RCPAQAP Molecular Genetics

**Quality use of Pathology Program**

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**The development of a mass spectrometry technical quality assurance program for detecting human disease-associated proteins.**

**Final Report**

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# Executive Summary

Genetic disease diagnostic testing is primarily focused on identifying gene DNA variations that are associated with a pathological disorder. However, accumulating evidence from whole genome (entire human DNA) and whole exome (gene coding DNA regions only) sequencing indicates that there are many 1000s of DNA variations found in healthy individuals and in individuals with a pathological disorder. Since many of the same DNA variants are found in healthy individuals and in a genetic pathology, the diagnostic evaluation of a small number of DNA gene variants alone may not necessarily indicate or confirm an underlying disease process. Such findings may therefore be difficult to fully clinically interpret. A more efficient process for aiding the clinician for interpretation of disease is to link DNA gene variation with both protein expression and protein variant identification. Such diagnostic data would provide evidence that a DNA gene variant is being expressed at the protein level and may therefore be functionally relevant to the disease process. This represents a novel approach for genetic disease diagnostics. Although protein testing is available for bacterial and viral infections, proteomic testing for genetic disease remains in its infancy. Importantly, laboratories using the technique of mass spectrometry have identified key proteins associated with various human genetic diseases. These data therefore provide evidence that proteomic diagnostic testing for genetic disorders are becoming a viable reality.

## Purpose

The purpose of this study was to therefore assess a small number of facilities using the technique of protein mass spectrometry for the detection of human specific proteins. The underlying principle for this pilot study was to identify in the short-term, key areas of problems associated with human protein testing and to determine the level of consistency of laboratory reporting for the proteins tested for. The data from this pilot can then be used to formulate a medium- to long-term strategy for the development of an external quality assurance (EQA) program for this state-of-the art application for human genetic disease diagnostics. Although funding from the Commonwealth for such an early phase pilot study may not be fully representative a standard business model for a QUPP award, the preliminary data produced are nonetheless pivotal for providing key information for the long-term development of a fully operation quality assurance program. Importantly, before any human protein genetic disease diagnostics can be offered and incorporated into the Medicare Benefits Schedule (MBS), an EQA proficiency testing program first needs to be available. Commonwealth funding for new areas of diagnostic developments that are of benefit for long-term patient care are therefore essential as this allows the Royal College of Pathologists of Australasia Quality Assurance Programs (RCPAQAP) to devise and develop novel EQAs alongside current pathological discoveries so that clinical and patient needs can be met and continually addressed.

For this RCPAQAP initiative pilot study, a total of 70 peptides (containing natural wild-type peptides and peptides isotopically labelled with 13C/15N at the C-terminal arginine or lysine peptide) were distributed to five mass spectrometry testing laboratories. 35 peptides (samples A, B, C) were uncorrelated (i.e., were a random generation of wild-type to labelled peptides), and 35 peptides (samples D, E, F) were correlated (i.e., known ratios of wild type to labelled peptide). Four laboratories (80%) successfully submitted data and one laboratory withdrew from the study due to unforeseen laboratory restructuring. The four laboratories that submitted data also encountered problems with the reference testing samples. For example, laboratories reported on high backpressure build-up on peptide filtering columns, and some peptides generally being of poor quality for adequate detection. These issues caused significant delays for result generation and for returning data to the RCPAQAP. However, such laboratory feedback is key information for the RCPAQAP since it allows for modification of future protein reference standard design.

For proficiency testing of the submitted peptide data, the RCPAQAP used a modified similarity and dissimilarity data mining statistical approach. This allowed for laboratory data to be directly compared against the reference standard data for (i) similarity testing (i.e., full peptide identification in comparison to the reference data) and (ii) dissimilarity testing (i.e., comparison to the reference data of the wild-type to labelled peptide ratios). Percentage values were then derived for final comparative analysis against the expected EQA reference data. In total, none of the four laboratories fully identified all peptides in all samples. For peptide similarity testing, laboratory peptide identification ranged from 66% to 97% similar in comparison to the reference data. For peptide ratio dissimilarity testing, ratio values ranged from 3% to 96% dissimilar in comparison to the reference data. In addition, the correlated samples (D, E, F) were more readily identified by laboratories than the uncorrelated samples (A, B, C) and may suggest an issue with the EQA reference samples, or with each laboratory’s mass spectrometer calibration setup. The advantage of using the similarity dissimilarity statistical approach for genetic protein diagnostic analysis is that it allows us to identify the performance of each laboratory for their ability to (i) detect and identify unknown peptides, and (ii) to determine each laboratories ability to characterise reference peptide ratio values. This strategy is key for providing constructive feedback to each laboratory’s performance so that areas of concern can be identified and improved.

The current Commonwealth funded project has allowed the RCPAQAP to identify key areas for improvement for the development an EQA for human protein diagnostics. In the short-term, the data generated from this study highlighted two primary areas for improvement. Firstly, the EQA reference material needs to be of better quality since each laboratory reported similar encountered problems. Secondly, there appears to be calibration issues relating to the mass spectrometry equipment used given that some laboratories were less proficient at detecting the unknown peptides and ratio values. Nonetheless, the data produced indicates that protein diagnostics using human peptides is certainly possible. In the mid-term, a second study is now required to encompass a larger cohort of laboratories. Such a second study allows the RCPAQAP to address the findings from the current study and improve on the development of a protein specific EQA. In the long-term, an EQA for protein mass spectrometry will be key for diagnosing the complex diseases of cancer (i.e., breast, lung, prostate gastrointestinal), neurological disorders (i.e., Alzheimer, Parkinson, Huntington, epilepsy), and age-related disease (i.e., cardiovascular disease). Thus, proteomic results in combination with identified genetic alterations will allow clinicians to make better informed decisions with respect to pharmaceutical intervention and patient management.

# Aim of the Study

(*What is the aim/purpose of the project?*)

The primary aim of this study was to assess facilities using the technique of protein mass spectrometry for the detection of human peptides. The reason being that key malfunctioning proteins have been detected in various human diseases and proteomic diagnostic testing is therefore becoming a viable reality. However, before any proteomic diagnostics can be offered, an EQA proficiency testing program needs to be available. The RCPAQAP therefore devised and performed an EQA trial study to determine the ability of mass spectrometry testing facilities to correctly identify a range of unknown reference testing protein standards.

# Background

*(What is the overview of the project and its importance for disease diagnostics?)*

Identifying abnormal proteins in response to genetic DNA variation, microbial infection, or as a consequence of another underlying disease pathology is key to understanding the human disease process. As such, key organisations are now aiming to identify human gene expressed proteins. In particular, the Human Proteome Organization (HUPO) are currently performing a study designed to map the entire human proteome using the technique of mass spectrometry together with new and emerging technologies. Completion of the HUPO project is expected to enhance our understanding of human biology at the cellular level and to provide a foundation for future developments of diagnostic, prognostic, therapeutic, and preventive medical applications. HUPO have currently identified 93% of the human proteome with 19,823 human proteins being categorised (https://www.hupo.org/).

In addition to the HUPO study, protein biomarkers are also being increasingly recognised in different human diseases. For example, rapid improvements in mass spectrometry technology has enabled the identification of key proteins associated with kidney disease, lung disease, and cancer (Picken 2015; Pontillo and Mischak 2017; Callejón-Leblic et al., 2016; Jones et al., 2016; Ohlmeier et al., 2016). Disease-specific biomarker proteins have also been found to be circulating in blood and to be excreted in urine (Bansal et al., 2016; Chen and Kim, 2016; Lu et al., 2016). These tissues are termed liquid biopsy tissue and the key advantage for proteomic diagnostic testing of these is that the invasive procedures of pathology tissue excision and tissue biopsy do not need to be performed which is more efficient and much less stressful for the patient. Importantly, a protein liquid biopsy test has now been developed for the detection of circulating blood proteins that are associated with cancer (https://www.biotechsupportgroup.com/Stroma-Liquid-Biopsy-s/287.htm). Tests such as these will further enhance a laboratory’s ability to detect cancer at a much earlier stage. Given the plethora of proteins being identified in disease, there is now a need and demand for the development of proteomic diagnostic testing. It is therefore essential that an EQA program be devised so that proficiency testing can be offered to laboratories for near future clinical proteomic diagnostics.

This project was therefore designed to quality assess the technical ability of laboratories to accurately detect and report on protein targets using the high-throughput sensitive technique of mass spectrometry. The development of a proteomic EQA will allow laboratories to enrol for proficiency testing in this new key area of clinical diagnostics.

# Addressing essential needs

(*What need/s will this project address?*)

Recent publications (including data presented at international and national conferences) reveal that there is a high need for quality metrics to be designed for monitoring of mass spectrometry proteomic analysis (Hoofnagle et al., 2016; Sanchez-Niño et al., 2017). Furthermore, with HUPO nearing completion of the human proteome, clinical proteomic diagnostic testing is becoming more likely (Sanchez-Niño et al., 2017). Importantly, combining genetic and proteomic analyses in the near future will enable greater clinical diagnostic power and disease prediction, and may also allow for early detection of underlying molecular disease processes (Cohen et al., 2017, 2018). There is therefore an essential requirement to meet technological developments and diagnostic improvements with an external quality assurance program so consistency of diagnoses can be monitored. This is of key concern especially if laboratories inadvertently miscall protein expression or post translational modifications and provide false negative or false positive reports to the referring clinician. In addition, there is an unmet need in post market surveillance for monitoring disease-specific proteins associated with treatment resistance in response to pharmaceutical intervention. This critical information will aid in the key decision-making process for patient treatment.

# Benefits

(*What benefit will the project be to consumers of pathology services?*)

Proteomic testing in disease pathology is growing and represents an essential strategy to determine key underlying molecular mechanisms involved in the disease process. The identification of protein biomarkers in blood serum and urine is causing a shift in thinking away from the traditional practise of tissue excision/biopsy to now focus on circulating or excreted proteins for diagnostic characterisation. This non-invasive nature of testing makes it a very attractive technique over the invasiveness of tissue excision/biopsy surgery and of the risks associated with this. A quality assurance program allows laboratories to directly compare data so that problems can be rectified, and levels of consistency can be maintained.

Proteomics tests can generate complex data and understanding their clinical significance is key as this will reflect in the clinical management of the patient. The ability to monitor the levels of circulating or excreted proteins in response to ongoing pharmaceutical intervention will help address the clinical significance between pharmaceutical treatment and patient response. This is particularly important in the monitoring of proteins that are associated with pharmaceutical intervention resistance (i.e., the epidermal growth factor receptor (EGFR) protein variant (p.Thr790Met) in non-small cell lung cancer) or in the monitoring of a patient’s response to compounds designed to overcome such tumour resistance (Kobayashi et al 2005; Ku et al., 2016). The need for quality assurance is therefore critical since the accuracy of these diagnostic tests will greatly aid the clinician for appropriate patient management. Furthermore, early patient diagnosis may improve clinical treatment and significantly reduce associated healthcare costs.

Quality monitoring disease-associated proteins will ultimately allow clinicians to make better informed decisions with respect to pharmaceutical intervention and patient management. The RCPAQAP also provide valuable educational components, which are produced in collaboration with key stakeholders (including pathologists) and members of the RCPAQAP genetics Advisory Committee. These are of benefit to pathology communities comprising scientists, clinical geneticists, genetics pathologists and oncologists.

# Reference samples

(*What were the samples used in the project*)

The reference testing peptide samples used for this project were designed and developed by MRM Proteomics (https://mrmproteomics.com/). Two sets of reference peptide mixes were produced. Each reference mix contained 35 standard synthetic peptides in two different isotopic forms: natural abundance (light peptide) and isotopically labelled with 13C/15N at the C-terminal arginine or lysine (Stable Isotope-labelled Standard peptide or SIS peptide). Each set of reference peptide mixes include three different samples, where the concentration of light peptides varies, but the concentration of SIS peptides remains constant. The concentrations of each peptide relative to other peptides vary. In the first set of peptides the light/SIS peptide ratios are uncorrelated, meaning that each peptide ratio varies with respect to other peptides in the sample and also between reference samples in the same set. The second set of reference samples are correlated, meaning that the light/SIS peptide ratios are similar between each peptide in the same reference sample and also vary similarly between reference samples. Table 1 below summarizes the reference samples used in the study and distributed to each laboratory. The proteins and peptide sequences are provided in the Appendix.

