; sample cone, 30 V; extraction cone, 5.0 V and source temperature, 80°C. Data acquisition occurred over 70 min, and the scan range was 50-2000 Da. The lockspray, used to ensure accuracy and reproducibility, was run with a [Glu1] fibrinopeptide solution (1 pmol/ μ L) at a flow rate of 1 μ L/min, as a reference ion in positive mode at m/z 785.8427. ProteinLynx Global Server (PLGS) version 3.0 was used to process and search the LC-MSE continuum data. Proteins were identified with the embedded ion accounting algorithm in the software and a search of the Homo sapiens database (reviewed only, UniProtKB/Swiss-Prot) downloaded on September 2015 from UniProtKB (http://www. uniprot.org/). The use of human database excludes the identification of bacterial proteins that could be

present in the saliva.

Results

In the first test, when the albumin and IgG depletion column was used, the total amount of protein recovered from the pooled samples after extraction was 8 μ g, while only 35 salivary proteins were identified. Among them are proteins typically found in saliva, such as alpha-amylase 1 and 2B,

cystatin isoforms, hemoglobin isoforms and mucin 7, among others (Table 1). When the depletion column was not used, the amount of protein recovered was much higher (48.0 μ g) and 248 proteins were identified, among them many typical components of saliva such as alpha-amylase 1 and 2B, many cystatin isoforms, carbonic anhydrase 6, lactotransferrin, lysozyme C, mucin 7, proline-rich protein 4, protein S100A9, serotransferrin, statherin, several hemoglobin isoforms, among others (Table 2).

In the second test, for comparison of analysis of pooled *versus* individual sample, the depletion column

Table 1- Salivary proteins identified when the albumin and IgG depletion column was used

Accession number	Protein name	score	Cover (%)
P04745	Alpha-amylase 1	7589.70	54.99
P19961	Alpha-amylase 2B	6833.20	47.75
P04280	Basic salivary proline-rich protein 1	488.14	43.88
P02812	Basic salivary proline-rich protein 2	3642.44	45.67
P49407	Beta-arrestin-1	158.66	9.09
P01036	Cystatin-S	1465.11	31.91
P09228	Cystatin-AS	516.59	24.11
P01037	Cystatin-SN	1378.19	21.28
Q9UGM3	Deleted in malignant brain tumors 1 protein	98.93	2.11
P14867	Gamma-aminobutyric acid receptor subunit alpha-1	92.53	7.46
G3V1N2	HCG1745306_ isoform CRA_a	456.20	22.73
P69905	Hemoglobin subunit alpha	1306.87	28.17
P68871	Hemoglobin subunit beta	1659.66	66.67
P02042	Hemoglobin subunit delta	497.84	25.17
A0A0G2JMB2	Ig alpha-2 chain C region (Fragment)	559.94	16.76
P01876	Immunoglobulin heavy constant alpha 1	912.82	30.59
P01877	Immunoglobulin heavy constant alpha 2	345.30	20.00
P01591	Immunoglobulin J chain	1363.63	36.48
P01834	Immunoglobulin kappa constant	333.71	51.40
P0CG04	Immunoglobulin lambda constant 1	136.40	14.15
P0DOY2	Immunoglobulin lambda constant 2	165.46	23.58
P0DOY3	Immunoglobulin lambda constant 3	153.74	23.58
P0CF74	Immunoglobulin lambda constant 6	136.40	14.15
B9A064	Immunoglobulin lambda-like polypeptide 5	136.40	7.01
P31025	Lipocalin-1	1181.01	26.70
Q8TAX7	Mucin-7	95.21	3.71
P04746	Pancreatic alpha-amylase	6723.99	41.49
P01833	Polymeric immunoglobulin receptor	305.15	15.58
P12273	Prolactin-inducible protein	1027.80	40.41
A0A0A0MT31	Proline-rich protein 4	8108.76	72.29
Q5VSP4	Putative lipocalin 1-like protein 1	958.48	6.79
P02810	Salivary acidic proline-rich phosphoprotein 1/2	8108.76	72.29
P02814	Submaxillary gland androgen-regulated protein 3B	2090.48	65.82
A0A087WZY1	Uncharacterized protein	7158.08	16.60
Q96DA0	Zymogen granule protein 16 homolog B	721.70	41.83

Table 2- Salivary	proteins iden	tified wher	n the albur	nin and Ig	gG depletion	column v	vas not	used

Accession number	Protein name	score	Cover (%)
Q15118	[Pyruvate dehydrogenase (acetyl-transferring)] kinase isozyme 1_ mitochondrial	89.50	8.26
P31946	14-3-3 protein beta/alpha	166.37	3.25
P62258	14-3-3 protein epsilon	177.85	3.14
Q04917	14-3-3 protein eta	166.37	3.25
P61981	14-3-3 protein gamma	166.37	3.24
P31947	14-3-3 protein sigma	166.37	3.23
P27348	14-3-3 protein theta	195.23	12.65
P63104	14-3-3 protein zeta/delta	166.37	3.27
Q6ZVK8	8-oxo-dGDP phosphatase NUDT18	138.11	19.50
E5KP25	A/G-specific adenine DNA glycosylase	242.24	5.28
P68032	Actin alpha cardiac muscle 1	10751.18	40.05
P68133	Actin alpha skeletal muscle	10681.87	33.95
P62736	Actin aortic smooth muscle	10396.48	37.14
P60709	Actin cytoplasmic 1	18715.02	66.67
P63261	Actin cytoplasmic 2	18715.02	66.67
P63267	Actin gamma-enteric smooth muscle	10327.17	31.12
Q6P461	Acyl-coenzyme A synthetase ACSM6 mitochondrial	399.16	13.33
Q9UIF7	Adenine DNA glycosylase	242.24	5.31
Q9Y6U3	Adseverin	51.66	5.17
C9JKR2	Albumin isoform CRA k	25004.47	77.94
P02763	Alpha-1-acid glycoprotein 1	259 49	7 46
P01009	Alpha-1-antitrypsin	114 17	14 59
P01023	Alpha-2-macroglobulin	195.37	14 25
P04745	Alpha-amylase 1	125762.3	77 69
P19961	Alpha-amylase 2B	85518 55	67.91
069YU3	Ankyrin repeat domain-containing protein 34A	213.80	23 19
Q5T3N1	Annexin (Fragment)	419.03	34 31
P04083	Annexin A1	454.28	33 53
P03973	Antileukonroteinase	822.96	40.15
O16671	Anti-Muellerian hormone type-2 recentor	646 30	18 32
P02647	Anolinoprotein A-I	436.68	32.58
B1APP8	ATP-dependent 6-phosphofructokinase platelet type	156 72	21.20
014965		187 17	8.93
P04280	Basic salivary proline-rich protein 1	13742 73	44 39
P02812	Basic salivary proline-rich protein 1	36329.24	69.23
06W/2.19	BCI -6 corepressor	171 50	2 34
P61769	Beta-2-microalobulin	7681.87	54 62
0562R1	Beta-actin-like protein 2	1631 58	17.02
096DR5	BPI fold-containing family A member 2	4054.46	40.56
	BPI fold-containing family R member 1	238 42	27.27
Q8N4E0	BPI fold-containing family B member 2	4941 71	32.97
	CA6 protein	236.85	1 17
D22280	Carbonic anhydraso 6	1027.33	4.47
P23200	Cathonic annyulase 0	153.05	45.05
	Cathepsin D	152.05	12.32
A0A087Y2R6	Call evels and apoptosis regulator protoin 2	196.22	12.52
060308	Centrosomal protein of 104 kDa	36.50	3 35
000000	Centrosomal protein of 152 kDa	24.18	5.03
034300	Clustered mitochondria protein homolog	24.10	0.03
D35606	Costomer subunit beta'	186.05	9.93 6.73
C3\/1A4		613.65	18 70
03V 1A4		612.65	16.79
P23320	Colled coil alpha balical rad protain 1	47.65	10.07
	Colled coll domain containing protein 167	170 22	2.40
Q9P0B0	Complement C2	170.32	15.40
PU1024	Complement company reporter 1 like protein	101.90	9.32
Q2VPA4	Complement component receptor 1-like protein	148.59	1.21
P04080		3144.06	55.10
P01034	Cystatin-C	1547.12	31.51

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P20032 Oystatin-S 47.09 P01036 Cystatin-SA 21107.61 53.90 P01037 Cystatin-SN 40764.24 68.09 P54108 Cystatin-erich secretory protein 3 371.45 26.94 Q9UGM3 Deleted in malignant brain tumors 1 protein 274.04 6.80 Q9UGM3 Deleted in malignant brain tumors 1 protein 274.04 6.80 Q9NVU0 DNA-directed RNA polymerase III subunit RPC5 187.74 4.66 Q1HG43 Dual oxidase maturation factor 1 248.89 13.12 Q95714 E3 ubiquitin-protein ligase HERC2 190.34 5.05 Q8NC27 E3 ubiquitin-protein ligase Praja-1 680.83 14.31 P43897 Elongator complex protein 6 63.64 13.53 Q9HW75 Epididymis secretory protein Li 109 337.33 22.86 Q0PRE2 Elongator complex protein 6 63.64 13.53 P02675 Fibrinogen bata chain 420.77 40.73 P02679 Fibrinogen gamma chain 453.82 22.52 Q
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P02679 Fibrinogen gamma chain 453.82 22.52 Q0PRL4 Forkhead box P2 variant 3 142.49 10.19 Q8N6B5 Forkhead box P2_isoform CRA_d (Fragment) 142.49 11.84 O15409 Forkhead box protein P2 199.65 12.45 O95872 G patch domain and ankyrin repeat-containing protein 1 268.32 17.70 P19526 Galactoside 2-alpha-L-fucosyltransferase 1 174.70 13.42 P48058 Glutamate receptor 4 50.22 2.55 P04406 Glyceraldehyde-3-phosphate dehydrogenase 190.90 16.72 P00738 Haptoglobin 349.21 24.88 G3V1N2 HCG1745306_isoform CRA_a 22783.57 58.18 P69905 Hemoglobin subunit alpha 27452.86 59.15 P68871 Hemoglobin subunit beta 49667.26 95.24 P02042 Hemoglobin subunit delta 9498.60 33.33 P02100 Hemoglobin subunit depsilon 1940.46 6.80
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P02100 Hemoglobin subunit epsilon 1940.46 6.80
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P69891 Hemoglobin subunit gamma-1 1940.46 6.80
P69892 Hemoglobin subunit gamma-2 1940.46 6.80
P02790 Hemopexin 460.96 22.51
P15515 Histatin-1 32092.25 36.84
P15516 Histatin-3 7558.25 13.73
P57058 Hormonally up-regulated neu tumor-associated kinase 218.10 3.50
Q9BS19 HPX protein 352.10 21.65
A0A0G2JMB2 Ig alpha-2 chain C region (Fragment) 22147.53 68.24
A0A0A0MS07 Ig gamma-1 chain C region (Fragment) 1490.66 45.76
A0A087WYJ9 Ig mu chain C region 2129.91 40.71
P04220 Ig mu heavy chain disease protein 1800.88 31.97
P01876 Immunoglobulin heavy constant alpha 1 25196 43 61 19
P01877 Immunoglobulin heavy constant alpha 2 18459.82 64.12
P01857 Immunoglobulin heavy constant gamma 1 3671 28 50.91
P01850 Immunoglobulin heavy constant gamma 2 729 35 38 34
P01960 Immunoglobulin heavy constant gamma 3 720.00 30.04 P01960 Immunoglobulin heavy constant gamma 3 487.81 24.03
P01000 Infinitulogiobulin heavy constant gamma 4
P01001 Infinitulogiobulin neavy constant gaining 4 599.47 20.16
P010/1 Infinitulogiobulin neavy constant mu 21/1.72 47.00
AUAU/5B/FU Immunoglobulin neavy variable 3/OR16-10 (non-functional) (Fragment) 378.41 9.48
S4R460 Immunoglobulin neavy variable 3/OR16-9 (non-functional) 5403.28 31.25
P01/62 Immunoglobulin heavy variable 3-11 3/8.41 9.40
P01766 Immunoglobulin heavy variable 3-13 378.41 9.48
AUAUC4DH32 Immunoglobulin heavy variable 3-20 (Fragment) 378.41 9.40
A0A0B4J1V1 Immunoglobulin heavy variable 3-21 378.41 9.40
A0A0B4J1X8Immunoglobulin heavy variable 3-43378.419.32
P01763 Immunoglobulin heavy variable 3-48 378.41 9.40
P01780 Immunoglobulin heavy variable 3-7 401.30 17.09
P01782 Immunoglobulin heavy variable 3-9 378.41 9.32
P01591 Immunoglobulin J chain 18415.28 42.14
P01834 Immunoglobulin kappa constant 16816.83 85.98
P0CG04 Immunoglobulin lambda constant 1 9338.45 77.36

