Enrichment and identification of glycoproteins in human saliva using lectin magnetic bead arrays.

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Abstract:

Aberrant glycosylation of proteins is a hallmark of tumorigenesis, and could provide diagnostic value in cancer detection. Human saliva is an ideal source of glycoproteins due to the relatively high proportion of glycosylated proteins in the salivary proteome. Moreover, saliva collection is non-invasive, technically straightforward and the sample collection and storage is relatively easy. Although, differential glycosylation of proteins can be indicative of disease states, identification of differential glycosylation from clinical samples is not trivial. To facilitate salivary glycoprotein biomarker discovery, we optimised a method for differential glycoprotein enrichment from human saliva based on lectin magnetic bead arrays (saLeMBA). Selected lectins from distinct reactivity groups were used in the saLeMBA platform to enrich salivary glycoproteins from healthy volunteer saliva. The technical reproducibility of saLeMBA was analysed with LC-MS/MS to identify the glycosylated proteins enriched by each lectin. Our saLeMBA platform enabled robust glycoprotein enrichment in a glycoprotein- and lectin-specific manner consistent with known protein-specific glycan profiles. We demonstrated that saLeMBA is a reliable method to enrich and detect glycoproteins present in human saliva.

***Keywords:** Saliva proteomics, Biomarker discovery, Glycosylation, Lectins, Magnetic Beads

Abbreviations: LeMBA- Lectin Magnetic Bead Array; saLeMBA- Saliva optimized Lectin Magnetic Bead Array; PTM - post-translational modification.

1. Introduction:

Glycosylation, the covalent addition of sugars to proteins, is a common post-translational modification (PTM) with more than 50% of all eukaryotic proteins thought to be glycosylated [1]. Glycoproteins and glycosylation play fundamental roles in many biological processes and have important cellular functions such as increasing protein stability, protecting proteins from degradation, increasing protein solubility and regulating protein activity [2]. Glycosylation is also known to be organism, tissue and cell type specific, regulated by the quantity and localization of glycosyltransferases enzyme and the available amount of substrate [3]. However, the equilibrium of this relationship can be disrupted in a disease state. Glycosylation is known to change within a cell from “healthy” to a “disease” state [4]. For instance, aberrant glycosylation is associated with cancer progression in breast [5], prostate [6], ovarian [7], lung [8] and hepatocellular carcinoma (HCC) [9].

Human saliva is emerging as a promising biological fluid for diagnostic testing. There is 20-30% overlap in protein content between saliva and blood/plasma, suggesting that saliva could be an attractive fluid for biomarker discovery for systemic diseases and may serve as a diagnostic alternative to blood tests [10]. Compared to other body fluids such as blood, cerebral spinal fluid (CSF) and urine, the collection of whole saliva is easy and relatively stress-free for the person donating it, non-invasive, and simple. As such it allows collection of multiple samples at one time without imposing too much discomfort, is relatively safe for hospital staff to handle, and is cost-effective [11, 12]. Saliva has been proposed as a diagnostic medium to detect many systemic diseases [13] such as breast cancers, lung cancers, pancreatic cancers [14], Sjögren’s syndrome [15], heart failure [16], and head and neck squamous cell carcinomas [17, 18].

While glycosylation is critical for modulating protein activity, efficient and sensitive analysis of protein glycosylation frequently requires enrichment of glycoproteins or glycopeptides. Current methods for glycoprotein enrichment include hydrazide chemistry [19-21], hexapeptide libraries [22], magnetic nano-probes [23], phenylboronic acid [24] and lectin based techniques including single lectin affinity chromatography, multiple lectin affinity chromatography [25], lectin microarrays [26], and lectin magnetic bead arrays [27, 28]. Lectins recognize specific glycan structures with high specificity and affinity, allowing targeted enrichment of proteins modified with specific subclasses of glycans. Methods such as single/multiple and serial lectin affinity chromatography can greatly reduce the complexity

of the sample being analysed, while simultaneously enriching for specific glycosylation structures of interest. However, the use of lectins for glycoprotein enrichment has drawbacks. The process can be time consuming, lectin affinity chromatography methods are not suitable for large population based screening studies, and no single lectin has the ability to enrich the entire glycoproteome within a biological sample [25, 29, 30]. Previously, we have reported [27, 28] the development of lectin magnetic bead arrays (LeMBA) for efficient high-throughput glycoprotein enrichment in serum samples using a panel of lectins.