**Table 1**. Reference testing samples used for mass spectrometry proficiency testing.

| Reference Set | Reference sample | Aliquots provided (tubes) | Aliquot volumes (µl) |
| --- | --- | --- | --- |
| Uncorrelated | A | 40 | 50 |
| Uncorrelated | B | 40 | 50 |
| Uncorrelated | C | 40 | 50 |
| Correlated | D | 40 | 50 |
| Correlated | E | 40 | 50 |
| Correlated | F | 40 | 50 |

# Methods

(*Technical background of the study*)

## Laboratories

A total of five mass spectrometry facilities agreed to participate in the RCPAQAP trial proteomic proficiency testing program. These facilities were based at Western Australia (two laboratories), New South Wales (two laboratories), and South Australia (one laboratory).

## Peptide mix preparation

Stocks of the various peptide standards (both light and SIS) were pooled together to produce the two sets of peptide reference samples. The mixes were prepared, lyophilized and rehydrated in digested bovine serum albumin (BSA) in 0.1% formic acid before aliquoting. The presence of peptides from digested BSA helps reduce the potential variability due to adsorption of peptide to labware and provides a more representative background matrix for the analysis of the proteomics reference sample. Briefly, the two sets of reference samples were prepared with the following guidelines:

### Uncorrelated Reference Samples (A, B, C)

1. Each sample contained 35 peptides in the SIS & light form.
2. The concentration of each SIS peptide was fixed across the three A, B, C reference samples.
3. The concentration of each light peptide varied across the three A, B, C reference samples.
4. The ratios of each SIS peptide to its corresponding light peptide for the three reference samples was quasi-random. There was an approximate 25-fold range for the concentration of each peptide across the three samples.
5. The concentrations for each peptide in a given reference sample are uncorrelated to those in the other reference samples.

### Correlated Reference Samples (D, E, F)

1. Each sample contained 35 peptides in the SIS & light forms.
2. The concentration of each SIS peptide was fixed across the three D, E, F reference samples.
3. The concentration of each light peptide varied across the three D, E, F reference samples.
4. The ratios of each SIS peptide to its corresponding light peptide for the three reference samples was approximately 1:0.2, 1:1, 1:5; giving a 25-fold range for the concentration of each peptide across the 3 samples.

## Reference testing of peptide samples

Five replicate injections of 10 µL of each reference sample were analysed by liquid chromatography–mass spectrometry (LC-MS). Samples were separated with a Zorbax Eclipse Plus RP-UHPLC column (2.1 x 150 mm, 1.8 µm particle diameter; Agilent) with a 1290 Infinity system (Agilent). Peptide separations were achieved at 0.4 mL/min over a 60 min run, via a multi-step LC gradient (2-80% mobile phase B; mobile phase compositions: A was 0.1% FA in water while B was 0.1% FA in acetonitrile). The column was maintained at 50°C. A post-gradient column re-equilibration of 4 min was used after each sample analysis.

The LC system was interfaced to a triple quadrupole mass spectrometer (Agilent 6495B) via a standard-flow AJS ESI source, operated in the positive ion mode. The general MRM acquisition parameters employed were as follows: 3.5 kV capillary voltage, 300 V nozzle voltage, 11 L/min sheath gas flow at a temperature of 250 °C, 15 L/min drying gas flow at a temperature of 150 °C, 30 psi nebulizer gas pressure, 380 V fragmentor voltage, 5 V cell accelerator potential, and unit mass resolution in the first and third quadrupole mass analysers. The high energy dynode (HED) multiplier was set to -20 kV for improved ion detection efficiency and signal-to-noise ratios. Specific LC-MS acquisition parameters were employed for optimal peptide ionization/fragmentation and scheduled MRM. Note that the peptide optimizations were empirically optimized previously by direct infusion of the purified SIS peptides. In the quantitative analysis, the targets were monitored over 1.5 min detection windows.

The MRM data was visualized and examined with Skyline Daily Quantitative Analysis software (version 3.7.1.11571, University of Washington). This involved peak inspection to ensure accurate selection, integration, and uniformity (in terms of peak shape and retention time) of the SIS and light peptides. The average light/SIS peptide ratios were calculated and the precision of the five measurements were tabulated (expressed as %CV) using the sum of the top three MRM transitions.

## RCPAQAP Proficiency test analysis

To determine the proficiency of each laboratory for detecting unknown protein peptides, each laboratory’s data were directly compared against the MRM Proteomic reference standard data to firstly confirm that the identity of each peptide was correct, and secondly, to identify that the ratios of the two sets of 35 peptides matched that of reference data. For analysis, an RCPAQAP proficiency test scoring system was devised using a modified similarity dissimilarity statistical test (Clarke, 1993). A percentage comparison to the reference standard data could therefore be derived for similarity testing (i.e., peptide identification) and dissimilarity testing (i.e., peptide ratio values). In this way, the proficiency of each laboratory for identifying unknown peptides and peptide ratio values could be fully determined. These data are key for diagnostic purposes given that novel peptides may be associated with different diseases.

Coefficients of similarity and dissimilarity calculation (Example):

**Protein Reference Ratio Laboratory Ratio Difference in Ratios Sum of Ratios**

Protein 1 0.098 0.111 0.013 0.209

Protein 2 0.063 0.065 0.002 0.128

Protein 3 0.042 0.049 0.007 0.091

Proteins

Detected 3 3

Sum 0.022 0.428

Similarity (protein identification) 3/3 = 1 (100%)

Dissimilarity (protein ratio identification) 0.022/0.428 = 0.05 (5%)

In the above example, the laboratory identified the same proteins as contained in the reference (i.e. were 100% similar) and their ratio values to the reference ratio values were only 5% dissimilar.

# Results

(*Results produced from the study*)

## Laboratories

Of the five laboratories agreeing to participate in this trial proficiency test, 80% (4/5) submitted data and one laboratory withdrew from the study.

## RCPAQAP Proficiency testing analysis

### Identification of peptide sequence for uncorrelated standards (Samples A, B, C)

Each laboratory submitted their peptide sequence data. For the uncorrelated proteins, Laboratory 1 could detect all reference sequences except for the Alpha-1-antichymotrypsin peptide (Table 2). Laboratory 2 could not perform a test for the uncorrelated samples and were not assessed, Laboratory 3 detected 23 peptides and Laboratory 4 detected 30 peptide sequences (Table 2).

### Identification of peptide sequence for correlated standards (Samples D, E, F)

For the correlated proteins, Laboratory 1 did not perform testing of Sample F but could detect all reference sequences in Samples D and E except for the Alpha-1-antichymotrypsin peptide (Table 3). Laboratory 2 detected 33 peptides, Laboratory 3 detected 30 peptides and Laboratory 4 detected 30 peptide sequences (Table 3).

### RCPAQAP proficiency test (PT) scoring of all protein standards (Samples A - F)

Laboratory similarity dissimilarity raw data and PT scores for the detection of each protein are listed in Tables 4 - 9. The percentage of similarity (protein identification) and dissimilarity (protein ratios) for each sample tested are presented in Table 10.

**Table 2**. Confirmation of laboratory detection for the reference testing of uncorrelated peptides.

| **Uncorrelated Proteins** | **Peptide Sequence** | **Protein Sequence** | **Protein Sequence** | **Protein Sequence** | **Protein Sequence** |
| --- | --- | --- | --- | --- | --- |
|  |  | **Lab 1** | **Lab 2** | **Lab 3** | **Lab 4** |
| Adiponectin | IFYNQQNHYDGSTGK | YES | NA | YES | YES |
| Afamin | DADPDTFFAK | YES | NA | NO | YES |
| Alpha-1-acid glycoprotein 1 | NWGLSVYADKPETTK | YES | NA | YES | YES |
| Alpha-1-antichymotrypsin | EIGELYLPK | NO | NA | NO | YES |
| Alpha-2-antiplasmin | LGNQEPGGQTALK | YES | NA | YES | YES |
| Alpha-2-macroglobulin | AIGYLNTGYQR | YES | NA | YES | YES |
| Apolipoprotein A-I | ATEHLSTLSEK | YES | NA | YES | YES |
| Apolipoprotein A-IV | LGEVNTYAGDLQK | YES | NA | YES | YES |
| Apolipoprotein B-100 | FPEVDVLTK | YES | NA | YES | YES |
| Apolipoprotein E | LGPLVEQGR | YES | NA | YES | YES |
| Attractin | SVNNVVVR | YES | NA | NO | YES |
| Beta-2-glycoprotein 1 | ATVVYQGER | YES | NA | YES | YES |
| Biotinidase | SHLIIAQVAK | YES | NA | NO | NO |
| Carbonic anhydrase 1 | VLDALQAIK | YES | NA | NO | YES |
| CD5 antigen-like | LVGGLHR | YES | NA | NO | YES |
| Cholinesterase | YLTLNTESTR | YES | NA | YES | NO |
| Clusterin | ELDESLQVAER | YES | NA | YES | YES |
| Coagulation factor XII | EQPPSLTR | YES | NA | NO | NO |
| Complement C1r subcomponent | GLTLHLK | YES | NA | NO | YES |
| Complement C3 | TGLQEVEVK | YES | NA | YES | YES |
| Complement component C9 | LSPIYNLVPVK | YES | NA | YES | YES |
| Complement factor B | EELLPAQDIK | YES | NA | NO | YES |
| Fibulin-1 | TGYYFDGISR | YES | NA | NO | YES |
| Hemoglobin subunit alpha | VGAHAGEYGAEALER | YES | NA | NO | YES |
| Hemopexin | NFPSPVDAAFR | YES | NA | YES | YES |
| Heparin cofactor 2 | TLEAQLTPR | YES | NA | YES | YES |
| Hyaluronan-binding protein 2 | VVLGDQDLK | YES | NA | YES | YES |
| Inter-alpha-trypsin inhibitor heavy chain H2 | SLAPTAAAK | YES | NA | YES | YES |
| Kininogen-1 | TVGSDTFYSFK | YES | NA | YES | YES |
| Pigment epithelium-derived factor | LQSLFDSPDFSK | YES | NA | YES | YES |
| Plasma protease C1 inhibitor | FQPTLLTLPR | YES | NA | YES | NO |
| Plasminogen | LFLEPTR | YES | NA | NO | YES |
| Prothrombin | ELLESYIDGR | YES | NA | YES | NO |
| Serotransferrin | DGAGDVAFVK | YES | NA | YES | YES |
| Vitronectin | FEDGVLDPDYPR | YES | NA | YES | YES |

\*NA (not assessed). Detection not applicable to Lab 2 as testing of the uncorrelated samples could not be performed.