P0DOY2	Immunoglobulin lambda constant 2	13921.14	77.36
P0DOY3	Immunoglobulin lambda constant 3	13921.14	77.36
P0CF74	Immunoglobulin lambda constant 6	13267.04	50.94
A0M8Q6	Immunoglobulin lambda constant 7	10499.89	36.79
B9A064	Immunoglobulin lambda-like polypeptide 5	9338.45	38.32
P08069	Insulin-like growth factor 1 receptor	32.75	5.63
P06870	Kallikrein-1	227.71	10.31
Q9Y5K2	Kallikrein-4	304.56	17.72
P13645	Keratin_type I cytoskeletal 10	297.80	2.05
Q99456	Keratin_type I cytoskeletal 12	421.18	14.17
P13646	Keratin_type I cytoskeletal 13	4810.33	46.94
P02533	Keratin_type I cytoskeletal 14	158.42	4.24
P19012	Keratin_ type I cytoskeletal 15	1164.86	14.25
P08779	Keratin_ type I cytoskeletal 16	158.42	4.23
Q04695	Keratin_ type I cytoskeletal 17	143.47	2.08
P08727	Keratin_ type I cytoskeletal 19	529.84	6.75
P35908	Keratin_ type II cytoskeletal 2 epidermal	300.25	22.07
Q01546	Keratin_ type II cytoskeletal 2 oral	165.14	12.07
P19013	Keratin_ type II cytoskeletal 4	876.71	42.13
P13647	Keratin_ type II cytoskeletal 5	489.99	7.97
P02538	Keratin_type II cytoskeletal 6A	794.78	31.56
P04259	Keratin_type II cytoskeletal 6B	765.88	28.01
P48668	Keratin_type II cytoskeletal 6C	765.88	28.01
O95678	Keratin_ type II cytoskeletal 75	190.38	3.81
Q5XKE5	Keratin_ type II cytoskeletal 79	190.38	3.93
014777	Kinetochore protein NDC80 homolog	410.89	9.03
P22079	Lactoperoxidase	1724.32	34.13
P02788	Lactotransferrin	382.65	32.11
Q9C099	Leucine-rich repeat and coiled-coil domain-containing protein 1	270.77	9.98
Q9NPC1	Leukotriene B4 receptor 2	209.15	4.37
P31025	Lipocalin-1	19334.38	57.95
P28330	Long-chain specific acyl-CoA dehydrogenase_ mitochondrial	137.44	9.07
Q8IYD9	Lung adenoma susceptibility protein 2	141.09	9.14
P61626	Lysozyme C	10190.75	70.27
Q14680	Maternal embryonic leucine zipper kinase	208.24	8.14
P42679	Megakaryocyte-associated tyrosine-protein kinase	156.39	10.85
P01033	Metalloproteinase inhibitor 1	858.61	44.44
Q2QL34	Mpv17-like protein	240.73	11.73
Q8TAX7	Mucin-7	11686.20	15.65
Q8NCY6	Myb/SANT-like DNA-binding domain-containing protein 4	176.81	11.30
P24158	Myeloblastin	175.85	4.69
Q8NCE2	Myotubularin-related protein 14	342.16	19.38
Q9NYA4	Myotubularin-related protein 4	234.57	15.82
F8WCT3	NEDD8-conjugating enzyme UBE2F	167.98	37.18
P59665	Neutrophil defensin 1	1037.46	17.02
P59666	Neutrophil defensin 3	1037.46	17.02
O00221	NF-kappa-B inhibitor epsilon	176.16	6.80
Q2L696	Nucb2 splice variant	337.33	24.62
Q14980	Nuclear mitotic apparatus protein 1	278.08	4.96
Q9Y618	Nuclear receptor corepressor 2	44.62	3.33
A0A087WSV8	Nucleobindin 2_ isoform CRA_b	337.33	22.86
P80303	Nucleobindin-2	337.33	22.86
075414	Nucleoside diphosphate kinase 6	140.72	14.52
C9JQB1	Nucleoside diphosphate kinase	140.72	19.15
Q9GZK3	Olfactory receptor 2B2	166.49	19.33
Q5SZR7	Ornithine decarboxylase antizyme 3	300.95	18.55
Q7RTY7	Ovochymase-1	190.59	10.05
P04746	Pancreatic alpha-amylase	79860.79	59.10
P13796	Plastin-2	364.90	18.02

P13797	Plastin-3	259.13	4.29
P01833	Polymeric immunoalobulin receptor	10715.77	41.62
Q6S8J3	POTE ankyrin domain family member E	7556.27	11.07
A5A3E0	POTE ankvrin domain family member F	7557.11	13.67
P0CG38	POTE ankyrin domain family member I	6915 24	6 79
P0CG39	POTE ankyrin domain family member J	2868 60	5.97
P51531	Probable global transcription activator SNE2L2	158 85	2 01
Q53EL6	Programmed cell death protein 4	138 40	8 74
P12273	Projectin_inducible protein	31682 10	76 71
016378	Proline-rich protein 4	312.60	21.64
	Pronionyl-CoA carboyylase alpha chain _mitochondrial (Fragment)	231 31	20.90
P07602	Prosanosin	205.84	9.35
D6RD72	Protein FAM103B (Fragment)	266.86	35 56
01/320	Protein FAM500	176 55	10.62
Q14320	Protein FAM78B	1/1.80	10.02
	Protein CV/OW/1	164.01	17.05
060592	Protein EVQW1	1162.24	20.00
	Protein LEGT homolog	270.50	29.09
	Protein Low 14 homolog A	270.30	3.00
QOWILS OFTUKI		322.12	3.91
	Protein PRR 14L	507.74	10.13
P00702	Protein Stindly	37 1.03	39.47
Q90EA4	Protein Spinory	130.75	2.04
	Puratrophin-i	100.93	2.00
	Putative bela-acim-like protein 3	1002.92	10.07
	Putative ripocalin 1-like protein 1	3906.17	11.11
A8K554	Putative protein ZNF815	163.67	20.15
	Pyndoxal phosphate phosphatase	92.62	11.15
H3BR70	Pyruvate kinase	336.60	18.03
P14618	Pyruvate kinase PKM	336.60	12.43
Q15276	Rab G Pase-binding effector protein 1	349.01	8.24
H3BPI9	Receptor protein serine/threonine kinase (Fragment)	641.71	47.67
P02810	Salivary acidic proline-rich phosphoprotein 1/2	40463.03	26.51
Q14074	Separini	32.00	4.39
		105.21	3.07
1201.04	Serine/Infectine-protein kinase	105.21	3.03
	Serine/threenine-protein kinase RiO3 (Fragment)	335.03	00.00
G3V5U6		107.01	24.55
P02787		0031.00	44.41
PU2768	Serum albumin	05771.02	81.28
P40763	Signal transducer and activator of transcription 3	43.20	6.10
	Small proline-rich protein 3	424.01	0.07
AIL4HI	Soluble scavenger receptor cysteine-rich domain-containing protein SSC5D	62.30	2.07
P02808	Statterin Schwarzeillen den den eine einer der stein OD	52769.28	53.23
P02814	Submaxiliary gland androgen-regulated protein 3B	52053.05	65.82
Q9UMS6	Synaptopodin-2	184.00	1.83
G5E9B5	I CF3 (E2A) fusion partner (in childhood Leukemia)_ isoform CRA_b	165.61	19.67
Q8777014	Teteri domain-containing protein 2	188.69	14.08
Q7Z6L1	Teteren entre 40	350.11	12.62
QYUKR8		313.97	27.35
P20061		230.38	20.32
A6H8Y1	Transcription factor TETIB component B" nomolog	167.29	2.82
095359	Transforming acidic colled-coll-containing protein 2	372.27	6.41
P00072	Transitional endoplasmic reticulum ATPase	236.03	10.92
	Tripuelootide report containing cone 64 protein	133.40	13.80
	Timucieolide repeat-containing gene 6A protein	180.44	3.98
	i yrosine-protein kinase	150.39	10.87
	Uncharacterized aar+ domain-containing protein kinase 1	1/4./6	10.57
	Uncharacterized protein (Fragment)	240.13	19.49
AUAUO/WZK3		409.24	43.09

A0A087WZY1	Uncharacterized protein	40463.03	16.60
A0A087WUV0	Uncharacterized protein	464.85	8.62
E7ESA3	Uncharacterized protein	188.69	18.87
Q9HB07	UPF0160 protein MYG1_ mitochondrial	435.46	12.23
Q9NY84	Vascular non-inflammatory molecule 3	540.71	10.58
Q14508	WAP four-disulfide core domain protein 2	1637.99	33.87
E9PDB0	WD repeat-containing protein 49	424.40	5.02
Q86UP3	Zinc finger homeobox protein 4	205.12	3.06
Q5FWF6	Zinc finger protein 789	138.52	9.41
Q17R98	Zinc finger protein 827	296.41	2.87
P25311	Zinc-alpha-2-glycoprotein	5026.17	55.03
Q96DA0	Zymogen granule protein 16 homolog B	47333.93	56.73

was not used. For the pooled sample, the amount of protein recovered after extraction was 54.02 μ g, which allowed the identification of 212 proteins, including alpha-amylase 1 and 2B, carbonic anhydrase 6, cystatin isoforms (B, C, D, S, SA, SN), histatin 1 and 3, lysozyme C, mucin 7, protein S100A8 and S100A9, statherin, several hemoglobin isoforms, among others (Table 3). In the analysis of the individual sample, 25.13 µg of total protein were obtained and 239 proteins were identified, among which are alphaamylase 1 and 2B, alpha-enolase, carbonic anhydrase 6, many cystatin isoforms (B, C-D, S, SA, SN), histatin 1 and 3, Ig alpha-2 chain C region, Ig a chain C region, lactotransferrin, lysozyme C, mucin 7, protein S1008 and S100A9, serotransferrin, statherin, among other proteins (Table 4).

Discussion

This study aimed at standardizing a protocol for proteomic analysis of saliva that is sensitive, easy to perform and of low cost, to be used in future experiments involving quantitative shotgun proteomics. The first issue to be solved was related to the necessity of depletion of highly abundant proteins in saliva, such as albumin and IgG^{8,14} that could mask and make difficult the identification of low abundance biomarkers. Krief and collaborators7 (2011) evaluated whether depletion of salivary amylase, albumin and IgGs could improve the ability to visualize proteins in two-dimensional gel electrophoresis (2-DE) in oral fluids. They observed 36 new spots after depletion, and 58 spots showed more than twofold increase intensity after depletion⁷. Therefore, we hypothesized that this better identification profile could occur not only in two-dimensional gel electrophoresis (2-DE), but also in shotgun proteomics, when albumin and IgG

were depleted. Thus, in the first test, we compared the use or not of the albumin and IgG depletion column after the extraction process of the salivary proteins. For this, we used a pool of ten saliva samples. When the column was used, only 35 proteins were identified (Table 1). This figure increased to 248 when the column was not used (Table 2). We believe this occurred because, when using the albumin and IgG depletion column, there was also depletion of other proteins, since using the column increases one more process in the methodology. We also believe that many proteins could bind to albumin and IgGs, thus being depleted together. Among the identified proteins, in both situations, are those typically found in saliva. By contrast, when the depletion column was used, classical salivary proteins such as 14-3-3 proteins, histatins, statherin, lactoperoxidase, lactotransferrin, lysozyme C, neutrophil defensins, protein S100A9, serotransferrin and some cystatin isoforms were not identified. Thus, contrary to what was observed in gel-based proteomics⁷, in shotgun proteomics the use of albumin and IgG depletion column impaired protein identification according to our workflow. Some studies, in spite of that, report advantages in using depletion columns when more than one workflow is employed¹⁴. However, this increases the time and cost of the analysis.

In the second test, we compared analysis of pooled samples (from ten individuals) *versus* individual analysis, without using the depletion column. In the individual analysis 239 proteins were identified (Table 4), while 212 proteins were identified in the pooled sample (Table 3). One-hundred and twenty three proteins were common to both groups (data not shown), and among them are most of the proteins typically found in saliva. The proteins exclusively found in the individual sample or in the pooled sample are not typically reported in saliva, which might be related to

Table 3- Proteins of the saliva identified in the pool analysis

Accession number	Protein name	score	Cover(%)
P16885	1-phosphatidylinositol 4 5-bisphosphate phosphodiesterase gamma-2	314.78	4.51
P68032	Actin_alpha cardiac muscle 1	6085.31	31.30
P68133	Actin alpha skeletal muscle	6085.31	31.30
P62736	Actin aortic smooth muscle	4676.94	28.38
P60709	Actin cytoplasmic 1	17496	67.20
P63261	Actin cytoplasmic 2	17496	67.20
P63267	Actin gamma-enteric smooth muscle	4676.94	28.46
Q01518	Adenvlvl cvclase-associated protein 1	440.27	26.11
C9JKR2	Albumin isoform CRA k	26466.72	74.82
P01009	Alpha-1-antitrypsin	2252.60	22.97
P01023	Alpha-2-macroglobulin	665.70	22.86
P04745	Alpha-amylase 1	153591 90	78 86
P19961	Alpha-amylase 2B	110753 50	58 51
P06733	Alpha-enolase	1637 76	33.87
001484	Ankyrin-2	52.62	2 75
P03973	Antileukonroteinase	701 53	28.03
P63010	AP-2 complex subunit beta	338.39	2.35
P02647	Anolinoprotein A-I	612 31	39.70
P02652	Anolinoprotein A-II	886.78	69.00
05EVB0		389.18	10.35
	ATP-dependent RNA belicase SLIP\/3 1 mitochondrial	235.17	6.23
Q01100	Basic salivary proline-rich protein 1	3025 20	58.67
P02812	Basic salivary proline-rich protein 7	73554.07	60.47
D61760	Basic salivary proline-nen protein 2	3725 17	49.74
O562P1	Beta actin like protoin 2	1532.83	40.74
Q302K1	Beta-actin-like protein 2	264 78	13.30
C06DP5	BPI fold containing family A member 2	204.70	F8 23
	BPI fold containing family R member 2	4501.10	20.20
	BPC 01 A complex suburit Abravas (Fragment)	332 77	16.03
	CA6 protoin	410.28	10.95
Q0IN404	Cancer/testis antigen 2	716 30	4.47
073030		15702.21	62.01
P23200		71.04	02.01
	Chromosomo 11 opon reading frame 58	258.60	15 20
E9F10192	Complement C3	230.09	15.29
D51160	Complement Co	232.14	11.07
		502.14	22.11
		502.10	22.11
002772	Cuplin dependent kingge like 2	457.07	24.95
Q92112		407.97	45.00
P04000	Cystalli-B	2200.27	43.92
P01034	Cystalin-C	3131.00	51.37 61.07
P20323		3340.32	01.97
P01030	Cystalli-S	34000.00	13.70
P09220	Cystalli-SA	24277.09	70.01
P01037	Cysteine rich accretery protein 2	23133.23	21.62
P04100	Cysteme-non secretory protein 5	204.30	21.03
P32320	Cylidine deaminase	1240.00	4.07
Q90GW3		300.82	4.97
	Deoxynbonuciease gamma	411.37	15.74
AUAUAUMI 168	Disperticule ase	411.37	10.07
P2/48/	Dipeptidayi peptidase 4	73.31	4.83
060216 D40N00	Double-strand-break repair protein rad21 nomolog	322.95	19.02
	Dual-specificity mitogen-activated protein kinase kinase 4	/80.16	97.56
V9HW75	Epialaymis secretory protein Li 109	954.67	25.48
B1AK53	Espin	277.28	4.80
Q01469	Fatty acid-binding protein_epidermal	4/5.76	30.37
Q8NCQ5	F-box only protein 15	465.73	3.73
P02679	Fibrinogen gamma chain	372.17	21.63