In the present study, we have optimised and developed the LeMBA methodology for use with complex biological matrices with relatively higher viscoelastic properties such as human saliva (saLeMBA). We describe an optimised protocol for saliva collection and processing, and demonstrate that the saLeMBA technology platform is robust, reproducible and can identify protein-specific differences in glycan structures in human saliva. We anticipate that saLeMBA will be useful in identifying modifications in human salivary glycoproteins, opening up new avenues of research in the field of salivary glycoproteomics.

2. Methods:

2.1 Study design:

This study was approved by the University of Queensland Medical Ethical Institutional Board and the Queensland University of Technology Ethics Committee (QUT 1400000617). We recruited 4 healthy, non-smoker volunteers, less than 30 years of age with no underlying medical conditions. Signed informed consent was obtained before saliva sample collection.

2.2 Saliva sample collection and processing:

Volunteers were requested not to eat food or drink (except water) for 1 h prior to donating saliva, adhering to our previously published saliva collection protocols [12, 31]. We asked the volunteers to rinse their mouths to remove any food debris, and then collected unstimulated whole mouth saliva as previously described [32]. In brief, unstimulated saliva was collected by asking volunteers to tilt their heads down and pool saliva in the front of their mouth for 2 min before the saliva sample was expectorated into a 50 mL Falcon Tube kept on ice. The saliva was then clarified at 500 rcf for 10 min at 4°C to remove cellular debris, and the supernatant was aliquoted into separate protein LoBind tubes (Eppendorf, Hamburg, Germany). All samples were stored at -80°C until required. Serum was obtained from whole

blood by centrifuging at 500 rcf for 15 min at 24°C. Samples were stored at -80°C until analysis.

2.3 Reagents

Dynabeads® MyOne™ Tosylactivated were purchased from Life Technologies (Carlsbad, CA, USA). Lectins: *Aleuria aurantia* (AAL), *Bauhinia purpurea* lectin (BPL), *Jacalin* (JAC), Soybean agglutinin (SBA), *Narcissus pseudonarcissus* lectin (NPL) and *Sambucus nigra* agglutinin (SNA) were purchased from Vector Laboratories (Burlingame, CA, USA) while lectins Concanavalin A (ConA) and *Erythrina cristagalli* (ECA) were purchased from Sigma. Bradford assay kit, Triton X-100, 30%-bis acrylamide, 5 x Laemmli sample buffer and TEMED were purchased from Bio-Rad (Hercules, CA, USA). All other chemicals were obtained from Sigma.

2.4 Coupling lectins to DynaBeads, enriching for specific glycoproteins and SDS-PAGE

Coupling of lectins to Dynabeads® MyOne™ Tosylactivated was performed according to previously established methods [27-29]. Saliva (50 µg of total protein, and typically a protein concentration of 1-2 µg/µL) was added to denaturing buffer (final concentration 20 mM Tris-HCl buffer pH 7.4, 1% SDS, 5% Triton x-100, and 20 mM DTT) to give a final volume of 75 µL, and incubated at 65°C for 30 min. Reduced cysteines in the denatured samples were alkylated by addition of iodoacetamide to a final concentration of 100 mM and incubated in the dark for 30 min. Reduced/alkylated saliva was diluted with 1425 µL of binding buffer (20 mM Tris-HCl buffer pH 7.4, 300 mM NaCl, 1 mM CaCl₂, 1 mM MnCl₂, 1% Triton X-100, 1 x Protease inhibitor cocktail) and incubated with 50 µL of lectin-coupled magnetic beads for 1 h at 4°C with constant rotation. The beads with captured salivary glycoproteins were washed 3 times in washing buffer (20 mM Tris-HCl buffer pH 7.4, 0.05% SDS, 1 mM DTT, 300 mM NaCl, 1 mM CaCl₂, 1 mM MnCl₂, 1% Triton X-100). For initial analysis and optimisation of saLeMBA, salivary glycoproteins were eluted from the beads by resuspending them in 20 µL of 2 x Laemmli sample buffer and incubating at 95 °C for 10 min. Eluted proteins were then separated by SDS-PAGE using hand-cast 1.0 mm thick 15% polyacrylamide gels using a Bio-Rad Mini-PROTEAN quad 4 system. The gels were electrophoresed at a constant 100 V until the bromophenol blue reached the bottom of the gel and stained overnight in colloidal Coomassie G-250 [33]. Gels were then de-stained in

1% glacial acetic acid until background colour was completely removed. Gels were scanned and protein bands were quantified using Image J (National Institute of Mental Health, Bethesda, Maryland, USA).