**Table 3**. Confirmation of laboratory detection for the reference testing of correlated peptides

| **Uncorrelated Proteins** | **Peptide Sequence** | **Protein Sequence** | **Protein Sequence** | **Protein Sequence** | **Protein Sequence** |
| --- | --- | --- | --- | --- | --- |
|  |  | **Lab 1** | **Lab 2** | **Lab 3** | **Lab 4** |
| Adiponectin | IFYNQQNHYDGSTGK | YES | YES | YES | YES |
| Afamin | DADPDTFFAK | YES | YES | YES | YES |
| Alpha-1-acid glycoprotein 1 | NWGLSVYADKPETTK | YES | YES | YES | YES |
| Alpha-1-antichymotrypsin | EIGELYLPK | NO | YES | YES | YES |
| Alpha-1-antitrypsin | LSITGTYDLK | YES | YES | NO | NO |
| Alpha-2-antiplasmin | LGNQEPGGQTALK | YES | YES | YES | YES |
| Alpha-2-macroglobulin | AIGYLNTGYQR | YES | YES | YES | YES |
| Antithrombin-III | DDLYVSDAFHK | YES | NO | NO | NO |
| Apolipoprotein A-I | ATEHLSTLSEK | YES | YES | YES | YES |
| Apolipoprotein A-IV | LGEVNTYAGDLQK | YES | YES | YES | YES |
| Apolipoprotein B-100 | FPEVDVLTK | YES | YES | YES | YES |
| Apolipoprotein E | LGPLVEQGR | YES | YES | YES | YES |
| Attractin | SVNNVVVR | YES | NO | YES | YES |
| Beta-2-glycoprotein 1 | ATVVYQGER | YES | YES | YES | YES |
| Carbonic anhydrase 1 | VLDALQAIK | YES | YES | YES | YES |
| CD5 antigen-like | LVGGLHR | YES | YES | NO | YES |
| Clusterin | ELDESLQVAER | YES | YES | YES | YES |
| Complement C1r subcomponent | GLTLHLK | YES | YES | NO | YES |
| Complement C3 | TGLQEVEVK | YES | YES | YES | YES |
| Complement component C9 | LSPIYNLVPVK | YES | YES | YES | YES |
| Complement factor B | EELLPAQDIK | YES | YES | NO | YES |
| Fibrinogen gamma chain | YEASILTHDSSIR | YES | YES | YES | NO |
| Fibulin-1 | TGYYFDGISR | YES | YES | YES | YES |
| Haptoglobin | DIAPTLTLYVGK | YES | YES | YES | NO |
| Hemoglobin subunit alpha | VGAHAGEYGAEALER | YES | YES | YES | YES |
| Hemopexin | NFPSPVDAAFR | YES | YES | YES | YES |
| Heparin cofactor 2 | TLEAQLTPR | YES | YES | YES | YES |
| Hyaluronan-binding protein 2 | VVLGDQDLK | YES | YES | YES | YES |
| Inter-alpha-trypsin inhibitor heavy chain H2 | SLAPTAAAK | YES | YES | YES | YES |
| Kininogen-1 | TVGSDTFYSFK | YES | YES | YES | YES |
| Pigment epithelium-derived factor | LQSLFDSPDFSK | YES | YES | YES | YES |
| Plasminogen | LFLEPTR | YES | YES | YES | YES |
| Serotransferrin | DGAGDVAFVK | YES | YES | YES | YES |
| Serum albumin | LVNEVTEFAK | YES | YES | YES | NO |
| Vitronectin | FEDGVLDPDYPR | YES | YES | YES | YES |

**Table 4**. Proficiency testing of each laboratory for the uncorrelated proteins in Sample A. (NA = peptide detected but ratio value not available, NP = not performed, 0 = peptide not detected).

| Proteins | Reference  Ratio | Lab 1  Ratio | Difference | Sum | Reference  Ratio | Lab 2  Ratio | Difference | Sum | Reference  Ratio | Lab 3  Ratio | Difference | Sum | Reference  Ratio | Lab 4  Ratio | Difference | Sum |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Adiponectin | 0.098 | 0.111 | 0.014 | 0.209 | 0.098 | NP | 0.098 | 0.098 | 0.098 | 0.01 | 0.088 | 0.108 | 0.098 | 0.09 | 0.004 | 0.191 |
| Afamin | 0.063 | 0.065 | 0.002 | 0.128 | 0.063 | NP | 0.063 | 0.063 | 0.063 | 0 | 0.063 | 0.063 | 0.063 | 0.07 | 0.009 | 0.135 |
| Alpha-1-acid glycoprotein 1 | 0.042 | 0.049 | 0.007 | 0.091 | 0.042 | NP | 0.042 | 0.042 | 0.042 | NA | 0.042 | 0.042 | 0.042 | 0.12 | 0.075 | 0.158 |
| Alpha-1-antichymotrypsin | 0.049 | 0 | 0.049 | 0.049 | 0.049 | NP | 0.049 | 0.049 | 0.049 | 0 | 0.049 | 0.049 | 0.049 | 0.06 | 0.010 | 0.107 |
| Alpha-2-antiplasmin | 2.054 | 2.083 | 0.029 | 4.137 | 2.054 | NP | 2.054 | 2.054 | 2.054 | 0.057 | 1.997 | 2.111 | 2.054 | 2.00 | 0.052 | 4.056 |
| Alpha-2-macroglobulin | 0.133 | 0.132 | 0.001 | 0.265 | 0.133 | NP | 0.133 | 0.133 | 0.133 | 0.01 | 0.123 | 0.143 | 0.133 | 0.15 | 0.017 | 0.282 |
| Apolipoprotein A-I | 0.240 | 0.230 | 0.010 | 0.470 | 0.240 | NP | 0.240 | 0.240 | 0.240 | 0.01 | 0.230 | 0.250 | 0.240 | 0.28 | 0.038 | 0.518 |
| Apolipoprotein A-IV | 2.480 | 2.351 | 0.128 | 4.831 | 2.480 | NP | 2.480 | 2.480 | 2.480 | 0.061 | 2.419 | 2.541 | 2.480 | NA | 2.480 | 2.480 |
| Apolipoprotein B-100 | 1.650 | 1.645 | 0.006 | 3.295 | 1.650 | NP | 1.650 | 1.650 | 1.650 | 0.043 | 1.607 | 1.693 | 1.650 | 1.57 | 0.083 | 3.218 |
| Apolipoprotein E | 0.372 | 0.400 | 0.028 | 0.773 | 0.372 | NP | 0.372 | 0.372 | 0.372 | NA | 0.372 | 0.372 | 0.372 | 0.41 | 0.040 | 0.785 |
| Attractin | 1.261 | 1.249 | 0.012 | 2.510 | 1.261 | NP | 1.261 | 1.261 | 1.261 | 0 | 1.261 | 1.261 | 1.261 | 1.24 | 0.023 | 2.499 |
| Beta-2-glycoprotein 1 | 1.317 | 1.221 | 0.096 | 2.538 | 1.317 | NP | 1.317 | 1.317 | 1.317 | 0.03 | 1.287 | 1.347 | 1.317 | 1.16 | 0.159 | 2.475 |
| Biotinidase | 0.046 | 0.073 | 0.027 | 0.119 | 0.046 | NP | 0.046 | 0.046 | 0.046 | 0 | 0.046 | 0.046 | 0.046 | 0 | 0.046 | 0.046 |
| Carbonic anhydrase 1 | 1.304 | 1.233 | 0.071 | 2.537 | 1.304 | NP | 1.304 | 1.304 | 1.304 | 0 | 1.304 | 1.304 | 1.304 | 0.66 | 0.639 | 1.968 |
| CD5 antigen-like | 0.044 | 0.047 | 0.002 | 0.091 | 0.044 | NP | 0.044 | 0.044 | 0.044 | 0 | 0.044 | 0.044 | 0.044 | 0.05 | 0.003 | 0.092 |
| Cholinesterase | 0.080 | 0.076 | 0.004 | 0.156 | 0.080 | NP | 0.080 | 0.080 | 0.080 | 0.01 | 0.070 | 0.090 | 0.080 | 0 | 0.080 | 0.080 |
| Clusterin | 0.039 | 0.040 | 0.001 | 0.079 | 0.039 | NP | 0.039 | 0.039 | 0.039 | NA | 0.039 | 0.039 | 0.039 | 0.14 | 0.105 | 0.182 |
| Coagulation factor XII | 1.533 | 1.544 | 0.011 | 3.077 | 1.533 | NP | 1.533 | 1.533 | 1.533 | 0 | 1.533 | 1.533 | 1.533 | 0 | 1.533 | 1.533 |
| Complement C1r subcomponent | 0.090 | 0.100 | 0.010 | 0.190 | 0.090 | NP | 0.090 | 0.090 | 0.090 | 0 | 0.090 | 0.090 | 0.090 | 0.16 | 0.065 | 0.245 |
| Complement C3 | 0.301 | 0.335 | 0.034 | 0.636 | 0.301 | NP | 0.301 | 0.301 | 0.301 | 0.01 | 0.291 | 0.311 | 0.301 | 0.31 | 0.007 | 0.609 |
| Complement component C9 | 0.045 | 0.047 | 0.003 | 0.092 | 0.045 | NP | 0.045 | 0.045 | 0.045 | 0.01 | 0.035 | 0.055 | 0.045 | 0.06 | 0.018 | 0.107 |
| Complement factor B | 0.035 | 0.038 | 0.004 | 0.073 | 0.035 | NP | 0.035 | 0.035 | 0.035 | 0 | 0.035 | 0.035 | 0.035 | 0.04 | 0.010 | 0.080 |
| Fibulin-1 | 0.043 | 0.043 | 0.001 | 0.086 | 0.043 | NP | 0.043 | 0.043 | 0.043 | 0 | 0.043 | 0.043 | 0.043 | 0.04 | 0.000 | 0.087 |
| Hemoglobin subunit alpha | 0.070 | 0.052 | 0.018 | 0.122 | 0.070 | NP | 0.070 | 0.070 | 0.070 | 0 | 0.070 | 0.070 | 0.070 | 0.69 | 0.617 | 0.757 |
| Hemopexin | 0.979 | 0.944 | 0.035 | 1.923 | 0.979 | NP | 0.979 | 0.979 | 0.979 | 0.029 | 0.950 | 1.008 | 0.979 | 1.01 | 0.031 | 1.989 |
| Heparin cofactor 2 | 0.034 | 0.038 | 0.004 | 0.072 | 0.034 | NP | 0.034 | 0.034 | 0.034 | 0.01 | 0.024 | 0.044 | 0.034 | NA | 0.034 | 0.034 |
| Hyaluronan-binding protein 2 | 0.058 | 0.073 | 0.015 | 0.131 | 0.058 | NP | 0.058 | 0.058 | 0.058 | 0.01 | 0.048 | 0.068 | 0.058 | 0.23 | 0.172 | 0.288 |
| Inter-alpha-trypsin inhibitor heavy chain H2 | 2.551 | 2.839 | 0.288 | 5.390 | 2.551 | NP | 2.551 | 2.551 | 2.551 | 0.062 | 2.489 | 2.613 | 2.551 | 2.44 | 0.113 | 4.989 |
| Kininogen-1 | 0.188 | 0.176 | 0.012 | 0.364 | 0.188 | NP | 0.188 | 0.188 | 0.188 | 0.01 | 0.178 | 0.198 | 0.188 | 0.20 | 0.014 | 0.390 |
| Pigment epithelium-derived factor | 0.156 | 0.180 | 0.023 | 0.336 | 0.156 | NP | 0.156 | 0.156 | 0.156 | 0.01 | 0.146 | 0.166 | 0.156 | 0.18 | 0.027 | 0.340 |
| Plasma protease C1 inhibitor | 0.683 | 0.633 | 0.051 | 1.316 | 0.683 | NP | 0.683 | 0.683 | 0.683 | 0.017 | 0.666 | 0.700 | 0.683 | 0 | 0.683 | 0.683 |
| Plasminogen | 1.200 | 1.185 | 0.015 | 2.385 | 1.200 | NP | 1.200 | 1.200 | 1.200 | 0 | 1.200 | 1.200 | 1.200 | 1.26 | 0.055 | 2.455 |
| Prothrombin | 0.446 | 0.554 | 0.108 | 1.000 | 0.446 | NP | 0.446 | 0.446 | 0.446 | 0.06 | 0.386 | 0.506 | 0.446 | 0 | 0.446 | 0.446 |
| Serotransferrin | 0.215 | 0.259 | 0.044 | 0.474 | 0.215 | NP | 0.215 | 0.215 | 0.215 | 0.01 | 0.205 | 0.225 | 0.215 | 0.23 | 0.011 | 0.441 |
| Vitronectin | 0.434 | 0.516 | 0.082 | 0.950 | 0.434 | NP | 0.434 | 0.434 | 0.434 | 0.012 | 0.422 | 0.446 | 0.434 | 0.49 | 0.054 | 0.922 |
| Peptides detected | **35** | **34** |  |  | **35** | **NP** |  |  | **35** | **23** |  |  | **35** | **30** |  |  |
| Sum |  |  | **1.25** | **40.89** |  |  | **20.33** | **20.33** |  |  | **19.85** | **20.81** |  |  | **7.75** | **35.67** |
| Similarity (peptide identification) | **0.97** |  |  |  | **NP** |  |  |  | **0.66** |  |  |  | **0.86** |  |  |  |
| Dissimilarity (peptide ratio identification) | **0.03** |  |  |  | **NP** |  |  |  | **0.95** |  |  |  | **0.22** |  |  |  |