Q08380	Galectin-3-binding protein	237.96	18.97
P06744	Glucose-6-phosphate isomerase	222.14	22.04
E7ETY7	Glutathione peroxidase	341.42	22.78
P09211	Glutathione S-transferase P	519.29	25.71
P04406	Glyceraldehyde-3-phosphate dehydrogenase	407 39	11 64
08IW.12	GRIP and coiled-coil domain-containing protein 2	718 24	4 81
P00738	Hantoglobin	960.32	41.87
G3V1N2	HCG1745306 isoform CRA a	11936.33	57 27
P60005	Hemoglobin subunit alpha	13598.42	54.93
P68871	Hemoglobin subunit apna	18402 54	89.80
P02042	Hemoglobin subunit delta	5838.80	63.05
P02100	Hemoglobin subunit ensilon	3895.00	6.80
P60801	Homoglobin subunit appma 1	3805.00	6.80
D60802	Homoglobin subunit gamma 2	3805.00	6.80
P 09092		16204 54	26.94
P 10010	Histotin 2	2621.50	12 72
016605	Historia 12.4t	2031.00	13.73
Q10095		524.06	23.55
Q05469	Hormone-sensitive lipase	43.08	5.30
	Hydrocephalus-Inducing protein homolog	15.21	1.93
AUAUG2JMB2	ig alpha-2 chain C region (Fragment)	43004.29	79.12
A0A0A0MS07	Ig gamma-1 chain C region (Fragment)	2528.80	42.37
A0A08/WYJ9	Ig mu chain C region	4012.85	48.67
P04220	Ig mu heavy chain disease protein	3190.64	37.85
P01876	Immunoglobulin heavy constant alpha 1	38140.46	73.65
P01877	Immunoglobulin heavy constant alpha 2	32255.84	65.29
P01857	Immunoglobulin heavy constant gamma 1	4336.06	47.88
P01859	Immunoglobulin heavy constant gamma 2	1181.17	37.42
P01860	Immunoglobulin heavy constant gamma 3	1276.14	14.59
P01861	Immunoglobulin heavy constant gamma 4	1489.84	38.23
P01871	Immunoglobulin heavy constant mu	4017.99	50.33
A0A075B7F0	Immunoglobulin heavy variable 3/OR16-10 (non-functional) (Fragment)	299.80	9.48
A0A075B7B8	Immunoglobulin heavy variable 3/OR16-12 (non-functional) (Fragment)	242.49	9.40
A0A075B7E8	Immunoglobulin heavy variable 3/OR16-13 (non-functional) (Fragment)	242.49	9.40
S4R460	Immunoglobulin heavy variable 3/OR16-9 (non-functional)	5489.71	31.25
P01762	Immunoglobulin heavy variable 3-11	299.80	9.40
P01766	Immunoglobulin heavy variable 3-13	299.80	9.48
A0A0C4DH32	Immunoglobulin heavy variable 3-20 (Fragment)	299.80	9.40
A0A0B4J1V1	Immunoglobulin heavy variable 3-21	299.80	9.40
P01764	Immunoglobulin heavy variable 3-23	242.49	12.82
P01768	Immunoglobulin heavy variable 3-30	242.49	31.62
P01772	Immunoglobulin heavy variable 3-33	242.49	31.62
A0A0B4J1X8	Immunoglobulin heavy variable 3-43	299.80	9.32
P01763	Immunoglobulin heavy variable 3-48	299.80	9.40
P01767	Immunoglobulin heavy variable 3-53	242.49	12.93
A0A0C4DH42	Immunoglobulin heavy variable 3-66	242.49	12.93
P01780	Immunoglobulin heavy variable 3-7	299.80	9.40
A0A0B4J1X5	Immunoglobulin heavy variable 3-74	242.49	9.40
P01782	Immunoglobulin heavy variable 3-9	299.80	9.32
P01591	Immunoglobulin J chain	20006.96	49.06
P01834	Immunoglobulin kappa constant	28856.88	82.24
A0A0C4DH90	Immunoglobulin kappa variable 3/OR2-268 (non-functional) (Fragment)	362.90	7.76
P04433	Immunoglobulin kappa variable 3-11	1198.54	26.09
P01624	Immunoglobulin kappa variable 3-15	362.90	7.83
A0A075B6H7	Immunoglobulin kappa variable 3-7 (non-functional) (Fragment)	362.90	7.76
A0A0A0MR78	Immunoglobulin kappa variable 3D-11	1198 54	26.09
A0A0C4DH55	Immunoglobulin kappa variable 3D-7	362.90	7.56
P06312	Immunoglobulin kappa variable 4-1	250 98	19.83
P0CG04	Immunoglobulin lambda constant 1	40610 55	77.36
	Immunoglobulin lambda constant ?	4471/ 51	93.40
	Immunoglobulin lambda constant 2	14.51	03.40
100013		77/14.31	33.40

P0CF74	Immunoglobulin lambda constant 6	23147.62	50.94
A0M8Q6	Immunoglobulin lambda constant 7	19435.36	36.79
P01715	Immunoglobulin lambda variable 3-1	344.58	38.26
B9A064	Immunoglobulin lambda-like polypeptide 5	40610.55	38.32
Q9BQU0	Inhibitory NK receptor	242.62	11.21
Q9NVH2	Integrator complex subunit 7	267.39	4.26
Q01638	Interleukin-1 receptor-like 1	304.24	7.01
H0YNL8	Iron-responsive element-binding protein 2	377.91	29.09
A0A0G2JPA6	Killer cell immunoglobulin-like receptor 3DL2	242.62	11.64
P22079	Lactoperoxidase	2259.91	35.11
P02788	Lactotransferrin	862.74	28.59
A6NMS7	Leucine-rich repeat-containing protein 37A	263.12	1.71
A6NM11	Leucine-rich repeat-containing protein 37A2	252.18	1.71
O60309	Leucine-rich repeat-containing protein 37A3	276.06	4.53
P31025	Lipocalin-1	14925.97	53.98
Q86W92	Liprin-beta-1	292.75	10.29
P00338	L-lactate dehydrogenase A chain	196.57	21.69
Q9BY66	Lysine-specific demethylase 5D	307.10	8.58
P61626	Lysozyme C	15283.53	66.89
P14174	Macrophage migration inhibitory factor	616.56	47.83
C9JF79	Malate dehvdrogenase (Fragment)	263.72	11.71
P40925	Malate dehydrogenase cytoplasmic	653.55	11.38
Q5HYA8	Meckelin	241.84	1.61
Q9Y4B5	Microtubule cross-linking factor 1	26.23	1.52
Q8TAX7	Mucin-7	13700.40	9.28
U3KPS2	Mveloblastin	554.69	17.67
P24158	Mveloblastin	631.43	28.52
Q9NYA4	Myotubularin-related protein 4	315 44	7 11
P59665	Neutrophil defensin 1	1789.52	25.53
P59666	Neutrophil defensin 3	1789 52	25.53
O9BYH8	NE-kappa-B inhibitor zeta	371 15	4 32
021 696	Nuch2 splice variant	663.95	25.13
A0A087WSV8	Nucleobindin 2 isoform CRA b	954 67	25.48
P80303	Nucleohindin-2	954.67	25.48
P04746	Pancreatic alpha-amylase	88276 59	55.97
P42338	Phosphatidylinositol 4, 5-bisphosphate 3-kinase catalytic subunit beta isoform	561 79	6.26
	Phosphainositide phosphalipase C	313.90	4 55
P13796	Plaetin_2	283.93	25.04
086717	Podonlanin	866.94	34.57
D110/0	Polyadenylate-binding protein 1	582 59	10.69
F 11340	Polyadenylate-binding protein	582.59	11.26
	Polyhomootic like protein 3	348 42	3.05
	Polymoria immunoglabulin recenter	12701.02	5.05
	Polymenc immunogiobulini receptor Polymenc immunogiobulini receptor Polymenc immunogiobulini receptor	32.40	310
065912	Polyhoonucleolide hucleolidylitansierase 1_ mitochondhar	JZ.49 4110 47	12.06
Q030J3	POTE ankyin domain family member E	4110.47	13.00
ADAJEU DOCC29	POTE ankyrin domain family member F	4040.70	11.72
PUCG30	POTE ankyrin domain family member I	3413.22	4.74
PUCG39	POTE ankynn domain fanniy member J	2790.00	3.65
	Probable glutathone peroxidase 8	341.42	17.22
	Probable sodium-coupled neutral amino acid transporter 6	426.92	6.80
K/EJ44	Proliin	4/0./8	37.50
PU//3/	Prolection inducible protein	910.82	49.29
P122/3	Prolactin-inducible protein	30448.27	/6./1
AUAUAUMT31	Proline-rich protein 4	23475.68	72.29
P07602	Prosaposin	510.46	39.12
Q5W0V3	Protein FAM160B1	862.81	23.66
Q6P5S2	Protein LEG1 homolog	6592.09	36.97
Q9H7Z3	Protein NRDE2 homolog	45.79	1.20
P05109	Protein S100-A8	3184.30	23.66

P06702	Protein S100-A9	1737.55	77.19
O14795	Protein unc-13 homolog B	59.05	1.19
H3BQ60	Puratrophin-1 (Fragment)	266.79	50.00
Q9BYX7	Putative beta-actin-like protein 3	2063.16	10.67
Q5VSP4	Putative lipocalin 1-like protein 1	3097.31	11.11
P52566	Rho GDP-dissociation inhibitor 2	1026.87	30.35
P35913	Rod cGMP-specific 3'_5'-cyclic phosphodiesterase subunit beta	374.14	8.08
P02810	Salivary acidic proline-rich phosphoprotein 1/2	4566.91	72.29
P02787	Serotransferrin	4566.92	48.42
P02768	Serum albumin	63281.61	75.04
O00193	Small acidic protein	258.69	13.11
P02808	Statherin	41653.6	48.39
P02814	Submaxillary gland androgen-regulated protein 3B	20898.6	65.82
Q9UH99	SUN domain-containing protein 2	70.82	1.67
A0A075B6V5	T cell receptor alpha variable 36/delta variable 7 (Fragment)	278.89	24.78
Q7Z6L1	Tectonin beta-propeller repeat-containing protein 1	384.23	7.12
F2Z350	Testis-expressed protein 29	447.37	32.14
Q7Z4L5	Tetratricopeptide repeat protein 21B	78.57	4.56
P20061	Transcobalamin-1	378.51	22.86
P29401	Transketolase	676.10	30.98
Q6ZMR5	Transmembrane protease serine 11A	281.15	11.16
P02766	Transthyretin	438.46	44.22
P60174	Triosephosphate isomerase	651.56	36.36
O43818	U3 small nucleolar RNA-interacting protein 2	297.84	16.00
A0A0J9YY99	Uncharacterized protein (Fragment)	242.49	12.82
H7C2Y3	Uncharacterized protein C2orf80 (Fragment)	318.87	16.41
H0Y8H3	Uncharacterized protein C3orf67 (Fragment)	590.54	74.68
A0A087WZY1	Uncharacterized protein	22581.8	16.60
A0A0G2JMZ2	Uncharacterized protein	252.18	1.71
A0A0G2JRT3	Uncharacterized protein	252.18	1.77
P02774	Vitamin D-binding protein	245.21	21.52
Q14508	WAP four-disulfide core domain protein 2	935.99	33.87
Q9UDV6	Zinc finger protein 212	424.39	16.97
P25311	Zinc-alpha-2-glycoprotein	2292.60	31.54
Q96DA0	Zymogen granule protein 16 homolog B	46355.09	58.17

individual variation. The analysis of individual samples is important to allow confident comparison among the groups under study, especially in quantitative shotgun proteomics.