2.5 Identification of glycoproteins enriched with saLeMBA using LC-MS/MS

For the identification of captured salivary glycoproteins, beads were washed 7 times with 20 µL of 50 mM ammonium bicarbonate (ABC), resuspended in 20 µL of 50 mM ABC with 1 µg trypsin (Promega, Madison, WI, USA) and incubated at 37 °C for 16 h with constant rotation. The supernatant was removed and kept and the beads were washed in 20 µL of 50 mM ABC, before combining supernatants of digested peptides. Peptide samples were transferred to u-bottom microtitre plates (Greiner Bio-One, Kremsmünster, Austria), dried in a speed vacuum at 45 °C for ~1 h and stored at -80°C until further use.

For LC-MS/MS, samples were resuspended in 20 µL of 0.1 % v/v formic acid. Digested peptides were detected using a 6520 QTOF (Agilent, Santa Clara, CA, USA) coupled with a Chip Cube and 1200 HPLC (Agilent). The nano pump was set at 0.3 µL/min and the capillary pump at 2.5 µL/min. The HPLC-chip used in this study contained a 160 nL C18 trapping column, and 150 mm C18 analytical column (Agilent, USA). Buffer A was 0.1 % v/v formic acid and Buffer B was 90 % v/v acetonitrile containing 0.1 % v/v formic acid. Peptides were eluted from the column using a gradient from 6 % B at 0 min to 46 % B at 45 min. Nano pump % B was increased to 95 % B at 45.5 min, maintained at that level till 53.5 min, and decreased to the original 6 % B at 55.5 min, with a total acquisition time of 60 min. The mass spectrometer was operated in a 2 GHz extended dynamic range and programmed to acquire 9 precursor MS1 spectra per second and 4 MS/MS spectra for each MS1 spectra. Dynamic exclusion applied after 2 MS/MS within 0.15 min. In addition, exclusion for lectin peptides was applied as reported previously [28].

Identification of peptides and proteins was achieved using Spectrum Mill (Agilent B.04.00.127) with Swiss-Prot human database (3/1/2012). A maximum of 2 missed cleavages was allowed, precursor mass tolerance was set at 20 ppm, and product mass tolerance was set at 50 ppm. Carbamidomethylation (C) was selected as a fixed modification and oxidized methionine was selected as a variable modification. Default settings were used for the automatic validation of the identified proteins and peptides. Results were then filtered with the following settings: protein score >15, peptide score >6 and % scored peak intensity (%SPI >60). False discovery rate was set to 1%.

3. Results and Discussion

3.1 Development of saLeMBA methodology to use with human saliva

Previously, LeMBA [27, 28] has been used for glycoprotein enrichment from serum. We optimized various parameters of this previously published LeMBA methodology to enrich salivary glycoproteins. Human saliva is a viscoelastic biofluid and contains an unusually high proportion of glycoproteins with very unusual complex carbohydrate structures. A key feature of saliva is that it has a much lower protein concentration than blood, which necessitated a larger volume of binding buffer. To determine whether the low concentrations of proteins in saliva affected the efficiency of glycoprotein enrichment by lectin-coupled beads, we enriched glycoproteins from serum containing 50 µg of total protein using the standard LeMBA method, and a simulated saLeMBA method in which the total incubation volume was increased to 1.5 mL to mimic the protein concentration in whole mouth unstimulated saliva, (labelled as diluted LeMBA in Figure 1A). SDS-PAGE analysis of eluted glycoproteins showed minimal differences between these treatments (Figure. 1A) indicating that the dilution effect of the larger binding buffer volume used in the saLeMBA methodology has no discernible influence on the efficiency of glycoprotein enrichment. As more dilute protein concentrations may affect the kinetics of glycoprotein-lectin interactions, we next investigated the optimal time for incubating saliva samples with ConA coupled beads using the saLeMBA methodology. This demonstrated that there are no significant changes in the intensity of eluted glycoproteins with varying incubation times (Figure. 1B). Therefore, an incubation time of 1h was used for all subsequent experiments, consistent with the previously published LeMBA methodology [27, 28]. To determine the optimum amount of salivary protein to use with lectin-coupled beads, we applied different protein amounts of saliva consisting of 25-100 µg of total protein to 50 µg beads coupled with JAC, WGA, and ConA lectins. These lectins were selected based on their distinct and diverse carbohydrate-binding specificities (Table 1).