**Table 5**. Proficiency testing of each laboratory for the uncorrelated proteins in Sample B. (NA = peptide detected but ratio value not available, NP = not performed, 0 = peptide not detected).

| Proteins | Reference  Ratio | Lab 1  Ratio | Difference | Sum | Reference  Ratio | Lab 2  Ratio | Difference | Sum | Reference  Ratio | Lab 3  Ratio | Difference | Sum | Reference  Ratio | Lab 4  Ratio | Difference | Sum |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Adiponectin | 0.865 | 0.852 | 0.013 | 1.717 | 0.865 | NP | 0.865 | 0.865 | 0.865 | 0.016 | 0.849 | 0.881 | 0.865 | 0.10 | 0.770 | 0.960 |
| Afamin | 0.476 | 0.450 | 0.026 | 0.926 | 0.476 | NP | 0.476 | 0.476 | 0.476 | 0 | 0.476 | 0.476 | 0.476 | 0.48 | 0.008 | 0.960 |
| Alpha-1-acid glycoprotein 1 | 0.625 | 0.661 | 0.036 | 1.285 | 0.625 | NP | 0.625 | 0.625 | 0.625 | NA | 0.625 | 0.625 | 0.625 | 0.68 | 0.053 | 1.302 |
| Alpha-1-antichymotrypsin | 0.367 | 0 | 0.367 | 0.367 | 0.367 | NP | 0.367 | 0.367 | 0.367 | 0 | 0.367 | 0.367 | 0.367 | 0.34 | 0.027 | 0.707 |
| Alpha-2-antiplasmin | 0.057 | 0.066 | 0.009 | 0.123 | 0.057 | NP | 0.057 | 0.057 | 0.057 | 0.01 | 0.047 | 0.067 | 0.057 | 0.07 | 0.015 | 0.129 |
| Alpha-2-macroglobulin | 0.024 | 0.028 | 0.004 | 0.053 | 0.024 | NP | 0.024 | 0.024 | 0.024 | 0.01 | 0.014 | 0.034 | 0.024 | 0.03 | 0.006 | 0.055 |
| Apolipoprotein A-I | 0.065 | 0.063 | 0.002 | 0.127 | 0.065 | NP | 0.065 | 0.065 | 0.065 | 0.01 | 0.055 | 0.075 | 0.065 | 0.08 | 0.011 | 0.140 |
| Apolipoprotein A-IV | 0.346 | 0.360 | 0.014 | 0.706 | 0.346 | NP | 0.346 | 0.346 | 0.346 | 0.014 | 0.332 | 0.360 | 0.346 | NA | 0.346 | 0.346 |
| Apolipoprotein B-100 | 0.038 | 0.130 | 0.092 | 0.167 | 0.038 | NP | 0.038 | 0.038 | 0.038 | 0.01 | 0.028 | 0.048 | 0.038 | 0.07 | 0.028 | 0.103 |
| Apolipoprotein E | 0.055 | 0.063 | 0.008 | 0.118 | 0.055 | NP | 0.055 | 0.055 | 0.055 | NA | 0.055 | 0.055 | 0.055 | 0.09 | 0.035 | 0.145 |
| Attractin | 0.153 | 0.165 | 0.012 | 0.318 | 0.153 | NP | 0.153 | 0.153 | 0.153 | 0 | 0.153 | 0.153 | 0.153 | NA | 0.153 | 0.153 |
| Beta-2-glycoprotein 1 | 0.228 | 0.221 | 0.007 | 0.449 | 0.228 | NP | 0.228 | 0.228 | 0.228 | 0.01 | 0.218 | 0.238 | 0.228 | 0.21 | 0.020 | 0.435 |
| Biotinidase | 0.082 | 0.108 | 0.026 | 0.190 | 0.082 | NP | 0.082 | 0.082 | 0.082 | 0 | 0.082 | 0.082 | 0.082 | 0 | 0.082 | 0.082 |
| Carbonic anhydrase 1 | 0.325 | 0.323 | 0.003 | 0.648 | 0.325 | NP | 0.325 | 0.325 | 0.325 | 0 | 0.325 | 0.325 | 0.325 | 0.25 | 0.073 | 0.578 |
| CD5 antigen-like | 0.185 | 0.196 | 0.012 | 0.381 | 0.185 | NP | 0.185 | 0.185 | 0.185 | 0 | 0.185 | 0.185 | 0.185 | 0.20 | 0.013 | 0.382 |
| Cholinesterase | 1.222 | 0.969 | 0.253 | 2.191 | 1.222 | NP | 1.222 | 1.222 | 1.222 | 0.031 | 1.191 | 1.253 | 1.222 | 0 | 1.222 | 1.222 |
| Clusterin | 1.669 | 1.636 | 0.033 | 3.305 | 1.669 | NP | 1.669 | 1.669 | 1.669 | NA | 1.669 | 1.669 | 1.669 | 1.67 | 0.004 | 3.334 |
| Coagulation factor XII | 0.264 | 0.287 | 0.023 | 0.551 | 0.264 | NP | 0.264 | 0.264 | 0.264 | 0 | 0.264 | 0.264 | 0.264 | 0 | 0.264 | 0.264 |
| Complement C1r subcomponent | 0.660 | 0.685 | 0.025 | 1.345 | 0.660 | NP | 0.660 | 0.660 | 0.660 | 0 | 0.660 | 0.660 | 0.660 | 0.63 | 0.028 | 1.292 |
| Complement C3 | 1.692 | 1.665 | 0.027 | 3.356 | 1.692 | NP | 1.692 | 1.692 | 1.692 | 0.044 | 1.648 | 1.736 | 1.692 | 1.44 | 0.252 | 3.131 |
| Complement component C9 | 1.882 | 1.752 | 0.130 | 3.633 | 1.882 | NP | 1.882 | 1.882 | 1.882 | 0.044 | 1.838 | 1.926 | 1.882 | 1.83 | 0.047 | 3.716 |
| Complement factor B | 0.184 | 0.189 | 0.005 | 0.373 | 0.184 | NP | 0.184 | 0.184 | 0.184 | 0 | 0.184 | 0.184 | 0.184 | 0.18 | 0.006 | 0.362 |
| Fibulin-1 | 0.706 | 0.662 | 0.045 | 1.368 | 0.706 | NP | 0.706 | 0.706 | 0.706 | 0 | 0.706 | 0.706 | 0.706 | 0.68 | 0.022 | 1.390 |
| Hemoglobin subunit alpha | 1.420 | 1.281 | 0.139 | 2.701 | 1.420 | NP | 1.420 | 1.420 | 1.420 | 0 | 1.420 | 1.420 | 1.420 | 0.58 | 0.843 | 1.997 |
| Hemopexin | 0.044 | 0.051 | 0.007 | 0.095 | 0.044 | NP | 0.044 | 0.044 | 0.044 | 0.01 | 0.034 | 0.054 | 0.044 | 0.07 | 0.026 | 0.113 |
| Heparin cofactor 2 | 0.263 | 0.254 | 0.009 | 0.517 | 0.263 | NP | 0.263 | 0.263 | 0.263 | 0.011 | 0.252 | 0.274 | 0.263 | NA | 0.263 | 0.263 |
| Hyaluronan-binding protein 2 | 0.537 | 0.588 | 0.052 | 1.125 | 0.537 | NP | 0.537 | 0.537 | 0.537 | 0.017 | 0.520 | 0.554 | 0.537 | 0.86 | 0.319 | 1.392 |
| Inter-alpha-trypsin inhibitor heavy chain H2 | 0.329 | 0.240 | 0.090 | 0.569 | 0.329 | NP | 0.329 | 0.329 | 0.329 | 0.01 | 0.319 | 0.339 | 0.329 | 0.32 | 0.007 | 0.651 |
| Kininogen-1 | 0.036 | 0.038 | 0.002 | 0.074 | 0.036 | NP | 0.036 | 0.036 | 0.036 | 0.01 | 0.026 | 0.046 | 0.036 | 0.05 | 0.011 | 0.083 |
| Pigment epithelium-derived factor | 1.053 | 1.000 | 0.054 | 2.053 | 1.053 | NP | 1.053 | 1.053 | 1.053 | 0.029 | 1.024 | 1.082 | 1.053 | 1.02 | 0.037 | 2.070 |
| Plasma protease C1 inhibitor | 0.014 | 0.018 | 0.005 | 0.032 | 0.014 | NP | 0.014 | 0.014 | 0.014 | 0.01 | 0.004 | 0.024 | 0.014 | 0 | 0.014 | 0.014 |
| Plasminogen | 0.031 | 0.043 | 0.011 | 0.074 | 0.031 | NP | 0.031 | 0.031 | 0.031 | 0 | 0.031 | 0.031 | 0.031 | 0.16 | 0.126 | 0.189 |
| Prothrombin | 0.129 | 0.181 | 0.051 | 0.310 | 0.129 | NP | 0.129 | 0.129 | 0.129 | 0.02 | 0.109 | 0.149 | 0.129 | 0 | 0.129 | 0.129 |
| Serotransferrin | 0.058 | 0.070 | 0.013 | 0.128 | 0.058 | NP | 0.058 | 0.058 | 0.058 | 0.01 | 0.048 | 0.068 | 0.058 | 0.07 | 0.008 | 0.123 |
| Vitronectin | 2.219 | 2.425 | 0.205 | 4.644 | 2.219 | NP | 2.219 | 2.219 | 2.219 | 0.061 | 2.158 | 2.280 | 2.219 | 2.22 | 0.001 | 4.440 |
| Peptides detected | **35** | **34** |  |  | **35** | **NP** |  |  | **35** | **23** |  |  | **35** | **30** |  |  |
| Sum |  |  | **1.81** | **36.02** |  |  | **18.30** | **18.30** |  |  | **17.92** | **18.69** |  |  | **5.27** | **32.65** |
| Similarity (peptide identification) | **0.97** |  |  |  | **NP** |  |  |  | **0.66** |  |  |  | **0.86** |  |  |  |
| Dissimilarity (peptide ratio identification) | **0.05** |  |  |  | **NP** |  |  |  | **0.96** |  |  |  | **0.16** |  |  |  |

**Table 6**. Proficiency testing of each laboratory for the uncorrelated proteins in Sample C. (NA = peptide detected but ratio value not available, NP = not performed, 0 = peptide not detected).