Continued from previous page

Generally, the methodologies used in proteomics are classified into two main categories: the bottom-up, which is also called shotgun proteomics, or top-down proteomics. Both methodologies have advantages and limitations, and their employment depends on the treatment given to the sample⁹. Shotgun proteomics is characterized by analyzing samples after proteolytic digestion in peptides, which is typically performed with trypsin^{2,9}, while the top-down proteome of a sample involves analysis of intact proteins⁹. In shotgun proteomics, proteins from a complex mixture are digested, and the resulting peptides are analyzed by mass spectrometry. One of the advantages of this strategy is to investigate a large number of proteins regardless of their size. The limitations are related to incomplete coverage of the protein sequence, loss

of post-translational modifications and degradation because of proteolytic digestion^{4,9}. The top-down proteomics differs from the shotgun as it explores intact proteins by injecting the proteins into the mass spectrometer without performing digestion, minimizing any change in the sample and allowing a better characterization of post-translational modifications, especially those related to naturally occurring cleavages and alternative splicing³, avoiding interference problems based on peptides and allowing deducing the primary structure of the protein^{4,9}. However, this technique is considered bounded by the collision energy required in protein fragmentation, which is insufficient for proteins greater than 50 KDa, and its application is restricted to the analysis of purified proteins^{4,9,11}. In addition, top-down proteomics method requires the use of one or more forms of separation prior to mass spectrometry analysis¹². Moreover, top-down platforms are intrinsically limited by the sample treatments required for use in mass

Table 4- Proteins of the saliva identified in only in the individual analysis

Accession number	Protein name	score	Cover(%)
P31947	14-3-3 protein siama	297.17	24.60
O00231	26S proteasome non-ATPase regulatory subunit 11	453.07	10.66
P68032	Actin alpha cardiac muscle 1	7799.84	26.53
P68133	Actin alpha skeletal muscle	7799.84	26.53
P62736	Actin aortic smooth muscle	7555.95	23.61
P60709	Actin cytoplasmic 1	17763 84	65.60
P63261	Actin cytoplasmic 2	17763 84	65.60
P63267	Actin gamma-enteric smooth muscle	7555.95	23.67
00/D77	ADAMTS-like protein 5	410.00	32.06
P00813		350.67	12.67
060503		471 53	5.69
000000	A-kinase anchor protein 9	34 16	3.58
COIKES		20220 48	74.82
D01000		413.67	11.24
P01009	Alpha 2 maaradabulin	415.07	15.24
P01023	Alpha 2 maaradabulin lika protain 1	445.71	10.33
A0K2UU	Alpha-2-macrogrobulin-like protein 1	146.31	10.32
P04745	Alpha-amylase T	97076.24	70.00
P19961	Alpha-amylase 2B	77429.32	62.82
P06733	Alpha-enolase	1439.59	49.08
Q8N6M6	Aminopeptidase O	261.58	10.13
Q01484	Ankyrin-2	39.24	4.22
P02652	Apolipoprotein A-II	941.64	47.00
Q14562	ATP-dependent RNA helicase DHX8	365.21	7.38
Q8IYB8	ATP-dependent RNA helicase SUPV3L1_ mitochondrial	331.22	7.00
P04280	Basic salivary proline-rich protein 1	8867.97	44.39
P02812	Basic salivary proline-rich protein 2	54196.77	69.71
I3L192	Basigin (Fragment)	185.70	16.88
P61769	Beta-2-microglobulin	2754.07	54.62
Q562R1	Beta-actin-like protein 2	1943.05	10.90
P13929	Beta-enolase	131.58	7.60
O95342	Bile salt export pump	495.58	8.18
Q96DR5	BPI fold-containing family A member 2	6426.16	43.37
Q8N4F0	BPI fold-containing family B member 2	6613.00	37.99
Q9NQY0	Bridging integrator 3	398.03	11.46
Q8N4G4	CA6 protein	294.75	4.47
O75808	Calpain-15	215.66	3.68
P23280	Carbonic anhydrase 6	9824.04	57.47
Q0P665	CCDC88C protein	188.41	0.00
Q8N163	Cell cycle and apoptosis regulator protein 2	573.49	11.05
O14647	Chromodomain-helicase-DNA-binding protein 2	250.16	2.84
H0Y7A8	Chromosome 9 open reading frame 3 (Fragment)	236.18	19.31
P35606	Coatomer subunit beta'	189.71	2.21
A2ABG0	Complement C2 (Fragment)	409.38	20.25
P01024	Complement C3	526.68	24.53
Q53SF7	Cordon-bleu protein-like 1	168.78	4.32
P04080	Cvstatin-B	1041 42	70.41
P01034	Cystatin-C	3437 76	51.37
P28325	Cystatin-D	2141 16	75.35
P01036	Cystatin-S	28189.63	76.60
P09228	Cystatin-SA	13641 19	67.38
P01037	Cystatin-SN	28293 31	70.21
P54108	Cysteine-rich secretory protein 3	373 11	34.20
08NE50	Dedicator of cytokinesis protein 8	351 74	5 72
	Delated in malignant brain tumore 1 protoin	295.05	7.05
OFTRUE	Delete in malignant prain tumors i protein	200.90	7.00
	Dinyuroxyacetone priospriate acylitaristerase (Fragment)	102.70 260.07	23.42
F 20340	DNA polymerase ueita catalylic suburilt	209.07	5.15
	Dive polyIIIelase	209.07	5.03
Q51457	E3 ubiquitin-protein ligase UBR4	22.83	2.70

002828	Estaduantasin A	059.64	15.96
Q92838		258.64	15.80
Q8N3D4	EH domain-binding protein 1-like protein 1	260.45	4.33
Q6P179	Endoplasmic reticulum aminopeptidase 2	522.88	7.92
Q7L775	EPM2A-interacting protein 1	277.97	2.80
Q9H501	ESF1 homolog	205.30	12.22
A0A1B0GUN9	Espin	59.79	6.02
Q8IXL6	Extracellular serine/threonine protein kinase FAM20C	322.36	5.48
Q01469	Fatty acid-binding protein_ epidermal	444.20	32.59
Q9BZK7	F-box-like/WD repeat-containing protein TBL1XR1	376.57	15.18
P02675	Fibrinogen beta chain	187.44	13.03
P15328	Folate receptor alpha	400.38	35.80
Q8NHY3	GAS2-like protein 2	287.31	6.14
P06396	Gelsolin	427.99	17.77
014893	Gem-associated protein 2	443 14	31.07
P53611	Geranylgeranyl transferase type-2 subunit beta	470.85	16.92
P06744	Glucose-6-nhosnhate isomerase	787.26	28.40
P04406	Glucose-o-phosphate isomerase	703.86	20.49
C05427	CDL athenalamina phaenhata transformer 1	793.00	39.40
095427	GPI ethanolamine phosphale transferase 1	200.92	1.13
Q8IVVJZ	GRIP and colled-coll domain-containing protein 2	22.31	1.25
P00738	Haptoglobin	1233.11	55.42
P00739	Haptoglobin-related protein	281.28	15.52
G3V1N2	HCG1745306_ isoform CRA_a	15851.36	94.55
E7BWR8	HCG2043595_ isoform CRA_a	252.74	7.76
P69905	Hemoglobin subunit alpha	16443.62	83.80
P68871	Hemoglobin subunit beta	22740.65	95.24
P02042	Hemoglobin subunit delta	5150.58	39.46
P02100	Hemoglobin subunit epsilon	2097.61	6.80
P69891	Hemoglobin subunit gamma-1	2097.61	6.80
P69892	Hemoglobin subunit gamma-2	2097.61	6.80
P15515	Histatin-1	5208.41	36.84
P15516	Histatin-3	4795.66	13.73
E9PRF4	Histone-lysine N-methyltransferase (Fragment)	316.72	3.89
Q15047	Histone-lysine N-methyltransferase SETDB1	316 72	3 80
P47902	Homeobox protein CDX-1	196.38	26.04
P31270	Homeobox protein Hox-A11	264.91	14 38
P09630	Homeobox protein Hox-C6	03.47	14.00
046083	Hudroconhalus inducing protoin homolog	264 63	2.46
	la alaba 2 abain C region (Fragment)	49202.07	2.40
AUAUGZJIVIBZ	la semine 4 sheire Creation (Fragment)	40303.27	19.12
	Ig gamma- i chain C region (Fragment)	3209.86	45.70
AUAU87WYJ9	Ig mu chain C region	3019.36	54.87
P04220	Ig mu heavy chain disease protein	2170.36	39.90
P01876	Immunoglobulin heavy constant alpha 1	40927.72	84.42
P01877	Immunoglobulin heavy constant alpha 2	28394.92	68.53
P01857	Immunoglobulin heavy constant gamma 1	5891.82	50.91
P01859	Immunoglobulin heavy constant gamma 2	1360.10	31.29
P01860	Immunoglobulin heavy constant gamma 3	1756.61	30.24
P01861	Immunoglobulin heavy constant gamma 4	1509.92	30.89
P01871	Immunoglobulin heavy constant mu	3019.36	54.75
A0A075B7D0	Immunoglobulin heavy variable 1/OR15-1 (non-functional) (Fragment)	252.28	10.26
A0A075B7F0	Immunoglobulin heavy variable 3/OR16-10 (non-functional) (Fragment)	3426.81	13.79
S4R460	Immunoglobulin heavy variable 3/OR16-9 (non-functional)	8502.51	36.46
P01762	Immunoglobulin heavy variable 3-11	3426.81	23.08
P01766	Immunoglobulin heavy variable 3-13	3426.81	13.79
A0A0C4DH32	Immunoglobulin heavy variable 3-20 (Fragment)	3426.81	13.68
A0A0B4J1V1	Immunoglobulin heavy variable 3-21	3426.81	23.08
A0A0B4.11X8	Immunoglobulin heavy variable 3-43	3426 81	13 56
P01763	Immunoglobulin heavy variable 3-48	3426.81	23.08
P01780	Immunoglobulin heavy variable 3-7	3426.81	23.00
D01782		3426.01	13 56
FU1/02	mmunoyiobulin neavy valiable 3-9	3420.01	13.30