Elutions from each condition were separated by SDS-PAGE and the total protein intensities were measured using Image J. For all lectins, protein intensity increased linearly up to 80 µg of applied salivary protein (Figure. 1C, Figure. 1D, Figure. 1E). We therefore selected 50 µg of salivary protein as a sufficient and non-saturating sample quantity. A summary of key features of the optimized saLeMBA method are shown in Table 2.

Figure 1: The optimization of SaLeMBA platform: (A) the comparison of LeMBA vs. the diluted LeMBA methodologies using 50 μ g of healthy volunteer serum on ConA coupled beads run on 10% SDS-PAGE stained in colloidal Coomassie, (B) The comparison of incubation times for saLeMBA. Each lectin was bound to tosyl activated magnetic beads with the same amount of protein (50 μ g). Titration of 25 μ g - 100 μ g unstimulated whole mouth saliva using the saLeMBA method across (C) ConA coupled beads, (D) JAC coupled beads, and (E) WGA coupled beads. The lectin pull downs were analysed using 10% SDS-PAGE stained in colloidal Coomassie.

225 *Reproducibility of saLeMBA platform*

226 Having optimized the experimental conditions for saLeMBA, we tested the utility of
227 this method using a panel of five lectins. Human saliva from healthy volunteers (n=4) were
228 pooled and used across 5 lectins (AAL, BPL, ECA, NPL, SNA) in three technical replicates.
229 These five lectins were selected because of their varying glycan binding specificities, with
230 each representing a different reactivity group: AAL (Fuc α 1-2, -3, -6 linked), BPL (Gal β 1-
231 3GalNAc), ECA (Gal β 1-4GlcNAc), NPL (Man α 1-6Man), and SNA (Neu5Ac α 2-6) (Table 1).
232 The subsequent pull down fractions were then analysed with LC-MS/MS. Supplementary
233 Table 1 shows the protein identifications generated by Spectrum Mill for all proteins found
234 across each replicate and lectin. Table 3 displays lectin binding distributions for proteins
235 consistently identified across technical triplicate analyses. Approximately 70% of proteins
236 bound across the 5 lectins were identified as glycosylated (as annotated in UniProt) and the
237 remaining 30% of the identified proteins were not glycosylated. These non-glycosylated
238 proteins were typically abundant salivary proteins which may account for their presence.
239 Alternatively, these findings may suggest new sites of glycosylation on proteins which have
240 not previously been shown to be glycosylated.

241 The highest number of glycoproteins were bound to AAL (46 unique proteins),
242 followed by NPL (45 unique proteins) and SNA (31 unique proteins). Importantly, many
243 proteins were found across more than one lectin, such as Cathepsin G, Alpha Amylase 1,
244 Zinc Alpha 2 glycoprotein, and Zymogen granule protein 16 homolog B. This suggests these
245 proteins (and others) are modified by multiple glycans at distinct sites. Specifically, our
246 saLeMBA results support the prevalence of Fuc, Man and Sialic Acid modifications in
247 human salivary glycoproteins, consistent with previous glycomic analyses which revealed
248 these monosaccharides are present in high relative abundance in human saliva [24, 34].

249 Abundant salivary proteins such as Alpha-1-amylase and Zymogen granule protein 16
250 homolog-B were also found across all five lectins. In contrast, proteins such as Azurocidin
251 (AAL), Haptoglobin (SNA), and Myeloperoxidase (NPL) were found specific to their
252 respective lectins. In agreement with binding to the Fucose-specific AAL, Azurocidin (or
253 Heparin binding protein) is an N-linked glycoprotein with abundant truncated neutral glycans
254 with core fucosylation (Fuc α 1-6GlcNAc) [35]. Haptoglobin, an N-linked glycoprotein, has
255 been reported to carry complex glycan structures with extensive α 2-6 and some α 2-3 linked
256 sialic acid [36], consistent with our identification of Haptoglobin after SNA enrichment
257 specific for Neu5Ac α 2-6. Finally, Myeloperoxidase has been reported to be modified with

high mannose glycans at five of its N-glycosylation sites [37] . Importantly, Mucin-5B was absent from lectin pull down using ECA coupled beads, whilst Mucin-5B was captured on AAL, BPL, NPL and SNA beads (Figure 2C). This is to be expected as Mucin-5B is extensively O-glycosylated with few non-modified lactosamine motifs and extensive terminal Fucose and sialic acid [34].