| Proteins | Reference  Ratio | Lab 1  Ratio | Difference | Sum | Reference  Ratio | Lab 2  Ratio | Difference | Sum | Reference  Ratio | Lab 3  Ratio | Difference | Sum | Reference  Ratio | Lab 4  Ratio | Difference | Sum |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Adiponectin | 0.026 | 0.034 | 0.008 | 0.060 | 0.026 | NP | 0.026 | 0.026 | 0.026 | 0.01 | 0.016 | 0.036 | 0.026 | 0.14 | 0.109 | 0.161 |
| Afamin | 0.018 | 0.020 | 0.002 | 0.038 | 0.018 | NP | 0.018 | 0.018 | 0.018 | 0 | 0.018 | 0.018 | 0.018 | 0.02 | 0.006 | 0.042 |
| Alpha-1-acid glycoprotein 1 | 0.005 | 0.011 | 0.006 | 0.017 | 0.005 | NP | 0.005 | 0.005 | 0.005 | NA | 0.005 | 0.005 | 0.005 | 0.06 | 0.056 | 0.067 |
| Alpha-1-antichymotrypsin | 1.422 | 0 | 1.422 | 1.422 | 1.422 | NP | 1.422 | 1.422 | 1.422 | 0 | 1.422 | 1.422 | 1.422 | 1.25 | 0.177 | 2.667 |
| Alpha-2-antiplasmin | 0.210 | 0.198 | 0.012 | 0.408 | 0.210 | NP | 0.210 | 0.210 | 0.210 | 0.01 | 0.200 | 0.220 | 0.210 | 0.21 | 0.005 | 0.424 |
| Alpha-2-macroglobulin | 0.724 | 0.842 | 0.118 | 1.567 | 0.724 | NP | 0.724 | 0.724 | 0.724 | 0.02 | 0.704 | 0.744 | 0.724 | 0.74 | 0.012 | 1.460 |
| Apolipoprotein A-I | 1.695 | 1.617 | 0.078 | 3.312 | 1.695 | NP | 1.695 | 1.695 | 1.695 | 0.042 | 1.653 | 1.737 | 1.695 | 1.51 | 0.186 | 3.204 |
| Apolipoprotein A-IV | 0.065 | 0.044 | 0.021 | 0.109 | 0.065 | NP | 0.065 | 0.065 | 0.065 | 0.01 | 0.055 | 0.075 | 0.065 | NA | 0.065 | 0.065 |
| Apolipoprotein B-100 | 0.217 | 0.247 | 0.030 | 0.464 | 0.217 | NP | 0.217 | 0.217 | 0.217 | 0.01 | 0.207 | 0.227 | 0.217 | 0.25 | 0.036 | 0.470 |
| Apolipoprotein E | 2.356 | 2.280 | 0.076 | 4.636 | 2.356 | NP | 2.356 | 2.356 | 2.356 | NA | 2.356 | 2.356 | 2.356 | 2.22 | 0.135 | 4.577 |
| Attractin | 0.060 | 0.064 | 0.005 | 0.124 | 0.060 | NP | 0.060 | 0.060 | 0.060 | 0 | 0.060 | 0.060 | 0.060 | NA | 0.060 | 0.060 |
| Beta-2-glycoprotein 1 | 0.052 | 0.052 | 0.000 | 0.104 | 0.052 | NP | 0.052 | 0.052 | 0.052 | 0.01 | 0.042 | 0.062 | 0.052 | 0.05 | 0.001 | 0.103 |
| Biotinidase | 0.385 | 0.457 | 0.072 | 0.842 | 0.385 | NP | 0.385 | 0.385 | 0.385 | 0 | 0.385 | 0.385 | 0.385 | 0 | 0.385 | 0.385 |
| Carbonic anhydrase 1 | 0.043 | 0.059 | 0.016 | 0.102 | 0.043 | NP | 0.043 | 0.043 | 0.043 | 0 | 0.043 | 0.043 | 0.043 | 0.11 | 0.069 | 0.154 |
| CD5 antigen-like | 1.348 | 1.361 | 0.013 | 2.709 | 1.348 | NP | 1.348 | 1.348 | 1.348 | 0 | 1.348 | 1.348 | 1.348 | 1.42 | 0.075 | 2.771 |
| Cholinesterase | 0.163 | 0.198 | 0.035 | 0.361 | 0.163 | NP | 0.163 | 0.163 | 0.163 | 0.01 | 0.153 | 0.173 | 0.163 | 0 | 0.163 | 0.163 |
| Clusterin | 0.201 | 0.227 | 0.026 | 0.429 | 0.201 | NP | 0.201 | 0.201 | 0.201 | NA | 0.201 | 0.201 | 0.201 | 0.35 | 0.146 | 0.549 |
| Coagulation factor XII | 0.034 | 0.050 | 0.016 | 0.084 | 0.034 | NP | 0.034 | 0.034 | 0.034 | 0 | 0.034 | 0.034 | 0.034 | 0 | 0.034 | 0.034 |
| Complement C1r subcomponent | 0.019 | 0.040 | 0.021 | 0.059 | 0.019 | NP | 0.019 | 0.019 | 0.019 | 0 | 0.019 | 0.019 | 0.019 | 0.07 | 0.054 | 0.093 |
| Complement C3 | 0.040 | 0.048 | 0.008 | 0.088 | 0.040 | NP | 0.040 | 0.040 | 0.040 | 0.01 | 0.030 | 0.050 | 0.040 | 0.05 | 0.012 | 0.092 |
| Complement component C9 | 0.279 | 0.273 | 0.005 | 0.552 | 0.279 | NP | 0.279 | 0.279 | 0.279 | 0.01 | 0.269 | 0.289 | 0.279 | 0.30 | 0.017 | 0.574 |
| Complement factor B | 1.178 | 1.154 | 0.024 | 2.332 | 1.178 | NP | 1.178 | 1.178 | 1.178 | 0 | 1.178 | 1.178 | 1.178 | 1.05 | 0.126 | 2.230 |
| Fibulin-1 | 0.144 | 0.132 | 0.012 | 0.275 | 0.144 | NP | 0.144 | 0.144 | 0.144 | 0 | 0.144 | 0.144 | 0.144 | 0.15 | 0.007 | 0.294 |
| Hemoglobin subunit alpha | 0.212 | 0.285 | 0.073 | 0.497 | 0.212 | NP | 0.212 | 0.212 | 0.212 | 0 | 0.212 | 0.212 | 0.212 | 0.91 | 0.703 | 1.127 |
| Hemopexin | 0.274 | 0.281 | 0.007 | 0.555 | 0.274 | NP | 0.274 | 0.274 | 0.274 | 0.01 | 0.264 | 0.284 | 0.274 | 0.31 | 0.032 | 0.581 |
| Heparin cofactor 2 | 1.892 | 1.775 | 0.117 | 3.667 | 1.892 | NP | 1.892 | 1.892 | 1.892 | 0.051 | 1.841 | 1.943 | 1.892 | NA | 1.892 | 1.892 |
| Hyaluronan-binding protein 2 | 0.123 | 0.136 | 0.013 | 0.260 | 0.123 | NP | 0.123 | 0.123 | 0.123 | 0.01 | 0.113 | 0.133 | 0.123 | 0.48 | 0.352 | 0.598 |
| Inter-alpha-trypsin inhibitor heavy chain H2 | 0.062 | 0.083 | 0.021 | 0.145 | 0.062 | NP | 0.062 | 0.062 | 0.062 | 0.01 | 0.052 | 0.072 | 0.062 | 0.07 | 0.013 | 0.137 |
| Kininogen-1 | 1.129 | NA | 1.129 | 1.129 | 1.129 | NP | 1.129 | 1.129 | 1.129 | 0.032 | 1.097 | 1.161 | 1.129 | 1.11 | 0.020 | 2.237 |
| Pigment epithelium-derived factor | 0.021 | 0.023 | 0.002 | 0.045 | 0.021 | NP | 0.021 | 0.021 | 0.021 | 0.01 | 0.011 | 0.031 | 0.021 | 0.03 | 0.013 | 0.056 |
| Plasma protease C1 inhibitor | 0.093 | 0.106 | 0.013 | 0.199 | 0.093 | NP | 0.093 | 0.093 | 0.093 | 0.01 | 0.083 | 0.103 | 0.093 | 0 | 0.093 | 0.093 |
| Plasminogen | 0.148 | 0.179 | 0.031 | 0.327 | 0.148 | NP | 0.148 | 0.148 | 0.148 | 0 | 0.148 | 0.148 | 0.148 | 0.19 | 0.038 | 0.333 |
| Prothrombin | 0.040 | 0.053 | 0.013 | 0.094 | 0.040 | NP | 0.040 | 0.040 | 0.040 | 0.01 | 0.030 | 0.050 | 0.040 | 0 | 0.040 | 0.040 |
| Serotransferrin | 1.360 | 1.206 | 0.154 | 2.566 | 1.360 | NP | 1.360 | 1.360 | 1.360 | 0.036 | 1.324 | 1.396 | 1.360 | 1.29 | 0.066 | 2.654 |
| Vitronectin | 0.068 | 0.089 | 0.021 | 0.158 | 0.068 | NP | 0.068 | 0.068 | 0.068 | 0.01 | 0.058 | 0.078 | 0.068 | 0.10 | 0.032 | 0.169 |
| Peptides detected | **35** | **34** |  |  | **35** | **NP** |  |  | **35** | **23** |  |  | **35** | **30** |  |  |
| Sum |  |  | **3.62** | **29.73** |  |  | **16.11** | **16.11** |  |  | **15.78** | **16.44** |  |  | **5.23** | **30.56** |
| Similarity (peptide identification) | **0.97** |  |  |  | **NP** |  |  |  | **0.66** |  |  |  | **0.86** |  |  |  |
| Dissimilarity (peptide ratio identification) | **0.12** |  |  |  | **NP** |  |  |  | **0.96** |  |  |  | **0.17** |  |  |  |

**Table 7**. Proficiency testing of each laboratory for the correlated proteins in Sample D. (NA = peptide detected but ratio value not available, 0 = peptide not detected).