P01591 Immungolobulin kappa constant 2120.25 85.85 P01334 Immungolobulin kappa variable 3-11 1303.46 26.09 P01519 Immungolobulin kappa variable 3-01 48.06 77.6 P04512 Immungolobulin kappa variable 3-01 423.92 198.31 P0512 Immungolobulin kappa variable 3-01 4057.07 77.36 P00CV3 Immungolobulin kappa variable 3-01 4057.07 77.36 P0DV3 Immungolobulin kappa variable 3-01 301.47 0.04 0.03 P0DV3 Immungolobulin kappa variable 3-02 301.90 77.36 P0DV3 Immungolobulin kappa variable 3-02 301.91 77.36 P0DV3 Immungolobulin kappa variable 3-02 301.91 77.36 P0DV3 Immungolobulin kappa variable 3-02 301.47.40 0.93.21 P0DV3 Immungolobulin kappa variable 3-02 301.91 77.36 P0DV3 Immungolobulin kappa variable 3-02 301.91 77.36 P0402 Kalikarin-1 92.02 10.31 4.33 P0452 <td< th=""><th>A0A0B4J1U7</th><th>Immunoglobulin heavy variable 6-1</th><th>294.24</th><th>5.79</th></td<>	A0A0B4J1U7	Immunoglobulin heavy variable 6-1	294.24	5.79
P01834 Immunoglobulin kappa centant 37052.12 85.86 P04433 Immunoglobulin kappa variable 3-10 1303.46 26.09 P01512 Immunoglobulin kappa variable 3-11 1303.46 26.09 P06512 Immunoglobulin kappa variable 3-1 1303.46 26.09 P0C504 Immunoglobulin lambda constant 1 3301.09 77.36 P0DOY2 Immunoglobulin lambda constant 3 4067.407 77.36 P0DOY3 Immunoglobulin lambda constant 7 2255.77 35.79 P0DC74 Immunoglobulin lambda constant 7 195.27 35.79 P0BA064 Immunoglobulin lambda constant 7 195.27 35.79 P0BA072 Killer cell immunoglobulin-like receptor 2D51 252.74 7.76 P0A302 Killer cell immunoglobulin-like receptor 2D51 252.74 7.76 P0A402 Kinesin-like protein KIF9 158.59 4.43 P0278 Lactotarsferrin 106.99 35.21 P04042 Kinesin-like protein KIF9 358.32 20.78 P04780 Lareletet derydrogenase A-chian	P01591	Immunoglobulin J chain	21280.25	68.55
P04433 Immunoglobulin kappa variable 3-20 866.06 7.76 ADAAAMRZ8 Immunoglobulin kappa variable 3-20 866.06 7.76 ADAAAMRZ8 Immunoglobulin kappa variable 3-11 130.34 26.09 P0512 Immunoglobulin kappa variable 3-11 33310.90 7.736 P0DCY3 Immunoglobulin lambda constant 1 40674.07 7.736 P0DCY3 Immunoglobulin lambda constant 3 40674.07 7.736 P0DCY4 Immunoglobulin lambda constant 7 22557.57 36.79 B8A064 Immunoglobulin lambda constant 7 22557.57 16.62 OP6670 Kallikerin-1 196.20 10.31 P43826 Killer cell immunoglobulin-like receptor 2D.1 225.75 16.62 OP4473 Killer cell immunoglobulin-like receptor 2D.1 25.75 16.62 OP44743 Killer cell immunoglobulin-like receptor 2D.1 25.55 13.33 P02785 Lactotransferm 1069.99 35.21 OP4784 Killer cell immunoglobulin-like receptor 2D.1 25.65 13.33 P02785 Lacto	P01834	Immunoglobulin kappa constant	37053.21	85.98
PD1619 Immunoplobulin kappa variable 3D-11 980.60 7.76 ADAAAM/R28 Immunoplobulin kappa variable 3D-11 303.46 26.09 PDC504 Immunoplobulin kappa variable 4-1 423.92 19.83 PDC504 Immunoplobulin ambda constant 1 3301.09 7.36 PDDV73 Immunoplobulin ambda constant 3 4067.407 7.73 PDC574 Immunoplobulin ambda constant 6 30147.40 60.94 ADM806 Immunoplobulin lambda constant 7 2255.75 8.79 PD6774 Immunoplobulin lambda kepy perglide 5 33910.9 38.32 PD8070 Kalikrein-1 196.20 10.31 PD4322 Kiler cell immunoplobulin-like receptor 2D51 25.74 7.76 ADAG23/INIG Kiler cell immunoplobulin-like receptor 2D51 25.75 14.33 PD2279 Lactoperoxidase 255.71 7.86 PD22079 Lactoperoxidase 25.55 19.31 PD413 KiR2D1 25.20 7.78 PD2207 Lactoperoxidase 25.56 19.31 <	P04433	Immunoglobulin kappa variable 3-11	1303.48	26.09
ADAGAMRZB Immunoglobulin kappa variable 30-11 103.44 26.69 P06312 Immunoglobulin lambda constant 1 3310.09 77.36 P00CV2 Immunoglobulin lambda constant 2 40674.07 77.36 P0DCV3 Immunoglobulin lambda constant 3 40674.07 77.36 P0CF74 Immunoglobulin lambda constant 6 30147.40 50.44 P0CF74 Immunoglobulin lambda constant 7 22557.57 36.79 B8A064 Immunoglobulin lambda constant 7 2257.4 77.66 P06870 Kallikern-1 196.20 10.31 P48382 Killer cell immunoglobulin-like receptor 2D.1 225.74 77.6 P04205 Knessin-like protein KIP9 185.69 4.43 B402K5 Kinessin-like protein 1 133.37 10.51 P0279 Lactoparoxidase 197.03 41.43 P0278 Lactoparoxidase 197.13 52.21 P0278 Lactoparoxidase 193.16 52.0 P04705 La-velated protein 1 196.65 20.78 P	P01619	Immunoglobulin kappa variable 3-20	868.06	7.76
P08512 Immunoglobulin kappa variable 4-1 423.92 19.33 P0C504 Immunoglobulin kappa variable 4-1 3391.09 77.36 P0DOY2 Immunoglobulin lambda constant 2 40674.07 77.36 P0DOY3 Immunoglobulin lambda constant 3 40674.07 77.36 P0DOY3 Immunoglobulin lambda constant 7 22557.7 36.79 BAA064 Immunoglobulin lambda constant 7 22557.7 36.79 BAA064 Immunoglobulin lambda key polypetide 5 3391.09 33.32 P08570 Kallivein-1 196.20 10.31 P43828 Killer cell immunoglobulin-like receptor 2D51 255.76 16.62 Q0HA02 Kinesin-like protein KIF9 135.37 10.51 Q6H213 KIR2DL 1 252.75 13.33 10.51 Q6PK00 La-related protein 1 135.82 20.73 225.55 19.31 P0238 Lactofransferrin 1066.99 52.1 7.76 Q0BK06 Lavatofranse A chain 296.52 20.73 Q0BK722 Lactofrans	A0A0A0MR78	Immunoglobulin kappa variable 3D-11	1303 48	26.09
P0C304 Immunoglobulin lambda constant 1 33910.90 77.36 P0D0Y2 Immunoglobulin lambda constant 2 40674.07 77.36 P0DY4 Immunoglobulin lambda constant 3 40674.07 77.36 P0CF74 Immunoglobulin lambda constant 6 30147.40 50.94 P0CF74 Immunoglobulin lambda-like potypoptide 5 33310.90 38.32 P06870 Kallicerin-1 196.20 10.31 P43826 Killer cell immunoglobulin-like receptor 2DS1 325.76 16.62 Q0HAQ2 Kinesin-like protein 153.77 10.51 Q0H2X5 Kinesin-like protein 199.99 35.21 Q0H2X6 Kinesin-like protein 199.99 35.21 Q0H2X1 Lactoperoxidase 197.78 0.78 Q0H22 Liactoperoxidase 193.18 6.20 Q0H22 Liactoperoxidase 193.18 6.20 Q0H22 Liactoperoxidase 193.18 6.20 Q0H22 Liactoperoxidase 193.18 6.20 Q0H22 Liactoperoxidase	P06312	Immunoglobulin kappa variable 4-1	423.92	19.83
PDDOY2 Immunoglobulin lambda constant 2 40674.07 77.36 PODCY3 Immunoglobulin lambda constant 3 40674.07 77.36 PODCY4 Immunoglobulin lambda constant 6 30147.40 77.36 ADM806 Immunoglobulin lambda constant 7 2255.77 36.79 BA0064 Immunoglobulin lambda constant 7 2252.74 7.76 MAR02 Killer coll immunoglobulin-like receptor 2D.1 252.74 7.76 ADA022 Kinesni-like protein KIF9 158.59 4.43 BAD2K5 Kinesni-like protein KIF9 158.59 4.43 P0279 Lactoperoxidase 157.70.3 41.43 BAD2K5 Kinesni-like protein KIF9 158.59 4.14.3 P0278 Lactoperoxidase 157.70 41.43 P0278 Lactoperoxidase 159.78 0.78 P030	P0CG04	Immunoglobulin lambda constant 1	33910.90	77.36
PODOY3 Immunoglobulin lambda constant 3 40674.07 77.38 POCF74 Immunoglobulin lambda constant 6 301474.00 50.94 AOM806 Immunoglobulin lambda constant 7 22567.57 36.79 B8A064 Immunoglobulin lambda-like poppedide 5 33310.9 38.32 PO6870 Kallkrein-1 196.20 10.31 PA3826 Killer cell immunoglobulin-like receptor 2D.1 252.74 7.76 AOM052.NIA6 Kinesin-like protein 133.37 10.51 B4DZK5 Kinesin-like protein 133.37 10.51 GPH278 Lactoperoxidase 157.70.3 41.43 P02788 Lactoperoxidase 129.16 6.20 OGFK00 La-related protein 1 199.16 6.20 17.78 P09600 Leukotrien A-4 hydrolase 223.86 8.66 23.36 8.66 OBFX20 L-lactate dehydrogenase A chain 966.52 2.0.78 0.78 O1303 L-lactate dehydrogenase A like 6B 23.36 8.66 60 OBFX20		Immunoglobulin lambda constant 2	40674.07	77.36
POCF74 Immunoglobulin lambda constant 6 30147.40 80.94 ADMR06 Immunoglobulin lambda constant 7 2255757 36.79 BAV064 Immunoglobulin lambda constant 7 2255757 36.79 PO6870 Kalikrein-1 196.20 10.31 PO6870 Kalikrein-1 252.74 7.76 ADAG22NIV6 Killer cell immunoglobulin-like receptor 2DS1 325.76 166.20 Q0HAQ2 Kinesin-like protein 133.37 10.51 Q0H213 Kil22DL1 252.74 7.76 Q0H214 Kil22DL1 252.74 7.76 Q0H214 Kil22DL1 252.74 7.76 Q0H214 Kil22DL1 255.51 13.31 P0279 Lactotransferrin 109.99 35.21 Q0FK20 Lactotransferrin 109.91 36.6 6.20 P09800 Leukotriene A-4 hydrolase 25.55 19.31 13.4 P0333 L-lactate dehydrogenase A chain 966.52 20.78 7.76 Q08V52 Lipactate	P0DOY3	Immunoglobulin lambda constant 2	40674.07	77.36
ADM/8026 Immunoglobulin landba constant 7 22567.27 36.79 BAA064 Immunoglobulin landba constant 7 22567.27 36.79 BAA064 Immunoglobulin landba constant 7 199.20 33.32 PO6870 Kallikrein-1 199.20 10.31 PA3826 Killer cell immunoglobulin-like receptor 2DS1 225.74 7.76 ADA022,NIX Killer cell immunoglobulin-like receptor 2DS1 225.74 7.76 ADA022,Killericht KIF9 158.69 4.43 B4DZK5 Kinesin-like protein 282.74 7.76 OGH2H3 KiR2DL1 282.74 7.76 P2279 Lactoperoxidase 255.55 19.31 P31025 Lipocalin-1 199.16 6.20 P09860 Lysion-specific demethylase 5D 59.78 0.78 P14174 Macrophage milleration inhibitory factor 226.62 15.13 O80408 Matrix metalloproteinase-9 226.62 15.13 O81030 Metalloproteinase-9 226.62 15.13 O8060X Metalloprotein	P0CF74	Immunoglobulin lambda constant 6	30147.40	50.94
Name of the second of the polypolide 5 339109 38.32 PO6870 Kallikrein-1 196.20 10.31 PA3826 Killer cell immunoglobulin-like receptor 2DL1 282.74 7.76 PO6870 Killer cell immunoglobulin-like receptor 2DL1 323.70 34.32 Q0HAQ2 Kinesin-like protein KIF9 158.69 4.43 B4DZK5 Kinesin-like protein KIF9 158.69 4.43 P22079 Lactoperoxidase 1577.03 41.43 P22781 Lactotransferrin 139.16 6.20 P09860 Leukotriene A-4 hydrolase 225.55 19.31 P31025 Lipocalin-1 139.16 6.20 P00338 Liactate dehydrogenase A-tain 986.52 20.78 P01032 Liactate dehydrogenase A-tain 987.80 7.76 P02360 Lysice-specific demethydrase 5D 9.78 0.78 P14174 Macrophage migration inhibitory factor 928.56 54.05 P14780 Matrix metalloproteinase-9 226.62 15.13 Q080/38		Immunoglobulin lambda constant 7	22557 57	36.79
Dataset Dataset Dataset Dataset PO6870 Kalliver1 199.20 10.31 PA3262 Killer cell immunoglobulin-ike receptor 2DS1 325.76 7.76 AA0G2.JNL6 Killer cell immunoglobulin-ike receptor 2DS1 325.76 16.62 Q6HAD2 Kinesin-like protein 105.11 25.74 7.76 AA0G2.JNL6 Killer cell immunoglobulin-ike receptor 2DS1 325.76 16.51 Q6HAD3 Killer cell immunoglobulin-ike receptor 2DS1 25.74 7.76 Q6HKG3 Lactoperoxidase 157.03 41.43 P02788 Lactotransferrin 1069.99 35.21 Q6FKG0 La-related protein 1 198.60 20.78 Q6BV22 L-lactate dehydrogenase A chain 986.52 20.78 Q6BV22 L-lactate dehydrogenase A-kike 6B 323.86 8.66 Q69V66 Lysine-specific demethylase 5D 59.78 0.78 P1470 Matrix metalloproteinase-9 225.62 15.13 Q69LG8 Metaloproteinase-104 150.96 60.07	R94064	Immunoglobulin lambda constant 7	33010.0	38.32
10010 Namedia 10020 10331 PA3626 Killer cell immunoglobulin-like receptor ZDS1 325.76 16.62 09HA02 Kinesin-like protein 135.87 16.62 09HA02 Kinesin-like protein 135.87 10.51 06H2H3 KIR2DL1 252.74 7.76 022079 Lactoperoxidase 157.10 41.43 020788 Lactoperoxidase 157.10 41.43 0208402 Lactoransferrin 109.99 35.21 026PKC0 Larcetale drologenase A chain 9806.52 20.78 029806 Luekotnere A-4 hydrolase 323.86 8.66 0981V66 Lysice-specific dembrydragenase A-like 6B 323.86 8.66 0981V66 Lysice-specific dembrydragenase A-like 6B 328.86 54.05 P14174 Macrophage migration inhibitory factor 254.18 55.65 P14700 Matrix metalloproteinase-9 225.62 16.13 096303 Melanoma-associated antigen D4 190.96 6.07 P14740 Matrix	D0A004	Kallikrein-1	196 20	10.31
Houze Houze Gammunoglobulin-like receptor ZDS1 222-74 F.16 Q9HAO2 Kinesin-like protein KIF9 158.59 4.43 MADG2LNAC Kinesin-like protein KIF9 158.59 4.43 Q9HAO2 Kinesin-like protein KIF9 252.74 7.76 P22079 Lactoperoxidase 252.74 7.76 P20788 Lactoperoxidase 255.5 19.31 Q9FKG0 La-related protein 1 106.99 35.21 Q9PK22 L-lactate dehydrogenase A chain 966.52 20.78 Q9PY22 L-lactate dehydrogenase A chain 986.52 20.78 Q9PY22 L-lactate dehydrogenase A chain 928.56 54.05 P14174 Macrophage migration inhibitory factor 254.18 55.65 P14174 Macrophage migration inhibitory factor 254.18 55.65 Q96308 Methydropenase inhibitor 1 445.25 29.95 Q96436 Metale dehydrogenase inhibitor 1 445.25 29.44 Q95050 Metaleproteinase inhibitor 1 445.25 29.43	P43626	Killer cell immunoglobulin-like recentor 2DI 1	252.74	7 76
Name Calminologio Datis Social S	A0A0G2 IN 16	Killer cell immunoglobulin-like receptor 2DE1	325.74	16.62
Optimuz2 Interainting public INT 9 100.39 4-4-5 BdDZK5 Kinesim-like protein 133.37 10.51 Q6H2H3 KIR2DL1 252.74 7.76 Q2079 Lactoproxidase 1577.03 41.43 P02788 Lactotransferrin 1069.99 35.21 Q6FK60 La-related protein 1 139.16 6.20 P09990 Leukotrine A-4 hydrolase 225.55 19.31 P31025 Lipocalin-1 986.52 20.78 QBYZ2 L-lactate dehydrogenase A chain 986.52 20.78 QBYZ2 L-lactate dehydrogenase A chain 986.52 20.78 QBYZ2 L-lactate dehydrogenase A-like 6B 323.86 8.66 QBYZ2 L-lactate dehydrogenase A-like 6B 323.86 8.61.05 P14174 Macrophage migration inhibitory factor 225.62 15.13 Q66US8 Melainoma-associated antigen D4 160.90 6.07 P0133 Metalloproteinase inhibitor 1 445.25 29.95 Q69GX9 Methylithoribulose-1		Kinesin like protoin KIEQ	158 50	10.02