Figure 2: LC-MS/MS triplicate analysis of 5 lectins used in saLeMBA enrichment of pooled human unstimulated whole mouth saliva. (A) Proportion of common proteins found across 3 replicates for each lectin (B) Correlations between intensities of common proteins determined by pairwise analysis (C) PCA plot, and (D) Venn diagram showing overlap of proteins identified by each lectin.

We next investigated the technical reproducibility of saLeMBA. Technical triplicate analysis showed that the enrichment of specific glycoproteins was highly consistent, with all lectins showing at least 79% common proteins across the triplicate enrichments (Figure. 2A, Table 3). These results represent the percentage of proteins found across each of the 3 replicates for each of the 5 different lectins (AAL, BPL, ECA, NPL, and SNA). It should be noted that these results do not indicate whether the identified proteins mentioned in (Table 3) share the same/similar abundances. For example Alpha 1 amylase is found on each of the 3 replicates and with each of the 5 lectins and would be considered a common protein for our study. Pairwise analysis and PCA comparison of the Spectrum Mill protein identification scores showed that the different lectins enriched distinct subsets of the salivary glycoproteome (Figure. 2B, Figure. 2C).

Pairwise comparisons of logged normalized glycoprotein abundance (Figure. 2B), demonstrated an excellent correlation between AAL vs NPL, AAL vs SNA and NPL vs SNA. While each lectin was able to identify unique proteins (with the exception of ECA), the correlation which AAL, NPL, SNA demonstrated the reproducibility of saLeMBA. In contrast, the lack of correlation between lectins with distinct binding specificities such as ECA vs NPL highlights the strength of using multiple diverse lectins in glycoprotein enrichment analysis.

PCA comparisons of logged normalized glycoprotein abundance (Figure. 2C) supported these findings, as arrows for SNA and BPL were almost perpendicular, with the strong correlation of AAL, NPL and SNA clearly visible, as well as highlighting the glycoproteins determining these various lectin-binding specificities. Literature [28] describes NPL (binds core mannose structures) as heavily influenced by the presence of branched sugars from the binding site suggesting that although NPL recognises core Man structures the presence of other branching glycans may affect its expected binding. This demonstrates that the lectins selected for reproducibility cover a diverse range of glycan binding activity, and supports the accuracy of our saLeMBA methodology. A Venn diagram (Figure. 2D) showing the overlap of proteins identified after enrichment with each lectin. We are able to distinctly identify proteins unique to each lectin as well as proteins which are identified by all 5 lectins. With reference to Figure. 2A, this qualitative analysis refers to the identification of a protein, whereas the results depicted in Figure. 2B and Figure.2C take into account the relative abundance of a particular protein. As with Figure 2A, this qualitative analysis refers to the identification of a protein, whereas the results depicted in Figure. 2B and Figure.2C take into

account the relative abundance of a particular protein. Reproducible identification of glycoproteins across a wide abundance range shows the robustness and specificity possible with saLeMBA for the enrichment of glycoproteins in saliva. Our current method allows relative quantification, which is appropriate for many discovery applications [38].

Figure 3: Abundance of selected salivary glycoproteins after saLeMBA enrichment with each lectin. Intensity is measured as log (10) total intensity (determined by Spectrum Mill) with bars representing the mean across the 3 repeat experiments and errors representing standard deviations.

Quantitative comparisons of the relative abundance of selected glycoproteins enriched by each lectin showed high technical reproducibility (Figure 3). For instance, Deleted in malignant brain tumors 1 protein is more abundant than Prolactin-inducible protein after enrichment by BPL, but the reverse is true after enrichment by NPL or SNA coupled beads. Reproducible identification of glycoproteins across a wide abundance range and shows the robustness and specificity possible with saLeMBA for the enrichment of glycoproteins in saliva.

4. Conclusion

The emergence of human saliva as a valuable diagnostic fluid for the identification and characterization of proteins and peptides involved in systemic diseases has led to the development and optimization of different techniques for their discovery. In this study, we have developed saLeMBA, a robust and reproducible methodology to enrich salivary glycoproteins based on differential lectin binding. We suggest our optimised saLeMBA may be useful in screening for potential glycoprotein biomarkers in human saliva to discern glycosylation changes that are specific for diseases.