| Proteins | Reference  Ratio | Lab 1  Ratio | Difference | Sum | Reference  Ratio | Lab 2  Ratio | Difference | Sum | Reference  Ratio | Lab 3  Ratio | Difference | Sum | Reference  Ratio | Lab 4  Ratio | Difference | Sum |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Adiponectin | 0.084 | 0.089 | 0.005 | 0.173 | 0.084 | 0.11 | 0.026 | 0.194 | 0.084 | 0.088 | 0.004 | 0.172 | 0.084 | 0.16 | 0.079 | 0.247 |
| Afamin | 0.115 | 0.115 | 0.001 | 0.230 | 0.115 | 0.12 | 0.005 | 0.235 | 0.115 | 0.118 | 0.003 | 0.233 | 0.115 | 0.12 | 0.010 | 0.239 |
| Alpha-1-acid glycoprotein 1 | 0.196 | 0.209 | 0.014 | 0.405 | 0.196 | 0.23 | 0.034 | 0.426 | 0.196 | NA | 0.196 | 0.196 | 0.196 | 0.25 | 0.058 | 0.450 |
| Alpha-1-antichymotrypsin | 0.342 | 0 | 0.342 | 0.342 | 0.342 | 0.36 | 0.018 | 0.702 | 0.342 | 0.399 | 0.057 | 0.741 | 0.342 | 0.29 | 0.055 | 0.629 |
| Alpha-1-antitrypsin | 0.168 | 0.166 | 0.002 | 0.333 | 0.168 | 0.17 | 0.002 | 0.338 | 0.168 | 0 | 0.168 | 0.168 | 0.168 | 0 | 0.168 | 0.168 |
| Alpha-2-antiplasmin | 0.265 | 0.279 | 0.014 | 0.544 | 0.265 | 0.28 | 0.015 | 0.545 | 0.265 | 0.296 | 0.031 | 0.561 | 0.265 | 0.28 | 0.013 | 0.543 |
| Alpha-2-macroglobulin | 0.128 | 0.140 | 0.011 | 0.268 | 0.128 | 0.15 | 0.022 | 0.278 | 0.128 | 0.161 | 0.033 | 0.289 | 0.128 | 0.14 | 0.011 | 0.268 |
| Antithrombin-III | 0.016 | 0.020 | 0.003 | 0.036 | 0.016 | 0 | 0.016 | 0.016 | 0.016 | 0 | 0.016 | 0.016 | 0.016 | 0 | 0.016 | 0.016 |
| Apolipoprotein A-I | 0.232 | 0.248 | 0.016 | 0.480 | 0.232 | 0.24 | 0.008 | 0.472 | 0.232 | 0.273 | 0.041 | 0.505 | 0.232 | 0.25 | 0.020 | 0.484 |
| Apolipoprotein A-IV | 0.300 | 0.312 | 0.013 | 0.612 | 0.300 | 0.3 | 0.000 | 0.600 | 0.300 | 0.351 | 0.051 | 0.651 | 0.300 | NA | 0.300 | 0.300 |
| Apolipoprotein B-100 | 0.337 | 0.338 | 0.001 | 0.675 | 0.337 | 0.36 | 0.023 | 0.697 | 0.337 | 0.378 | 0.041 | 0.715 | 0.337 | 0.35 | 0.015 | 0.689 |
| Apolipoprotein E | 0.300 | 0.320 | 0.020 | 0.620 | 0.300 | 0.33 | 0.030 | 0.630 | 0.300 | 0.299 | 0.001 | 0.599 | 0.300 | 0.33 | 0.027 | 0.627 |
| Attractin | 0.155 | 0.177 | 0.022 | 0.331 | 0.155 | 0 | 0.155 | 0.155 | 0.155 | NA | 0.155 | 0.155 | 0.155 | 0.19 | 0.033 | 0.342 |
| Beta-2-glycoprotein 1 | 0.312 | 0.328 | 0.016 | 0.639 | 0.312 | 0.36 | 0.048 | 0.672 | 0.312 | 0.355 | 0.043 | 0.667 | 0.312 | 0.34 | 0.026 | 0.650 |
| Carbonic anhydrase 1 | 0.228 | 0.246 | 0.018 | 0.473 | 0.228 | 0.26 | 0.032 | 0.488 | 0.228 | 0.246 | 0.018 | 0.474 | 0.228 | 0.24 | 0.016 | 0.471 |
| CD5 antigen-like | 0.164 | 0.179 | 0.016 | 0.343 | 0.164 | 0.19 | 0.026 | 0.354 | 0.164 | 0 | 0.164 | 0.164 | 0.164 | 0.19 | 0.024 | 0.352 |
| Clusterin | 0.083 | 0.095 | 0.012 | 0.178 | 0.083 | 0.11 | 0.027 | 0.193 | 0.083 | 0.095 | 0.012 | 0.178 | 0.083 | 0.10 | 0.020 | 0.187 |
| Complement C1r subcomponent | 0.089 | 0.108 | 0.019 | 0.197 | 0.089 | 0.12 | 0.031 | 0.209 | 0.089 | 0 | 0.089 | 0.089 | 0.089 | 0.08 | 0.004 | 0.173 |
| Complement C3 | 0.238 | 0.240 | 0.001 | 0.478 | 0.238 | 0.25 | 0.012 | 0.488 | 0.238 | 0.278 | 0.040 | 0.516 | 0.238 | 0.23 | 0.008 | 0.469 |
| Complement component C9 | 0.196 | 0.200 | 0.005 | 0.396 | 0.196 | 0.23 | 0.034 | 0.426 | 0.196 | 0.214 | 0.018 | 0.410 | 0.196 | 0.21 | 0.017 | 0.408 |
| Complement factor B | 0.257 | 0.260 | 0.003 | 0.518 | 0.257 | 0.83 | 0.573 | 1.087 | 0.257 | 0 | 0.257 | 0.257 | 0.257 | 0.24 | 0.013 | 0.502 |
| Fibrinogen gamma chain | 0.152 | 0.172 | 0.020 | 0.324 | 0.152 | 0.18 | 0.028 | 0.332 | 0.152 | 0.178 | 0.026 | 0.330 | 0.152 | 0 | 0.152 | 0.152 |
| Fibulin-1 | 0.105 | 0.116 | 0.012 | 0.221 | 0.105 | 0.12 | 0.015 | 0.225 | 0.105 | 0.094 | 0.011 | 0.199 | 0.105 | 0.11 | 0.007 | 0.217 |
| Haptoglobin | 0.166 | 0.169 | 0.003 | 0.336 | 0.166 | 0.22 | 0.054 | 0.386 | 0.166 | 0.199 | 0.033 | 0.365 | 0.166 | 0 | 0.166 | 0.166 |
| Hemoglobin subunit alpha | 0.288 | 0.297 | 0.008 | 0.585 | 0.288 | 0.31 | 0.022 | 0.598 | 0.288 | 0.268 | 0.020 | 0.556 | 0.288 | 1.10 | 0.807 | 1.384 |
| Hemopexin | 0.200 | 0.205 | 0.005 | 0.405 | 0.200 | 0.23 | 0.030 | 0.430 | 0.200 | 0.225 | 0.025 | 0.425 | 0.200 | 0.23 | 0.033 | 0.432 |
| Heparin cofactor 2 | 0.266 | 0.270 | 0.004 | 0.535 | 0.266 | 0.26 | 0.006 | 0.526 | 0.266 | 0.274 | 0.008 | 0.540 | 0.266 | 0.27 | 0.000 | 0.532 |
| Hyaluronan-binding protein 2 | 0.134 | 0.137 | 0.003 | 0.270 | 0.134 | 0.13 | 0.004 | 0.264 | 0.134 | 0.136 | 0.002 | 0.270 | 0.134 | 0.17 | 0.037 | 0.304 |
| Inter-alpha-trypsin inhibitor heavy chain H2 | 0.361 | 0.370 | 0.009 | 0.731 | 0.361 | 0.37 | 0.009 | 0.731 | 0.361 | 0.385 | 0.024 | 0.746 | 0.361 | 0.36 | 0.000 | 0.722 |
| Kininogen-1 | 0.123 | 0.118 | 0.004 | 0.241 | 0.123 | 0.13 | 0.008 | 0.253 | 0.123 | 0.141 | 0.019 | 0.264 | 0.123 | 0.13 | 0.004 | 0.249 |
| Pigment epithelium-derived factor | 0.128 | 0.132 | 0.004 | 0.260 | 0.128 | 0.14 | 0.012 | 0.268 | 0.128 | 0.137 | 0.009 | 0.265 | 0.128 | 0.14 | 0.010 | 0.266 |
| Plasminogen | 0.179 | 0.199 | 0.020 | 0.378 | 0.179 | 0.23 | 0.051 | 0.409 | 0.179 | NA | 0.179 | 0.179 | 0.179 | 0.17 | 0.008 | 0.349 |
| Serotransferrin | 0.247 | 0.243 | 0.004 | 0.490 | 0.247 | 0.27 | 0.023 | 0.517 | 0.247 | 0.247 | 0.000 | 0.494 | 0.247 | 0.26 | 0.016 | 0.510 |
| Serum albumin | 0.242 | 0.232 | 0.010 | 0.474 | 0.242 | 0.27 | 0.028 | 0.512 | 0.242 | 1.43 | 1.188 | 1.672 | 0.242 | 0 | 0.242 | 0.242 |
| Vitronectin | 0.600 | 0.633 | 0.032 | 1.233 | 0.600 | 0.6 | 0.000 | 1.200 | 0.600 | 0.582 | 0.018 | 1.182 | 0.600 | 0.59 | 0.013 | 1.188 |
| Peptides detected | **35** | **34** |  |  | **35** | **33** |  |  | **35** | **30** |  |  | **35** | **30** |  |  |
| Sum |  |  | **0.69** | **14.75** |  |  | **1.43** | **15.85** |  |  | **3.0** | **15.24** |  |  | **2.43** | **14.93** |
| Similarity (peptide identification) | **0.97** |  |  |  | **0.94** |  |  |  | **0.86** |  |  |  | **0.86** |  |  |  |
| Dissimilarity (peptide ratio identification) | **0.05** |  |  |  | **0.09** |  |  |  | **0.19** |  |  |  | **0.16** |  |  |  |

**Table 8**. Proficiency testing of each laboratory for the correlated proteins in Sample E. (NA = peptide detected but ratio value not available, 0 = peptide not detected).