DH2LAG Ninesimine protein 133.31 10.31 DeH2AH3 KIRZDL1 25.274 7.76 P22079 Lactoperoxidase 166.09 35.21 OGPKG0 La-related protein 1 139.16 6.20 P09960 Leukotriene A-4 hydrolase 225.55 19.31 P01025 Lipocolin-1 8361.36 651.14 P003038 L-lactate dehydrogenase A-like 6B 323.86 8.66 OBPY66 Lysics-specific demethylase 5D 59.78 0.78 P01626 Lysozyme C 928.65 54.05 P14174 Macrophage migration inhibitory factor 225.62 15.13 OB90466 Melanoma-associated antigen D4 150.96 6.07 P114780 Matrix metalloproteinase-10hitorin in-protein kinase 4 168.04 3.43 OB5021 Microtubule-associated serine/threonine-protein kinase 4 168.04 3.43 O15021 Microtubule-associated serine/threonine-protein kinase kinase 13 533.35 8.70 O84283 Mitogin recepin 138.12.31 2.32		Kinesin-like protein Kines	122.27	4.45
Contaria NIXCUL 202.179 Lactoperoxidase 170 41.43 P02788 Lactotransferrin 1009.99 35.21 Q6PKG0 La-related protein 1 139.16 6.20 P09960 Leukotriene A-4 hydrolase 225.55 19.31 P31025 Lipocalin-1 8361.36 51.14 P00338 Liactate dehydrogenase A-taike 6B 323.86 8.66 O9BYC2 L-lactate dehydrogenase A-taike 6B 323.86 8.66 O9BYC6 Lysine-specific demethylase 5D 59.78 0.78 P14174 Macrophage migration inhibitory factor 254.18 55.65 P14174 Macrophage migration inhibitory factor 254.2 15.13 O9BJC6 Melanoma-associated antigen D4 109.96 6.07 P01033 Metalloproteinase inhibitor 1 445.25 29.95 Q96GX9 Methylthioribulose-1-phosphate dehydratase 198.99 23.14 Q15021 Multiple coagulation factor deficiency protein 2 260.43 32.397 Q75970 Multiple coagulation factor defici			133.37	7.76
P22079 Lactoperoutase 1577.03 4143 Q6FKG0 La-related protein 1 139.16 6.20 P09960 Leukotransferrin 830.13 651.14 P03025 Lipocalin-1 833.136 651.14 P03033 L-lactate dehydrogenase A-klike 6B 323.86 8.66 Q9BYZ2 L-lactate dehydrogenase A-klike 6B 323.86 8.66 Q9BYG2 L-lactate dehydrogenase A-like 6B 323.86 8.66 Q9BYG2 L-lactate dehydrogenase A-like 6B 323.86 8.66 Q9BYG2 L-lactate dehydrogenase A-like 6B 323.86 8.66 Q9BYG6 Lysin-especific demethylase 5D 59.78 0.78 P14174 Macrophage migration inhibitor factor 255.62 51.33 Q96JG8 Melanoma-associated antigen D4 150.96 6.07 P01033 Metalloproteinase-19 25.62 29.95 Q96GX9 Methylthioribulose-1-phosphate dehydratase 188.92 3.14 O15021 Microtubule-associated serine2/trepolien kinase kinase 13 533.35 8.70			252.74	1.10
PI2/783 Lactotransem 1009/997 53/21 QGPKGO La-related protein 1 139/16 6.20 P09960 Leukotriene A-4 hydrolase 225.55 19.31 P31025 Lipocalin-1 8361.36 51.14 P00338 L-lactate dehydrogenase A chain 896.52 20.78 Q9BYZ2 L-lactate dehydrogenase A chain 928.56 54.05 P14174 Macrophage migration inhibitory factor 928.56 54.05 P14174 Macrophage migration inhibitory factor 225.62 15.13 Q9GJG8 Melanoma-associated antigen D4 150.96 6.07 P11303 Metaloproteinase inhibitor 1 445.25 29.95 Q9GQS4 Methythioribulose-i-phosphate dehydratase 188.94 3.43 O45021 Microtubule-associated serine/threonine-protein kinase 4 168.04 3.43 Q43283 Mitogen-activated protein finase kinase 13 53.35 8.70 Q87N2 Multiple Coagulation factor deficiency protein 2 260.43 23.97 Q75970 Multiple Coagulation factor deficiency	P22079		1577.03	41.43
CorrAcu Larletated price 139.16 6.20 P099960 Leukotrien A.4 hydrolase 225.55 19.31 P31025 Lipocalin-1 8361.36 51.14 P00338 L-lactate dehydrogenase A-like 6B 323.86 8.66 Q98YZZ L-lactate dehydrogenase A-like 6B 928.56 54.05 P16126 Lysozyme C 928.56 54.05 P14174 Macrophage migration inhibitory factor 254.18 55.65 P14700 Matrix metalloproteinase-9 225.62 15.13 Q96JG8 Melanoma-associated anligen D4 150.96 6.07 P01033 Metalloproteinase inhibitor 1 445.25 29.95 Q96GX9 Methylthioribulose-1-phosphate dehydratase 198.99 23.14 O15021 Microtbule-associated senner/threonine-protein kinase 4 168.04 3.43 Q43283 Mitogen-activated protein kinase Kinase 13 533.35 8.70 Q81AX7 Mucin-7 10429.01 15.65 Q81A22 Multiple coagulation factor deficiency protein 2 206.43 2	PU2788		1069.99	35.21
P09800 Leukontene A-4 inydrolase 225,55 19,31 P01025 Lipocalin-1 8361,36 51,14 P00338 L-lactate dehydrogenase A chain 986,52 20,78 Q9BYY2 L-lactate dehydrogenase A-like 6B 323,86 8,66 Q9BY66 Lysine-specific demethylase 5D 59,78 0,78 P61626 Lysozyme C 9288,56 54,05 P14174 Macrophage migration inhibitory factor 256,82 15,13 Q96JG8 Melanoma-associated antigen D4 150,96 6,07 P01033 Metalloproteinase inhibitor 1 445,25 29,95 Q96GX9 Methylthioribuloes-1-phosphate dehydratase 198,99 23,14 O15021 Microthulue-associated serine/threonine-protein kinase 4 166,04 3,43 O43283 Mitogen-activated protein kinase kinase 13 533,35 8,70 Q8TAX7 Mucin-7 260,43 23,97 O75970 Multiple PD2 domain protein 341,23 17,19 P59666 Neutrophil defensin 1 253,04 15,96 </td <td>QOPKGU</td> <td></td> <td>139.16</td> <td>0.20</td>	QOPKGU		139.16	0.20
P31025 Lipocaln-1 9361.36 51.14 P00338 L-lactate dehydrogenase A chain 986.52 20.78 Q9BYZ2 L-lactate dehydrogenase A-like 6B 323.86 8.66 Q9BY66 Lysine-specific demethylase 5D 59.78 0.78 P61626 Lysozyme C 9288.56 64.05 P14174 Macrophage migration inhibitory factor 254.18 55.65 P14780 Matrix metalloproteinase-9 225.62 15.13 096JG68 Metalloproteinase inhibitor 1 445.25 29.95 096GX9 Methylthioribulose-1-phosphate dehydratase 198.99 23.14 043283 Mitogen-activated protein kinase kinase 13 533.35 8.70 05501 Mitrofubule-associated serine/threonine-protein kinase 4 166.04 3.43 043283 Mitogen-activated protein kinase kinase 13 533.35 8.70 075970 Multiple coagulation factor deficiency protein 2 266.43 23.97 075970 Multiple D2 domain protein 2353.04 15.96 P04746 Pancreatic alpha-amylase <td>P09960</td> <td>Leukotriene A-4 hydrolase</td> <td>225.55</td> <td>19.31</td>	P09960	Leukotriene A-4 hydrolase	225.55	19.31
P00338 L-laciate dehydrogenase A chain 986.5.2 20.78 09BYZ2 L-laciate dehydrogenase A chain 938.5.6 8.66 09BYZ6 Lysozyme C 9285.56 54.05 P61626 Lysozyme C 9285.52 15.13 09BYZ6 Matrix metalloproteinase-9 225.62 15.13 0960X9 Methylthiorbulose-1-phosphate dehydratase 189.9 23.14 015021 Microtubule-associated antigen D4 168.04 3.43 045023 Metalloproteinase inhibitor 1 445.25 29.95 0960X9 Methylthiorbulose-1-phosphate dehydratase 188.99 23.14 015021 Microtubule-associated serine/threonine-protein kinase 4 168.04 3.43 043283 Mitogen-activated protein kinase kinase 13 533.35 8.70 081N22 Multiple D2 domain protein 2353.04 15.65 08NI22 Multiple PDZ domain protein 2353.04 15.96 P59665 Neutrophil defensin 1 2353.04 15.96 P59666 Neutrophil defensin 3 2351.41 <	P31025	Lipocalin-1	8361.36	51.14
OgeY22 L-laciate dehydrogenase A-likk eb 323.86 8.66 OgeX66 Lysin-specific demydrogenase A-likk eb 59.78 0.78 P61626 Lysozyme C 9288.56 54.05 P14174 Macrophage migration inhibitory factor 254.18 55.65 P14780 Matrix metalloproteinase-9 225.62 15.13 OgeGX64 Melanoma-associated antigen D4 150.96 6.07 P01033 Metalloproteinase inhibitor 1 445.25 29.95 O960C6X9 Methylitoribulose-1-phosphate dehydratase 198.99 23.14 O15021 Microtubule-associated serin/Phreonine-protein kinase 4 166.04 3.43 O43283 Mitogen-activated protein kinase kinase 13 533.35 8.70 O8TAX7 Mucin-7 10429.01 15.65 Q8NI22 Multiple coagulation factor deficiency protein 2 260.43 23.97 O75970 Multiple PD2 domain protein 2353.04 15.96 P04766 Pancreatic alpha-amylase 64829.77 60.27 P06752 Petidyl-prolyl cis-trans isomerase D	P00338	L-lactate denydrogenase A chain	986.52	20.78
UB9176b Lysine-specific demetrylase 5D 59.78 0.78 P61626 Lysozyme C 9288.56 55.65 P14174 Macrophage migration inhibitory factor 254.18 55.65 P14780 Matrix metalloproteinase-9 225.62 15.13 Q96JG8 Metalloproteinase inhibitor 1 445.25 29.95 Q96GX9 Methylthoribulose-1-phosphate dehydratase 198.99 23.14 O15021 Microtubule-associated serine//threonine-protein kinase 4 168.04 3.43 O43283 Mitogen-activated protein kinase kinase 13 533.35 8.70 Q8FAX7 Mucin-7 10429.01 15.65 Q8HX2 Multiple coagulation factor deficiency protein 2 260.43 23.97 O75970 Multiple DDZ domain protein 341.23 17.19 P59666 Neutrophil defensin 3 2353.04 15.96 P04746 Pancreatic alpha-amylase 6482.77 60.27 Q0852 Peptidyl-prolyl cis-trans isomerase D 470.08 17.57 P13766 Plastin-2 531.41 <td< td=""><td>Q9BYZZ</td><td>L-lactate denydrogenase A-like 6B</td><td>323.86</td><td>8.66</td></td<>	Q9BYZZ	L-lactate denydrogenase A-like 6B	323.86	8.66
Profice Lysozyme C Sozyme C	Q9BY66		59.78	0.78
P141/4 Macrophage migration inhibitory factor 254.18 55.65 P14780 Matrix metalloproteinase-9 225.62 15.13 096JG8 Melanoma-associated antigen D4 150.96 6.07 P01033 Metalloproteinase inhibitor 1 445.25 29.95 096GX9 Methylthioribulose-1-phosphate dehydratase 198.99 23.14 015021 Microtubule-associated serine/threonine-protein kinase 4 168.04 3.43 043283 Mitogen-activated protein kinase kinase kinase 13 533.35 8.70 08TAX7 Mucin-7 10429.01 15.65 08N122 Multiple coagulation factor deficiency protein 2 260.43 23.97 075970 Multiple PDZ domain protein 43.13 2.32 P24158 Myeloblastin 2353.04 15.96 P59666 Neutrophil defensin 1 2353.04 15.96 P04746 Pancreatic alpha-amylase 64829.77 60.27 008752 Peptidyl-prolyl cis-trans isomerase D 470.08 17.57 P13796 Plastin-2 531.41 28.87 P04783 Polymetric immunoglobulin receptor <td>P61626</td> <td>Lysozyme C</td> <td>9288.56</td> <td>54.05</td>	P61626	Lysozyme C	9288.56	54.05
P14780 Matrix metalloproteinase-9 226.62 15.13 Q96JG8 Melanoma-associated antigen D4 150.96 6.07 P01033 Metalloproteinase inhibitor 1 445.25 29.95 Q96GX9 Methylthioribulose-1-phosphate dehydratase 198.99 23.14 O15021 Microtubule-associated serine/threonine-protein kinase 4 168.04 3.43 O43283 Mitogen-activated protein kinase kinase 13 533.35 8.70 Q8TAX7 Mucin-7 10429.01 15.65 Q8NI22 Multiple coagulation factor deficiency protein 2 260.43 23.97 O75970 Multiple PDZ domain protein 43.13 2.32 P24158 Myeloblastin 341.23 17.19 P59666 Neutrophil defensin 3 2353.04 15.96 P04746 Pancreatic alpha-amylase 64829.77 60.27 Q08752 Peptidyl-prolyt cis-trans isomerase D 470.08 17.57 P13796 Plastin-2 531.41 2.887 P01833 PoYmeric immunoglobulin receptor 16305.42 4	P14174		254.18	55.65
OpsDGS Metalloproteinase inhibitor 1 100.96 6.07 P01033 Metalloproteinase inhibitor 1 445.25 29.95 096CX9 Methylthioribulose-1-phosphate dehydratase 198.99 23.14 015021 Microtubule-associated serine/threonine-protein kinase 4 168.04 3.43 043283 Mitogen-activated protein kinase kinase kinase 13 533.35 8.70 08TAX7 Mucin-7 10429.01 15.65 08N122 Multiple coagulation factor deficiency protein 2 260.43 23.97 075970 Multiple Coagulation factor deficiency protein 2 260.43 23.97 075970 Multiple coagulation factor deficiency protein 2 260.43 23.97 075970 Multiple coagulation factor deficiency protein 2 260.43 23.97 075970 Multiple coagulation factor deficiency protein 2 2353.04 15.96 P04746 Pancreatic alpha-amylase 64829.77 60.27 008752 Peptidyl-prolyl cis-trans isomerase D 470.08 17.57 P13796 Plastin-2 355.10 10.14 2691.4	P14780	Matrix metalloproteinase-9	225.62	15.13
P01033 Metalloptotelnase inhibitor 1 443.25 29.95 Q96CX9 Methylthoribulose-1-phosphate dehydratase 198.99 23.14 Q15021 Microtubule-associated serine/threonine-protein kinase 4 168.04 3.43 Q43283 Mitogen-activated protein kinase kinase 13 533.35 8.70 Q8TAX7 Mucin-7 10429.01 15.65 Q8NI22 Multiple coagulation factor deficiency protein 2 260.43 23.97 Q75970 Multiple PDZ domain protein 43.13 2.32 P24158 Myeloblastin 341.23 17.19 P59666 Neutrophil defensin 3 2353.04 15.96 P04746 Pancreatic alpha-amylase 64829.77 60.27 Q08752 Peptidyl-prolyl cis-trans isomerase D 470.08 17.57 P13796 Plastin-2 531.41 28.87 P01833 POITE ankyrin domain family member E 3659.07 9.49 A5A3E0 POTE ankyrin domain family member J 16305.42 45.42 P0C638 POTE ankyrin domain family member J 1368.07	Q96JG8	Melanoma-associated antigen D4	150.96	0.07
Operation Methylthiorinouose 1-phosphate denydratase 198.99 23.14 O15021 Microtubule-associated serine/threonine-protein kinase 4 168.04 3.43 O43283 Mitogen-activated protein kinase kinase 13 533.35 8.70 Q8TAX7 Mucin-7 10429.01 15.65 Q8NI22 Multiple coagulation factor deficiency protein 2 260.43 23.97 O75970 Multiple PDZ domain protein 341.23 17.19 P59665 Neutrophil defensin 1 2353.04 15.96 P59666 Neutrophil defensin 3 2353.04 15.96 P04746 Pancreatic alpha-amylase 64829.77 60.27 Q08752 Peptidyl-prolyl cis-trans isomerase D 470.08 17.57 P13796 Plastin-2 531.41 28.87 P01833 POTE ankyrin domain family member F 3659.07 9.49 A5A3E0 POTE ankyrin domain family member J 1382.79 4.82 P17844 Probable ATP-dependent RNA helicase DDX5 220.36 4.89 P17844 Probable ATP-dependent RNA helicase D	P01033	Metalloproteinase Innibitor 1	445.25	29.95
O15021 Microubule-associated serification environme-protein kinase 4 165.04 3.43 O43283 Mitogen-activated protein kinase kinase 13 533.35 8.70 O8TAX7 Mucin-7 10429.01 15.65 Q8NI22 Multiple coagulation factor deficiency protein 2 260.43 23.97 O75970 Multiple PDZ domain protein 43.13 2.32 P24158 Myeloblastin 341.23 17.19 P59665 Neutrophil defensin 1 2353.04 15.96 P04746 Pancreatic alpha-amylase 64829.77 60.27 Q08752 Peptidyl-prolyl cis-trans isomerase D 470.08 17.57 P13796 Plastin-2 531.41 28.87 Q688J3 POTE ankyrin domain family member E 3659.07 9.49 A5A3E0 POTE ankyrin domain family member F 3575.10 10.14 P0CG38 POTE ankyrin domain family member J 1362.79 4.82 P17844 Probable ATP-dependent RNA helicase DDX5 220.36 4.89 I31.305 Profilin (Fragment) 1209.81	Q96GX9	Mernylthioribulose-1-phosphate denydratase	198.99	23.14