Supporting Information

Table S1 shows the MS data generated for the reproducibility testing of saLeMBA (triplicate experiment using 5 different lectins).

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500 Table 1:
501 Carbohydrate binding specificities of lectins used in this study.
502

Lectin	Sugar Specificity
Aleuria aurantia (AAL)	Fucα1-6/3GlcNAc
Bauhinia purpurea lectin (BPL)	Galβ1-3GalNAc
Concanavalin A (ConA)	α-Man, α-Glc, α-GlcNAc
Erythrina cristagalli agglutinin (ECA)	Galβ4GlcNAc
Jacalin (JAC)	Galα1-6GalNAc and Galβ1-3GalNAc
Narcissus pseudonarcissus lectin (NPL)	High Mannose, Manα1-6Man
Sambucus nigra agglutinin (SNA)	Neu5Acα2-6Gal/GalNAc
Wheat germ agglutinin (WGA)	GlcNAcβ1-4GlcNAc and Neu5Ac

Table 2:

Key features of the optimized SaLeMBA method

	LeMBA	SaLeMBA
Biological sample used	Serum	Saliva
Amount of sample protein per pull down	50 µg	50 µg
Average sample protein concentrations (± stdev)	384.8 ± 9.2 µg/µL	2.11 ± 0.035 µg/µL
Initial sample treatment buffer (Denaturing Buffer) volume	15 µL	75 µL
Sample to Lectin coupled bead binding buffer volume	85 µL	1425 µL
Total volume (Denaturing buffer + Binding buffer)	100 µL	1500 µL
Total incubation time	1 h at 4°C	1 h at 4°C

Table 3: Proteins robustly identified after saLeMBA enrichment.

Identified Protein	Accession number	AAL	BPL	ECA	NPL	SNA
Actin, cytoplasmic 2	P63261	4.67 ± 1.15				
Alpha-2-macroglobulin	P04745				3.67 ± 1.53	
Alpha-2-macroglobulin-like protein 1	A8K2U0				19.33 ± 1.15	
Alpha-amylase 1	P04745	49.00 ± 2.65	20.67 ± 8.39	18.33 ± 8.62	46.27 ± 3.21	33.67 ± 12.74
Annexin A1	P04083	8.00 ± 3.00	11.67 ± 2.08	5.67 ± 3.21	7.67 ± 1.53	9.67 ± 3.06
Azurocidin	P20160	6.67 ± 1.53				
Beta-2-glycoprotein 1	P02749					1.67 ± 0.58
BPI fold-containing family A member 2	Q8N4F0	5.53 ± 1.15				
BPI fold-containing family B member 2	Q96DR5	7.67 ± 0.58			7.00 ± 1.00	6.33 ± 1.53
Carbonic anhydrase 6	P23280	8.33 ± 0.58	2.33 ± 0.58		10.67 ± 2.52	11.67 ± 3.06
Cathepsin G	P08311	3.00 ± 1.00			6.00 ± 1.00	5.33 ± 1.15
Complement C3	P01024				2.67 ± 0.58	
Cornulin	Q9UBG3	3.33 ± 2.08	2.67 ± 0.58		6.00 ± 1.00	

Cystatin-S	P01036	9.00 ± 2.00			5.33 ± 0.58	
Cytoplasmic dynein 1 heavy chain 1	Q14204		1.67 ± 0.58			
Deleted in malignant brain tumors 1 protein	Q9UGM3	15.00 ± 2.52	14.33 ± 0.58	1.33 ± 0.58	8.00 ± 2.00	5.33 ± 1.21
Galectin-3-binding protein	Q08380	5.67 ± 0.58			2.00 ± 0.00	
Glyceraldehyde-3-phosphate dehydrogenase	P04406				2.33 ± 0.58	
Haptoglobin	P00738					3.33 ± 1.53
Heat shock cognate 71 kDa protein	P11142		3.33 ± 0.58			
Hemopexin	P02790					1.67 ± 0.58
Histatin-1	P15515	1.67 ± 0.58				
Ig alpha-1 chain C region	P01876	23.00 ± 4.36	10.33 ± 1.15	7.33 ± 2.89	32.67 ± 0.58	22.00 ± 3.00
Ig gamma-1 chain C region	P01857	4.00 ± 0.00	1.67 ± 0.58		10.00 ± 0.00	6.00 ± 0.00
Ig gamma-2 chain C region	P01859	5.00 ± 1.00				
Ig heavy chain V-III region BRO	P01766	2.67 ± 0.58			3.00 ± 0.00	2.33 ± 0.58
Ig kappa chain C region	P01834	15.00 ± 1.00	7.00 ± 1.00	14.33 ± 7.23	17.33 ± 1.53	13.00 ± 3.61
Ig kappa chain V-II region RPMI 6410	P06310	1.00 ± 0.00				