| Proteins | Reference  Ratio | Lab 1  Ratio | Difference | Sum | Reference  Ratio | Lab 2  Ratio | Difference | Sum | Reference  Ratio | Lab 3  Ratio | Difference | Sum | Reference  Ratio | Lab 4  Ratio | Difference | Sum |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Adiponectin | 0.538 | 0.541 | 0.002 | 1.079 | 0.538 | 0.57 | 0.032 | 1.108 | 0.538 | 0.259 | 0.279 | 0.797 | 0.538 | 0.89 | 0.352 | 1.429 |
| Afamin | 0.705 | 0.674 | 0.031 | 1.379 | 0.705 | 0.69 | 0.015 | 1.395 | 0.705 | 0.335 | 0.370 | 1.040 | 0.705 | 0.94 | 0.237 | 1.647 |
| Alpha-1-acid glycoprotein 1 | 1.189 | 1.266 | 0.077 | 2.455 | 1.189 | 1.34 | 0.151 | 2.529 | 1.189 | NA | 1.189 | 1.189 | 1.189 | 2.01 | 0.824 | 3.202 |
| Alpha-1-antichymotrypsin | 2.050 | 0.000 | 2.050 | 2.050 | 2.050 | 1.98 | 0.070 | 4.030 | 2.050 | 0.938 | 1.112 | 2.988 | 2.050 | 2.34 | 0.291 | 4.392 |
| Alpha-1-antitrypsin | 0.989 | 0.975 | 0.014 | 1.964 | 0.989 | 1.01 | 0.021 | 1.999 | 0.989 | 0 | 0.989 | 0.989 | 0.989 | 0 | 0.989 | 0.989 |
| Alpha-2-antiplasmin | 1.550 | 1.633 | 0.083 | 3.182 | 1.550 | 1.6 | 0.051 | 3.150 | 1.550 | 0.8 | 0.750 | 2.350 | 1.550 | 2.16 | 0.615 | 3.714 |
| Alpha-2-macroglobulin | 0.797 | 0.799 | 0.002 | 1.596 | 0.797 | 0.85 | 0.053 | 1.647 | 0.797 | 0.393 | 0.404 | 1.190 | 0.797 | 1.15 | 0.353 | 1.947 |
| Antithrombin-III | 0.100 | 0.111 | 0.011 | 0.211 | 0.100 | 0 | 0.100 | 0.100 | 0.100 | 0 | 0.100 | 0.100 | 0.100 | 0 | 0.100 | 0.100 |
| Apolipoprotein A-I | 1.448 | 1.429 | 0.019 | 2.877 | 1.448 | 1.42 | 0.028 | 2.868 | 1.448 | 0.755 | 0.693 | 2.203 | 1.448 | 2.07 | 0.618 | 3.514 |
| Apolipoprotein A-IV | 1.864 | 1.915 | 0.051 | 3.780 | 1.864 | 1.75 | 0.114 | 3.614 | 1.864 | 0.852 | 1.012 | 2.716 | 1.864 | NA | 1.864 | 1.864 |
| Apolipoprotein B-100 | 2.055 | 1.913 | 0.142 | 3.969 | 2.055 | 1.99 | 0.065 | 4.045 | 2.055 | 0.953 | 1.102 | 3.008 | 2.055 | 3.35 | 1.299 | 5.409 |
| Apolipoprotein E | 1.841 | 1.794 | 0.047 | 3.635 | 1.841 | 1.88 | 0.039 | 3.721 | 1.841 | 0.925 | 0.916 | 2.766 | 1.841 | 2.63 | 0.790 | 4.472 |
| Attractin | 0.967 | 0.991 | 0.024 | 1.958 | 0.967 | 0 | 0.967 | 0.967 | 0.967 | NA | 0.967 | 0.967 | 0.967 | 1.44 | 0.469 | 2.403 |
| Beta-2-glycoprotein 1 | 1.959 | 1.880 | 0.079 | 3.839 | 1.959 | 1.93 | 0.029 | 3.889 | 1.959 | 0.957 | 1.002 | 2.916 | 1.959 | 2.60 | 0.642 | 4.560 |
| Carbonic anhydrase 1 | 1.497 | 1.444 | 0.053 | 2.942 | 1.497 | 1.41 | 0.087 | 2.907 | 1.497 | 0.704 | 0.793 | 2.201 | 1.497 | 1.89 | 0.388 | 3.383 |
| CD5 antigen-like | 0.957 | 1.039 | 0.082 | 1.997 | 0.957 | 1.16 | 0.203 | 2.117 | 0.957 | 0 | 0.957 | 0.957 | 0.957 | 1.43 | 0.471 | 2.386 |
| Clusterin | 0.546 | 0.552 | 0.006 | 1.098 | 0.546 | 0.58 | 0.034 | 1.126 | 0.546 | 0.302 | 0.244 | 0.848 | 0.546 | 0.87 | 0.321 | 1.413 |
| Complement C1r subcomponent | 0.575 | 0.650 | 0.075 | 1.226 | 0.575 | 0.61 | 0.035 | 1.185 | 0.575 | 0 | 0.575 | 0.575 | 0.575 | 0.72 | 0.145 | 1.295 |
| Complement C3 | 1.411 | 1.387 | 0.024 | 2.798 | 1.411 | 1.35 | 0.061 | 2.761 | 1.411 | 0.739 | 0.672 | 2.150 | 1.411 | 1.83 | 0.415 | 3.238 |
| Complement component C9 | 1.155 | 1.136 | 0.019 | 2.291 | 1.155 | 1.2 | 0.045 | 2.355 | 1.155 | 0.559 | 0.596 | 1.714 | 1.155 | 1.80 | 0.641 | 2.952 |
| Complement factor B | 1.577 | 1.491 | 0.086 | 3.068 | 1.577 | 2.06 | 0.483 | 3.637 | 1.577 | 0 | 1.577 | 1.577 | 1.577 | 2.04 | 0.464 | 3.618 |
| Fibrinogen gamma chain | 0.967 | 0.978 | 0.011 | 1.945 | 0.967 | 0.97 | 0.003 | 1.937 | 0.967 | 0.48 | 0.487 | 1.447 | 0.967 | 0 | 0.967 | 0.967 |
| Fibulin-1 | 0.648 | 0.640 | 0.008 | 1.288 | 0.648 | 0.66 | 0.012 | 1.308 | 0.648 | 0.363 | 0.285 | 1.011 | 0.648 | 0.94 | 0.296 | 1.592 |
| Haptoglobin | 1.034 | 0.992 | 0.042 | 2.026 | 1.034 | 1.07 | 0.036 | 2.104 | 1.034 | 0.532 | 0.502 | 1.566 | 1.034 | 0 | 1.034 | 1.034 |
| Hemoglobin subunit alpha | 1.770 | 1.716 | 0.054 | 3.486 | 1.770 | 1.78 | 0.010 | 3.550 | 1.770 | 0.933 | 0.837 | 2.703 | 1.770 | 1.07 | 0.696 | 2.844 |
| Hemopexin | 1.214 | 1.192 | 0.023 | 2.406 | 1.214 | 1.21 | 0.004 | 2.424 | 1.214 | 0.586 | 0.628 | 1.800 | 1.214 | 1.79 | 0.573 | 3.002 |
| Heparin cofactor 2 | 1.631 | 1.536 | 0.095 | 3.166 | 1.631 | 1.38 | 0.251 | 3.011 | 1.631 | 0.771 | 0.860 | 2.402 | 1.631 | 2.09 | 0.462 | 3.723 |
| Hyaluronan-binding protein 2 | 0.845 | 0.815 | 0.029 | 1.660 | 0.845 | 0.75 | 0.095 | 1.595 | 0.845 | 0.408 | 0.437 | 1.253 | 0.845 | 1.12 | 0.271 | 1.961 |
| Inter-alpha-trypsin inhibitor heavy chain H2 | 2.191 | 1.969 | 0.221 | 4.160 | 2.191 | 2.08 | 0.111 | 4.271 | 2.191 | 0.998 | 1.193 | 3.189 | 2.191 | 2.88 | 0.693 | 5.075 |
| Kininogen-1 | 0.736 | 0.728 | 0.008 | 1.464 | 0.736 | 0.73 | 0.006 | 1.466 | 0.736 | 0.364 | 0.372 | 1.100 | 0.736 | 1.03 | 0.289 | 1.761 |
| Pigment epithelium-derived factor | 0.773 | 0.765 | 0.008 | 1.538 | 0.773 | 0.8 | 0.027 | 1.573 | 0.773 | 0.379 | 0.394 | 1.152 | 0.773 | 1.16 | 0.386 | 1.931 |
| Plasminogen | 1.077 | 1.102 | 0.025 | 2.179 | 1.077 | 1.06 | 0.017 | 2.137 | 1.077 | NA | 1.077 | 1.077 | 1.077 | 1.24 | 0.162 | 2.316 |
| Serotransferrin | 1.500 | 1.483 | 0.016 | 2.983 | 1.500 | 1.44 | 0.060 | 2.940 | 1.500 | 0.717 | 0.783 | 2.217 | 1.500 | 2.02 | 0.516 | 3.516 |
| Serum albumin | 1.371 | 1.374 | 0.003 | 2.745 | 1.371 | 1.26 | 0.111 | 2.631 | 1.371 | 1.219 | 0.152 | 2.590 | 1.371 | 0 | 1.371 | 1.371 |
| Vitronectin | 3.643 | 3.627 | 0.016 | 7.270 | 3.643 | 3.29 | 0.353 | 6.933 | 3.643 | 1.941 | 1.702 | 5.584 | 3.643 | 5.43 | 1.785 | 9.071 |
| Peptides detected | **35** | **34** |  |  | **35** | **33** |  |  | **35** | **30** |  |  | **35** | **30** |  |  |
| Sum |  |  | **3.54** | **87.71** |  |  | **3.78** | **89.03** |  |  | **26.0** | **64.33** |  |  | **21.79** | **98.09** |
| Similarity (peptide identification) | **0.97** |  |  |  | **0.94** |  |  |  | **0.86** |  |  |  | **0.86** |  |  |  |
| Dissimilarity (peptide ratio identification) | **0.04** |  |  |  | **0.04** |  |  |  | **0.4** |  |  |  | **0.22** |  |  |  |

**Table 9**. Proficiency testing of each laboratory for the correlated proteins in Sample F. (NA = peptide detected but ratio value not available, NP = not performed, 0 = peptide not detected).

| Proteins | Reference  Ratio | Lab 1  Ratio | Difference | Sum | Reference  Ratio | Lab 2  Ratio | Difference | Sum | Reference  Ratio | Lab 3  Ratio | Difference | Sum | Reference  Ratio | Lab 4  Ratio | Difference | Sum |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Adiponectin | 3.012 | NP | 3.012 | 3.012 | 3.012 | 3.15 | 0.138 | 6.162 | 3.012 | 0.366 | 2.646 | 3.378 | 3.012 | 1.74 | 1.274 | 4.749 |
| Afamin | 3.956 | NP | 3.956 | 3.956 | 3.956 | 3.68 | 0.276 | 7.636 | 3.956 | 0.47 | 3.486 | 4.426 | 3.956 | 3.34 | 0.617 | 7.295 |
| Alpha-1-acid glycoprotein 1 | 7.068 | NP | 7.068 | 7.068 | 7.068 | 6.72 | 0.348 | 13.788 | 7.068 | NA | 7.068 | 7.068 | 7.068 | 5.81 | 1.254 | 12.883 |
| Alpha-1-antichymotrypsin | 11.446 | NP | 11.446 | 11.446 | 11.446 | 8.7 | 2.746 | 20.146 | 11.446 | 1.394 | 10.052 | 12.840 | 11.446 | 8.37 | 3.072 | 19.819 |
| Alpha-1-antitrypsin | 5.734 | NP | 5.734 | 5.734 | 5.734 | 4.93 | 0.804 | 10.664 | 5.734 | 0 | 5.734 | 5.734 | 5.734 | 0 | 5.734 | 5.734 |
| Alpha-2-antiplasmin | 9.131 | NP | 9.131 | 9.131 | 9.131 | 7.3 | 1.831 | 16.431 | 9.131 | 1.116 | 8.015 | 10.247 | 9.131 | 8.11 | 1.019 | 17.244 |
| Alpha-2-macroglobulin | 4.489 | NP | 4.489 | 4.489 | 4.489 | 4.2 | 0.289 | 8.689 | 4.489 | 0.573 | 3.916 | 5.062 | 4.489 | 4.09 | 0.395 | 8.583 |
| Antithrombin-III | 0.601 | NP | 0.601 | 0.601 | 0.601 | 0 | 0.601 | 0.601 | 0.601 | 0 | 0.601 | 0.601 | 0.601 | 0 | 0.601 | 0.601 |
| Apolipoprotein A-I | 8.082 | NP | 8.082 | 8.082 | 8.082 | 7.15 | 0.932 | 15.232 | 8.082 | 0.974 | 7.108 | 9.056 | 8.082 | 7.15 | 0.935 | 15.228 |
| Apolipoprotein A-IV | 10.185 | NP | 10.185 | 10.185 | 10.185 | 9.63 | 0.555 | 19.815 | 10.185 | 1.349 | 8.836 | 11.534 | 10.185 | NA | 10.185 | 10.185 |
| Apolipoprotein B-100 | 11.957 | NP | 11.957 | 11.957 | 11.957 | 10.77 | 1.187 | 22.727 | 11.957 | 1.537 | 10.420 | 13.494 | 11.957 | 9.65 | 2.305 | 21.608 |
| Apolipoprotein E | 10.649 | NP | 10.649 | 10.649 | 10.649 | 9.53 | 1.119 | 20.179 | 10.649 | 1.333 | 9.316 | 11.982 | 10.649 | 9.34 | 1.308 | 19.989 |
| Attractin | 5.296 | NP | 5.296 | 5.296 | 5.296 | 0 | 5.296 | 5.296 | 5.296 | NA | 5.296 | 5.296 | 5.296 | 4.98 | 0.319 | 10.274 |
| Beta-2-glycoprotein 1 | 10.713 | NP | 10.713 | 10.713 | 10.713 | 9.84 | 0.873 | 20.553 | 10.713 | 1.401 | 9.312 | 12.114 | 10.713 | 9.10 | 1.609 | 19.816 |
| Carbonic anhydrase 1 | 8.206 | NP | 8.206 | 8.206 | 8.206 | 7.35 | 0.856 | 15.556 | 8.206 | 0.987 | 7.219 | 9.193 | 8.206 | 6.87 | 1.340 | 15.072 |
| CD5 antigen-like | 5.667 | NP | 5.667 | 5.667 | 5.667 | 5.38 | 0.287 | 11.047 | 5.667 | 0 | 5.667 | 5.667 | 5.667 | 5.02 | 0.650 | 10.685 |
| Clusterin | 3.180 | NP | 3.180 | 3.180 | 3.180 | 3.23 | 0.050 | 6.410 | 3.180 | 0.39 | 2.790 | 3.570 | 3.180 | 2.65 | 0.530 | 5.829 |
| Complement C1r subcomponent | 3.299 | NP | 3.299 | 3.299 | 3.299 | 2.95 | 0.349 | 6.249 | 3.299 | 0 | 3.299 | 3.299 | 3.299 | 2.84 | 0.455 | 6.142 |
| Complement C3 | 7.875 | NP | 7.875 | 7.875 | 7.875 | 7 | 0.875 | 14.875 | 7.875 | 1.03 | 6.845 | 8.905 | 7.875 | 6.72 | 1.157 | 14.592 |
| Complement component C9 | 5.994 | NP | 5.994 | 5.994 | 5.994 | 5.12 | 0.874 | 11.114 | 5.994 | 0.829 | 5.165 | 6.823 | 5.994 | 6.07 | 0.071 | 12.059 |
| Complement factor B | 9.149 | NP | 9.149 | 9.149 | 9.149 | 7.95 | 1.199 | 17.099 | 9.149 | 0 | 9.149 | 9.149 | 9.149 | 7.05 | 2.099 | 16.199 |
| Fibrinogen gamma chain | 5.361 | NP | 5.361 | 5.361 | 5.361 | 5.31 | 0.051 | 10.671 | 5.361 | 0.844 | 4.517 | 6.205 | 5.361 | 0 | 5.361 | 5.361 |
| Fibulin-1 | 3.705 | NP | 3.705 | 3.705 | 3.705 | 3.37 | 0.335 | 7.075 | 3.705 | 0.549 | 3.156 | 4.254 | 3.705 | 3.28 | 0.426 | 6.984 |
| Haptoglobin | 5.652 | NP | 5.652 | 5.652 | 5.652 | 5.97 | 0.318 | 11.622 | 5.652 | 0.782 | 4.870 | 6.434 | 5.652 | 0 | 5.652 | 5.652 |
| Hemoglobin subunit alpha | 9.957 | NP | 9.957 | 9.957 | 9.957 | 9.32 | 0.637 | 19.277 | 9.957 | 1.293 | 8.664 | 11.250 | 9.957 | 2.31 | 7.646 | 12.269 |
| Hemopexin | 6.889 | NP | 6.889 | 6.889 | 6.889 | 5.57 | 1.319 | 12.459 | 6.889 | 0.838 | 6.051 | 7.727 | 6.889 | 6.27 | 0.621 | 13.157 |
| Heparin cofactor 2 | 8.838 | NP | 8.838 | 8.838 | 8.838 | 7.41 | 1.428 | 16.248 | 8.838 | 1.077 | 7.761 | 9.915 | 8.838 | 7.83 | 1.011 | 16.665 |
| Hyaluronan-binding protein 2 | 4.928 | NP | 4.928 | 4.928 | 4.928 | 3.88 | 1.048 | 8.808 | 4.928 | 0.642 | 4.286 | 5.570 | 4.928 | 4.08 | 0.846 | 9.010 |
| Inter-alpha-trypsin inhibitor heavy chain H2 | 12.329 | NP | 12.329 | 12.329 | 12.329 | 10.6 | 1.729 | 22.929 | 12.329 | 1.415 | 10.914 | 13.744 | 12.329 | 10.29 | 2.035 | 22.624 |
| Kininogen-1 | 4.013 | NP | 4.013 | 4.013 | 4.013 | 3.73 | 0.283 | 7.743 | 4.013 | 0.541 | 3.472 | 4.554 | 4.013 | 3.46 | 0.557 | 7.468 |
| Pigment epithelium-derived factor | 4.329 | NP | 4.329 | 4.329 | 4.329 | 4.2 | 0.129 | 8.529 | 4.329 | 0.549 | 3.780 | 4.878 | 4.329 | 3.94 | 0.394 | 8.264 |
| Plasminogen | 6.114 | NP | 6.114 | 6.114 | 6.114 | 6.44 | 0.326 | 12.554 | 6.114 | NA | 6.114 | 6.114 | 6.114 | 4.93 | 1.180 | 11.048 |
| Serotransferrin | 8.524 | NP | 8.524 | 8.524 | 8.524 | 6.43 | 2.094 | 14.954 | 8.524 | 1.011 | 7.513 | 9.535 | 8.524 | 7.31 | 1.217 | 15.832 |
| Serum albumin | 5.717 | NP | 5.717 | 5.717 | 5.717 | 4.23 | 1.487 | 9.947 | 5.717 | 1.117 | 4.600 | 6.834 | 5.717 | 0 | 5.717 | 5.717 |
| Vitronectin | 19.969 | NP | 19.969 | 19.969 | 19.969 | 16.84 | 3.129 | 36.809 | 19.969 | 2.444 | 17.525 | 22.413 | 19.969 | 16.59 | 3.377 | 36.561 |
| Peptides detected | **35** | **NP** |  |  | **35** | **33** |  |  | **35** | **30** |  |  | **35** | **30** |  |  |
| Sum |  |  | **252.01** | **252.01** |  |  | **35.80** | **469.89** |  |  | **225.16** | **278.86** |  |  | **72.97** | **431.20** |
| Similarity (peptide identification) | **NP** |  |  |  | **0.94** |  |  |  | **0.86** |  |  |  | **0.86** |  |  |  |
| Dissimilarity (peptide ratio identification) | **NP** |  |  |  | **0.08** |  |  |  | **0.81** |  |  |  | **0.17** |  |  |  |