O43283 Mildigen-activated protein kinase kinase kinase in a 533.33 8.70 Q8TAX7 Mucin-7 10429.01 15.65 Q8NJ22 Multiple coagulation factor deficiency protein 2 260.43 23.97 O75970 Multiple PDZ domain protein 341.23 17.19 P59665 Neutrophil defensin 1 2353.04 15.96 P59666 Neutrophil defensin 3 2353.04 15.96 P04746 Pancreatic alpha-amylase 64829.77 60.27 Q08752 Peptidyl-prolyl cis-trans isomerase D 470.08 17.57 P13796 Plastin-2 531.41 28.87 P01833 Polymeric immunoglobulin receptor 16305.42 45.42 Q688J3 POTE ankyrin domain family member F 3575.10 10.14 P0CG38 POTE ankyrin domain family member J 1362.79 4.82 P17844 Probable ATP-dependent RNA helicase DDX5 220.36 4.89 I3L3D5 Profilin (Fragment) 1209.81 20.71 P07737 Profilin-1 1209.81 20.71 <td>015021</td> <td>Microtubule-associated senie/threonine-protein kinase 4</td> <td>108.04</td> <td>3.43</td>	015021	Microtubule-associated senie/threonine-protein kinase 4	108.04	3.43
Q8 N/22 Multiple coagulation factor deficiency protein 2 260.43 23.97 O75970 Multiple PDZ domain protein 43.13 2.32 P24158 Myeloblastin 341.23 17.19 P59665 Neutrophil defensin 1 2353.04 15.96 P59666 Neutrophil defensin 3 2353.04 15.96 P04746 Pancreatic alpha-amylase 64829.77 60.27 Q08752 Peptidyl-prolyl cis-trans isomerase D 470.08 17.57 Q08752 Peptidyl-prolyl cis-trans isomerase D 470.08 17.57 P01833 Polymeric immunoglobulin receptor 16305.42 45.42 Q6S8J3 POTE ankyrin domain family member E 3659.07 9.49 A5A3E0 POTE ankyrin domain family member J 10.14 P0CG38 POTE ankyrin domain family member J 1362.79 4.82 P17844 Probale ATP-dependent RNA helicase DDX5 220.36 4.89 P1273 Profilin (Fragment) 120.981 10.91 P07737 Profilin (Fragment) 22984.41 89.04	043263		030.00	0.70
Construct Construction Construction <td></td> <td>Multiple coossulation factor deficiency protein 2</td> <td>10429.01</td> <td>15.05</td>		Multiple coossulation factor deficiency protein 2	10429.01	15.05
Or Syr0 Multiple PD2 domain protein 43.13 2.32 P24158 Myeloblastin 341.23 17.19 P59665 Neutrophil defensin 1 2353.04 15.96 P59666 Neutrophil defensin 3 2353.04 15.96 P04746 Pancreatic alpha-amylase 64829.77 60.27 Q08752 Peptidyl-prolyl cis-trans isomerase D 470.08 17.57 P13796 Plastin-2 531.41 28.87 P01833 Polymeric immunoglobulin receptor 16305.42 45.42 Q6S8J3 POTE ankyrin domain family member E 3659.07 9.49 A5A3E0 POTE ankyrin domain family member F 3575.10 10.14 P0CG38 POTE ankyrin domain family member J 1362.79 4.82 P17844 Probable ATP-dependent RNA helicase DDX5 220.36 4.89 I3L3D5 Profilin (Fragment) 1209.81 10.91 P07737 Profilin 1 1209.81 20.71 P12273 Prolactin-inducible protein 4 52615.69 72.29 P07602<	QONIZZ	Multiple Coagulation factor denciency protein 2	200.43	23.97
P24158 Myeloblastin 341.23 17.19 P59665 Neutrophil defensin 1 2353.04 15.96 P59666 Neutrophil defensin 3 2353.04 15.96 P04746 Pancreatic alpha-amylase 64829.77 60.27 Q08752 Peptidyl-prolyl cis-trans isomerase D 470.08 17.57 P13796 Plastin-2 531.41 28.87 P01833 Polymeric immunoglobulin receptor 16305.42 45.42 Q6S8J3 POTE ankyrin domain family member E 3659.07 9.49 A5A3E0 POTE ankyrin domain family member F 3575.10 10.14 P0CG38 POTE ankyrin domain family member J 2591.40 5.67 P0CG39 POTE ankyrin domain family member J 1362.79 4.82 P17844 Probable ATP-dependent RNA helicase DDX5 220.36 4.89 I3J.3D5 Profilin (Fragment) 1209.81 20.71 P1737 Profilin 1 22984.41 89.04 A0A0A0MT31 Proline-rich protein 4 52615.69 72.29	075970	Multiple PDZ domain protein	43.13	2.32
P5965 Neturophil defensin 1 2353.04 15.96 P59666 Neutrophil defensin 3 2353.04 15.96 P04746 Pancreatic alpha-amylase 64829.77 60.27 Q08752 Peptidyl-prolyl cis-trans isomerase D 470.08 17.57 P13796 Plastin-2 531.41 28.87 P01833 Polymeric immunoglobulin receptor 16305.42 45.42 Q6S8J3 POTE ankyrin domain family member E 3659.07 9.49 A5A3E0 POTE ankyrin domain family member F 3575.10 10.14 P0CG38 POTE ankyrin domain family member J 2591.40 5.67 P0CG39 POTE ankyrin domain family member J 1362.79 4.82 P17844 Probable ATP-dependent RNA helicase DDX5 220.36 4.89 I3J3D5 Profilin (Fragment) 1209.81 20.71 P12733 Prolactin-inducible protein 2284.41 89.04 A0A0A0MT31 Proline-rich protein 4 52615.69 72.29 P07602 Prosaposin 316.92 22.52	P24158	Nyeloblastin Neutrophil defensie 1	341.23	17.19
P39000 Neutrophil defensin 3 2333.04 13.96 P04746 Pancreatic alpha-amylase 64829.77 60.27 Q08752 Peptidyl-prolyl cis-trans isomerase D 470.08 17.57 P13796 Plastin-2 531.41 28.87 P01833 Polymeric immunoglobulin receptor 16305.42 45.42 Q6S8J3 POTE ankyrin domain family member E 3659.07 9.49 A5A3E0 POTE ankyrin domain family member F 3575.10 10.14 P0CG38 POTE ankyrin domain family member J 220.36 4.82 P17844 Probable ATP-dependent RNA helicase DDX5 220.36 4.89 I3L3D5 Profilin (Fragment) 1209.81 10.91 P07737 Profilin-1 1209.81 20.71 P12273 Prolactin-inducible protein 2284.41 89.04 A0A0A0MT31 Protein Daple 206.07 0.69 P49354 Protein farmesyltransferase/geranylgeranyltransferase type-1 subunit alpha 1184.15 17.41 Q6FSS2 Protein NRDE2 homolog 339.41 6	P39003	Neutrophil defensin 2	2353.04	15.90
PO4746 Particleatic appla-anylase 6429.77 60.27 Q08752 Peptidyl-prolyl cis-trans isomerase D 470.08 17.57 P13796 Plastin-2 531.41 28.87 P01833 Polymeric immunoglobulin receptor 16305.42 45.42 Q6S8J3 POTE ankyrin domain family member E 3659.07 9.49 A5A3E0 POTE ankyrin domain family member F 3575.10 10.14 P0CG38 POTE ankyrin domain family member J 2591.40 5.67 P0CG39 POTE ankyrin domain family member J 1362.79 4.82 P17844 Probable ATP-dependent RNA helicase DDX5 220.36 4.89 3L3D5 Profilin (Fragment) 1209.81 10.91 P17273 Profilin-1 1209.81 20.71 P12273 Prolactin-inducible protein 2284.41 89.04 A0A0A0MT31 Proline-rich protein 4 52615.69 72.29 P07602 Prosaposin 316.92 22.52 Q9P219 Protein Daple 206.07 0.69 P49354<	P09000	Repercetie alpha amulasa	2303.04	10.90
Close Peptidy-problets isobilities b 470.06 17.37 P13796 Plastin-2 531.41 28.87 P01833 Polymeric immunoglobulin receptor 16305.42 45.42 Q6S8J3 POTE ankyrin domain family member E 3659.07 9.49 A5A3E0 POTE ankyrin domain family member F 3575.10 10.14 P0CG38 POTE ankyrin domain family member I 2591.40 5.67 P0CG39 POTE ankyrin domain family member J 1362.79 4.82 P17844 Probable ATP-dependent RNA helicase DDX5 220.36 4.89 I3L3D5 Profilin (Fragment) 1209.81 10.91 P07737 Profilin-1 1209.81 20.71 P12273 Prolactin-inducible protein 22984.41 89.04 A0A0A0MT31 Protein Daple 206.07 0.69 P49354 Protein Daple 206.07 0.69 P49354 Protein LEG1 homolog 7928.19 40.00 Q9H7Z3 Protein NRDE2 homolog 339.41 6.79	C09752	Pancieduc alpha-amylase	470.09	17.57
P13790 P13811-2 20.07 P01833 Polymeric immunoglobulin receptor 16305.42 45.42 Q6S8J3 POTE ankyrin domain family member E 3659.07 9.49 A5A3E0 POTE ankyrin domain family member F 3575.10 10.14 P0CG38 POTE ankyrin domain family member I 2591.40 5.67 P0CG39 POTE ankyrin domain family member J 1362.79 4.82 P17844 Probable ATP-dependent RNA helicase DDX5 220.36 4.89 I3L3D5 Profilin (Fragment) 1209.81 10.91 P07737 Profilin-1 1209.81 20.71 P12273 Prolactin-inducible protein 22984.41 89.04 A0A0A0MT31 Proline-rich protein 4 52615.69 72.29 P07602 Prosaposin 316.92 22.52 Q9P219 Protein Daple 206.07 0.69 P49354 Protein farnesyltransferase/geranylgeranyltransferase type-1 subunit alpha 1184.15 17.41 Q6P5S2 Protein NRDE2 homolog 339.41 6.79	Q00732		470.00 521.41	20.07
Poiss of polymenc infinition of polymenic minitor globulin receptor 10303.42 43.42 Q6S8J3 POTE ankyrin domain family member E 3659.07 9.49 A5A3E0 POTE ankyrin domain family member F 3575.10 10.14 POCG38 POTE ankyrin domain family member I 2591.40 5.67 POCG39 POTE ankyrin domain family member J 1362.79 4.82 P17844 Probable ATP-dependent RNA helicase DDX5 220.36 4.89 I3L3D5 Profilin (Fragment) 1209.81 10.91 P07737 Profilin-1 1209.81 20.71 P12273 Prolactin-inducible protein 22984.41 89.04 A0A0A0MT31 Proline-rich protein 4 52615.69 72.29 P07602 Prosaposin 316.92 22.52 Q9P219 Protein Daple 206.07 0.69 P49354 Protein farnesyltransferase/geranylgeranyltransferase type-1 subunit alpha 1184.15 17.41 Q6P5S2 Protein LEG1 homolog 7928.19 40.00 Q9H7Z3 Protein NRDE2 homolog 339.41	P 13790	Plasui-2	16305 42	20.07
A50353POTE ankyrin domain family member E3039.079.49A5A3E0POTE ankyrin domain family member F3575.1010.14P0CG38POTE ankyrin domain family member I2591.405.67P0CG39POTE ankyrin domain family member J1362.794.82P17844Probable ATP-dependent RNA helicase DDX5220.364.89I3L3D5Profilin (Fragment)1209.8110.91P07737Profilin-11209.8120.71P12273Prolactin-inducible protein22984.4189.04A0A0A0MT31Proline-rich protein 452615.6972.29P07602Prosaposin316.9222.52Q9P219Protein Daple206.070.69P49354Protein farnesyltransferase/geranylgeranyltransferase type-1 subunit alpha1184.1517.41Q6P5S2Protein NRDE2 homolog339.416.79	065813	POTE ankurin domain family member E	3650.07	43.42
ACAGEFOTE ankyrin domain family member 15070.1010.14POCG38POTE ankyrin domain family member 12591.405.67POCG39POTE ankyrin domain family member J1362.794.82P17844Probable ATP-dependent RNA helicase DDX5220.364.89I3L3D5Profilin (Fragment)1209.8110.91P07737Profilin-11209.8120.71P12273Prolactin-inducible protein22984.4189.04A0A0A0MT31Proline-rich protein 452615.6972.29P07602Prosaposin316.9222.52Q9P219Protein Daple206.070.69P49354Protein farnesyltransferase/geranylgeranyltransferase type-1 subunit alpha1184.1517.41Q6P5S2Protein LEG1 homolog7928.1940.00Q9H7Z3Protein NRDE2 homolog339.416.79	Q030J3	POTE ankyrin domain family member E	3575 10	9.49 10.14
POCG39POTE ankyrin domain family member J1362.794.82P17844Probable ATP-dependent RNA helicase DDX5220.364.89I3L3D5Profilin (Fragment)1209.8110.91P07737Profilin-11209.8120.71P12273Prolactin-inducible protein22984.4189.04A0A0A0MT31Proline-rich protein 452615.6972.29P07602Prosaposin316.9222.52Q9P219Protein Daple206.070.69P49354Protein farnesyltransferase/geranylgeranyltransferase type-1 subunit alpha1184.1517.41Q6P5S2Protein NRDE2 homolog339.416.79	POCG38		2501 40	5.67
P17844 Probable ATP-dependent RNA helicase DDX5 220.36 4.89 I3L3D5 Profilin (Fragment) 1209.81 10.91 P07737 Profilin-1 1209.81 20.71 P12273 Prolactin-inducible protein 22984.41 89.04 A0A0A0MT31 Proline-rich protein 4 52615.69 72.29 P07602 Prosaposin 316.92 22.52 Q9P219 Protein Daple 206.07 0.69 P49354 Protein farnesyltransferase/geranylgeranyltransferase type-1 subunit alpha 1184.15 17.41 Q6P5S2 Protein NRDE2 homolog 339.41 6.79	P0CG30	POTE ankyrin domain family member 1	1362 70	1.82
Introduct Profilin (Fragment) 1209.81 10.91 I3L3D5 Profilin (Fragment) 1209.81 10.91 P07737 Profilin-1 1209.81 20.71 P12273 Prolactin-inducible protein 22984.41 89.04 A0A0A0MT31 Proline-rich protein 4 52615.69 72.29 P07602 Prosaposin 316.92 22.52 Q9P219 Protein Daple 206.07 0.69 P49354 Protein farnesyltransferase/geranylgeranyltransferase type-1 subunit alpha 1184.15 17.41 Q6P5S2 Protein NRDE2 homolog 339.41 6.79	P178//		220.36	4.02
P07737 Profilin-1 1209.81 20.71 P12273 Prolactin-inducible protein 22984.41 89.04 A0A0A0MT31 Proline-rich protein 4 52615.69 72.29 P07602 Prosaposin 316.92 22.52 Q9P219 Protein Daple 206.07 0.69 P49354 Protein farnesyltransferase/geranylgeranyltransferase type-1 subunit alpha 1184.15 17.41 Q6P5S2 Protein NRDE2 homolog 339.41 6.79	131 305		1200.81	10.01
P1000000000000000000000000000000000000	P07737	Profilin_1	1203.01	20.71
AOAOAOMT31Proline-rich protein22501.4105.04P07602Prosaposin52615.6972.29Q9P219Protein Daple206.070.69P49354Protein farnesyltransferase/geranylg	P12273	Prolactin-inducible protein	22084 41	89.04
PO7602Prosaposin316.9222.52Q9P219Protein Daple206.070.69P49354Protein farnesyltransferase/geranylgeranyltransferase type-1 subunit alpha1184.1517.41Q6P5S2Protein NRDE2 homolog7928.1940.00Q9H7Z3Protein NRDE2 homolog339.416.79	ΔΟΔΟΔΟΜΤ31	Proline_rich protein 4	52615.69	72 29
Q9P219Protein Daple206.070.69P49354Protein farnesyltransferase/geranylgeranyltransferase type-1 subunit alpha1184.1517.41Q6P5S2Protein LEG1 homolog7928.1940.00Q9H7Z3Protein NRDE2 homolog339.416.79	P07602	Prosanosin	316 92	22 52
P49354Protein farnesyltransferase/geranylgeranylgeranyltransferase type-1 subunit alpha1184.1517.41Q6P5S2Protein LEG1 homolog7928.1940.00Q9H7Z3Protein NRDE2 homolog339.416.79	09P219	Protein Danle	206.07	0.69
Q6P5S2Protein LEG1 homolog7928.1940.00Q9H7Z3Protein NRDE2 homolog339.416.79	P49354	Protein farnesyltransferase/geranylgeranyltransferase type-1 subunit alpha	1184 15	17 41
Q9H7Z3 Protein NRDE2 homolog 339.41 6.79	Q6P5S2	Protein LEG1 homolog	7928 19	40.00
· · · · · · · · · · · · · · · · · · ·	Q9H7Z3	Protein NRDE2 homolog	339.41	6.79