Ig lambda-2 chain C regions	P0CG05	8.00 ± 1.73				
Ig lambda-7 chain C region	A0M8Q6				3.33 ± 0.58	
Ig mu chain C region	P01871	8.67 ± 1.53			17.67 ± 3.21	6.33 ± 1.15
Immunoglobulin J chain	P01591					2.67 ± 0.58
Integrin alpha-M	P11215				12.33 ± 2.52	
Kallikrein-1	P06870	3.67 ± 0.58				3.00 ± 1.00
Keratin, type I cytoskeletal 13	P13646					26.33 ± 6.03
Keratin, type I cytoskeletal 9	P35527	17.67 ± 1.53	7.67 ± 5.50	14.67 ± 7.09	5.67 ± 1.15	
Keratin, type II cytoskeletal 1	P04264			18.33 ± 3.51	15.33 ± 2.31	
Keratin, type II cytoskeletal 4	P19013		19.67 ± 5.13	20.67 ± 6.43		
Keratin, type II cytoskeletal 6A	P02538				11.67 ± 3.79	
Lactoperoxidase	P22079	4.33 ± 0.58			12.33 ± 1.15	
Lactotransferrin	P02788	37.67 ± 0.58	10.00 ± 2.00	30.00 ± 6.56	31.33 ± 5.69	42.67 ± 2.08
Lysozyme C	P61626	1.33 ± 0.58	1.67 ± 0.58		1.67 ± 0.58	
Mucin-5B	Q9HC84	59.00 ± 4.36	11.00 ± 4.58		25.00 ± 2.65	56.67 ± 2.52
Mucin-7	Q8TAX7	5.00 ± 2.00	8.00 ± 2.65	2.33 ± 0.58	3.33 ± 1.53	

Myeloblastin	P24158	2.33 ± 0.58				
Myeloperoxidase	P05164				32.33 ± 2.52	
Neuroblast differentiation-associated protein AHNAK	Q09666					2.00 ± 0.00
Neutrophil defensin 3	P59666			2.00 ± 0.00		
Polymeric immunoglobulin receptor	P01833	34.67 ± 3.06	13.00 ± 1.73		40.33 ± 4.73	27.67 ± 2.89
Profilin-1	P07737	3.00 ± 1.00			2.67 ± 0.58	
Prolactin-inducible protein	P12273	15.67 ± 3.51	5.00 ± 1.00	7.67 ± 3.21	11.00 ± 1.00	11.67 ± 3.21
Protein S100-A8	P05109	4.00 ± 0.00	3.33 ± 0.58	3.33 ± 1.15	4.33 ± 0.58	4.00 ± 1.00
Protein S100-A9	P06702	2.67 ± 0.58				
Protein-glutamine gamma-glutamyltransferase E	Q08188	2.67 ± 0.58			3.67 ± 1.15	
Serotransferrin	P02787	5.33 ± 1.53			3.00 ± 1.00	6.33 ± 1.53
Serum albumin	P02768	14.00 ± 0.00			5.33 ± 1.15	
Small proline-rich protein 2G	Q9BYE4			1.67 ± 1.15		
Small proline-rich protein 3	Q9UBC9	2.33 ± 0.58	3.00 ± 1.73	3.67 ± 1.15	3.33 ± 0.58	

Statherin	P02808					2.00 ± 0.00
Transcobalamin-1	P20061					1.00 ± 0.00
Transketolase	P29401				7.33 ± 0.58	
UPF0762 protein C6orf58	Q6P552	4.33 ± 0.58			2.00 ± 0.00	3.67 ± 1.53
Zinc-alpha-2-glycoprotein	P25311	16.67 ± 1.15			5.00 ± 1.00	14.67 ± 0.58
Zymogen granule protein 16 homolog B	Q96DA0	17.00 ± 3.61	8.00 ± 1.00		17.00 ± 0.00	12.67 ± 0.58

Accession numbers are from Swiss-Prot. The table shows the average number of spectra ± standard deviation.