Continued	from	nroviouo	0000
Continueu	nom	previous	page

Q8WYL5	Protein phosphatase Slingshot homolog 1	286.92	2.38
Q43663	Protein regulator of cytokinesis 1	83 55	7 42
P05109	Protein S100-A8	1391 46	31.18
P06702	Protein S100-A9	2043.00	78.07
09NOW1	Protein transport protein Sec31B	442 02	7.63
002054	Proteonlycan 4	188 50	2.78
Q92934		287.95	8.50
OOBVX7	Putative heta-actin-like protein 3	1353.87	29.07
	Putative beta-actin-like protein 3	3005.80	29.07
	Putative upiculity conjugating onzume E2 N like	341 70	32.03
	Putative updataterized protoin ancoded by LINC01553	101.02	10.53
A4QN01	Putative uncharacterized protein encoded by EINCO1555	211 70	0 50
Q15270		211.79	0.00
Q912J0	Rabpinini-5A Dofflin	706.05	17.30
Q14099	Ratuil	790.05	12.94
G3AAJ0	Rait-inking protein_isoionin CRA_C	251 72	10.64
P02000	RIIO GDP-uissociation Infinition I	201.72	19.01
	RNA-binding protein 12B	203.79	0.39
K4DI92	RWD domain containing 4A	636.75	30.48
Q6NW29	RWD domain-containing protein 4	030.75	30.32
P02810	Salivary acidic proline-rich phosphoprotein 1/2	52615.69	72.29
Q9BZL6	Serine/threenine-protein kinase D2	403.28	9.68
B4D152	Serine/threenine-protein kinase	401.26	9.57
P02787	Serotransterrin	4390.41	39.26
P02768	Serum albumin	64055.35	79.80
P02808	Statherin	25654.54	48.39
P02814	Submaxillary gland androgen-regulated protein 3B	50678.11	65.82
P00441	Superoxide dismutase [Cu-Zn]	1005.47	45.45
H0YN01		197.30	34.55
Q92609	TBC1 domain family member 5	344.39	5.16
Q7Z6L1	Tectonin beta-propeller repeat-containing protein 1	62.51	2.49
Q6N022	Ieneurin-4	64.41	4.15
P10599	I hioredoxin	300.36	32.38
Q96J01	THO complex subunit 3	335.46	20.51
Q5J1D0	light junction-associated protein 1	432.54	3.95
P37837	Transaldolase	676.70	23.74
P20061	Transcobalamin-1	670.49	33.26
A6H8Y1	Transcription factor TFIIIB component B" homolog	67.01	6.17
P29401	Transketolase	1109.18	29.05
Q9C0B7	Transport and Golgi organization protein 6 homolog	101.09	8.78
P60174	Triosephosphate isomerase	582.07	15.73
P07437	Tubulin beta chain	251.86	5.86
Q13885	Tubulin beta-2A chain	268.91	5.84
Q9BVA1	Tubulin beta-2B chain	251.86	5.84
P04350	Tubulin beta-4A chain	242.62	5.86
P68371	Tubulin beta-4B chain	242.62	5.84
H3BLT7	Tubulin monoglycylase TTLL3 (Fragment)	205.55	1.15
Q9NVE5	Ubiquitin carboxyl-terminal hydrolase 40	49.55	6.15
Q70EL2	Ubiquitin carboxyl-terminal hydrolase 45	709.84	12.04
D6RC01	Ubiquitinyl hydrolase 1	685.20	10.14
B4DSH7	UDP-galactose translocator	296.27	22.16
H7C2Y3	Uncharacterized protein C2orf80 (Fragment)	203.05	50.78
Q9H1L0	Uncharacterized protein MIR1-1HG	440.61	32.48
A0A087WZY1	Uncharacterized protein	50162.86	16.60
J3QRI8	UPF0183 protein C16orf70 (Fragment)	350.13	32.65
Q13488	V-type proton ATPase 116 kDa subunit a isoform 3	105.99	9.40
Q14508	WAP four-disulfide core domain protein 2	2122.26	33.87
Q9NXC5	WD repeat-containing protein mio	208.07	1.94
Q9BUG6	Zinc finger and SCAN domain-containing protein 5A	97.41	13.71
Q8N8U3	Zinc finger CCHC domain-containing protein 5	189.02	7.79

Q9H0M4	Zinc finger CW-type PWWP domain protein 1	242 57	7 10
Q01101111			
Q9NWS9	Zinc finger protein 446	77.75	7.56
P25311	Zinc-alpha-2-glycoprotein	1420.80	28.19
Q96DA0	Zymogen granule protein 16 homolog B	32673.11	56.73

spectrometry, involving the use of acids such as formic and trifluoroacetic acid^{9,12,19}, which inevitably exclude proteins that are insoluble in acidic solutions. In addition, intact high molecular weight proteins and heterogeneous glycosylated proteins are not accessible in their naturally occurring form, even to the best level of mass spectrometry².

Previous studies demonstrated that top-down platforms cannot achieve the same coverage of shotgun platforms for different reasons, such as: i) the intact protein must be soluble in the acid solution compatible with an ESI-MS analysis; (ii) the protein should not be heterogeneous (glycosylated isoforms), because in this case the intact protein mass cannot be deduced by the ESI spectrum; (iii) protein dimensions have to be limited, because MS-MS fragmentation spectra are too complex to be interpreted^{3,15}. Nonetheless, the topdown strategy may reveal the richness of the isoform and the diversity of post-translational modifications, which in the shotgun proteomics strategy may result in the relevant loss of this molecular information^{2,3}. Thus, shotgun proteomics may exhibit this deficiency in the human saliva proteome, in which many proteins such as basic PRPs and acids are not very susceptible to the proteolytic enzymes action and reveal very similar sequences. Therefore, many fragments cannot be related to a specific original protein. However, the shotgun platforms showed the best performance in terms of number of components detected, because the sensitivity of mass spectrometry is sufficient to reveal thousands of peptides in a single analysis. In this way, shotgun proteomics covers the largest variety of detectable components, regardless of their mass, due to the proteolytic digestion of large proteins almost always generates peptides that can disclose the presence of the protein in a complex mixture. Due to these reasons, the number of salivary components currently detectable by shotgun proteomics approaches is more than five times greater than that of components detected by any other platform^{2,10}. Thus, in this study we employed shotgun proteomics.

Based on the results of the two tests, the protocol for salivary shotgun proteomic analysis was

satisfactory, since it allowed the identification of many proteins, including those typically found in saliva. Moreover, it is easy to perform and cheaper than the methods previously described, since it does not require the use of depletion columns. Furthermore, it allows individual analysis of the samples, which is very important in quantitative proteomics. Thus, this protocol could be used in future studies involving shotgun proteomic analysis of saliva.

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