**Table 10**. Proficiency testing scores of each laboratory in comparison to the reference laboratory data for all samples analysed.

| Sample | Laboratory | % Similarity (peptide identification) | % Dissimilarity (peptide ratio identification) |
| --- | --- | --- | --- |
| A | **Reference Laboratory** | **100** | **0** |
| A | Laboratory 1 | 97 | 3 |
| A | Laboratory 2\* | NA | NA |
| A | Laboratory 3 | 66 | 95 |
| A | Laboratory 4 | 86 | 22 |
| B | **Reference Laboratory** | **100** | **0** |
| B | Laboratory 1 | 97 | 5 |
| B | Laboratory 2\* | NA | NA |
| B | Laboratory 3 | 66 | 96 |
| B | Laboratory 4 | 86 | 16 |
| C | **Reference Laboratory** | **100** | **0** |
| C | Laboratory 1 | 97 | 12 |
| C | Laboratory 2\* | NA | NA |
| C | Laboratory 3 | 66 | 96 |
| C | Laboratory 4 | 86 | 17 |
| D | **Reference Laboratory** | **100** | **0** |
| D | Laboratory 1 | 97 | 5 |
| D | Laboratory 2 | 94 | 9 |
| D | Laboratory 3 | 86 | 19 |
| D | Laboratory 4 | 86 | 16 |
| E | **Reference Laboratory** | **100** | **0** |
| E | Laboratory 1 | 97 | 4 |
| E | Laboratory 2 | 94 | 4 |
| E | Laboratory 3 | 86 | 40 |
| E | Laboratory 4 | 86 | 22 |
| F | **Reference Laboratory** | **100** | **0** |
| F | Laboratory 1\*\* | NA | NA |
| F | Laboratory 2 | 94 | 8 |
| F | Laboratory 3 | 86 | 81 |
| F | Laboratory 4 | 86 | 17 |

\* NA (not assessed). Detection not applicable to Lab 2 as testing of the uncorrelated samples (A, B, C) could not be performed. Laboratory 2 were therefore not assessed for these samples. \*\* Laboratory 1 could not test Sample F and were not assessed for this sample.

# Project findings

The use of both uncorrelated and correlated peptide standards was aimed at challenging laboratories for their ability to detect and identify each unknown peptide. Five laboratories were sent samples for analysis with four laboratories returning data. One laboratory did not perform any analyses due to unforeseen restructuring in their laboratory and therefore had to withdraw from the trial.

The data generated from this study indicates that protein diagnostics using human peptides is certainly possible. Laboratories appeared to detect the protein peptides in the correlated samples (D, E, F) with greater efficiency in comparison to the protein peptides in the uncorrelated samples (A, B, C) (Table 10). However, from laboratory feedback and from analysis of the laboratory generated data, two primary areas for improvement of an EQA program were identified. Firstly, the current EQA reference testing material needs to be improved since laboratories reported the same (or similar) problems encountered (see below). Secondly, there appears to be technical issues relating to the testing columns used for mass spectrometry given that some laboratories could not detect the unknown peptides. For example, Laboratory 2 were the only testing facility that could not perform any protein assay in the uncorrelated samples (A, B, C), and Laboratory 1 could not perform a protein assay for the correlated Sample F (see Table 10). However, the identification of these shortcomings is essential information to relay back to laboratories since it allows for the laboratory to troubleshoot their testing pipeline so that data analysis and performance can be improved. These data demonstrate the importance of participation in an EQA program. This study therefore achieved its aim in identifying areas of concern for laboratory testing and in highlighted technological shortcomings.

# Problems encountered

Key problems were encountered in this study which resulted in a delay for data submission. A major hold-up was that each laboratory upon performing peptide measurements encountered difficulties in measuring several of the RCPAQAP reference testing peptides. There were subsequently multiple requests from laboratories for more samples to be sent so that a complete analysis could be made. Laboratories reported that upon measuring certain peptides, their mass spectrometer liquid chromatography peptide columns were damaged and had to be replaced from overseas suppliers. This was due to high pressures building up on the testing columns which resulted in blockages. This had an unforeseen impact in holding up each laboratory’s diagnostic samples for clinical analyses. Subsequently, the RCPAQAP peptide samples were put on hold until the backlog of the clinical samples could be completed. Laboratories also reported issues relating to the quality of some samples since peptides could not be readily detected with consistent efficiency. However, these encountered issues were precisely what this trial project was aimed at discovering given that this is a new potential area for human diagnostics.

# Future diagnostics

(*Does the proposed project complement other similar services, activities and resources?*)

The data from this project directly complements another funded QUPP project. In 2017, QUPP funding (Agreement id: 4-4YYPT91) was awarded for developing EQA proficiency testing of, (i) DNA extraction, (ii) circulating free DNA for cancer-associated biomarkers and for non-invasive prenatal testing (NIPT), and (iii) leukaemia-associated DNA variants. The identification of protein biomarkers (that are potentially circulating in the blood) are therefore directly complementary. For example, future protein diagnostics may be used with DNA analyses to identify circulating levels of both proteins and DNA that are associated with different human diseases (Cohen et al 2017). Such analyses would be for more informative for the clinician and may help identify disease development early, so that appropriate clinical management can be initiated.

The development of future multi-protein biomarker EQA programs will also complement our existing programs. For example, we have molecular quality assurance programs in the area of inherited haematological disorders (i.e., haemochromatosis, thrombosis, and thalassaemia) and in the qualitative and quantitative detection of nucleic acids from infectious micro-organisms including viruses and bacteria. Quality assuring protein mass spectrometry will add disease-associated proteins/peptides to this EQA list.

# Long term outcomes

(*Does the project provide value for money including: Economy, Efficiency and Effectiveness - delivering a better service or getting a better return for the same amount of expense, time or effort?*)

The potential for diagnostic applications using mass spectrometry is rapidly growing owing to the technology having increased levels of diagnostic sensitivity and high throughput capacity for testing. However, the technology has not yet been fully adopted for human genetic-associated disease testing partly due to the lack of quality assurance programs. This trial was therefore initiated to challenge mass spectrometer testing facilities to determine if disease-associated protein diagnostics is viable. The data produced from this study suggests that protein diagnostics using human peptides is possible. Thus, the long-term benefit of an EQA for mass spectrometry is that it will allow the RCPAQAP to develop proteomic EQA programs for (i) cancer (i.e., breast, lung, prostate gastrointestinal); (ii) for neurological disorders (i.e., Alzheimer, Parkinson, Huntington, epilepsy); and (iii) for age-related disease (i.e., cardiovascular disease). Importantly, proteomic results in combination with identified genetic alterations will allow clinicians to make better informed decisions with respect to pharmaceutical intervention and patient management.

In terms of near future multi-protein biomarker testing, the development of quality assessments will allow diagnostic laboratories to detect protein variation or abnormal protein expression in a much shorter time-period with high accuracy and sensitivity. The cost of healthcare and treatment plans can therefore be significantly reduced and implemented earlier. Reduced patient invasiveness (due to only a blood sample being required) will increase patient care by allowing earlier diagnoses which will benefit patient management strategies and thus increase cost and time effectiveness.

The development of a proteomic EQA will allow the establishment of cross functional and inter-discipline collaboration. For example, the RCPAQAP Biosecurity, Serology and Microbiology disciplines will benefit from analyses of disease-associated protein targets. This combined discipline approach will result in the production of highly-developed quality assurance programs for future cost-effective analyses across the RCPAQAP.

# The next step

The next step for the development of an RCPAQAP proteomic proficiency testing program is to perform a follow-on study that encompasses a larger cohort of laboratories. The challenges identified from this study need to be rectified and put in to practice before this EQA program can be offered for the proficiency testing of mass spectrometry. However, the current data will nonetheless allow us to improve on the development of a protein specific EQA. A larger cohort of data will additionally allow the RCPAQAP to apply for NATA accreditation. Importantly, the data from this study has been peer-reviewed and published (Horan et al., 2019).

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# Appendix

Proteins and peptide sequences used in the reference testing samples.

| **Uncorrelated Protein** | **Peptide Sequence** | **Correlated Protein** | **Peptide Sequence** |
| --- | --- | --- | --- |
| Adiponectin | IFYNQQNHYDGSTGK | Adiponectin | IFYNQQNHYDGSTGK |
| Afamin | DADPDTFFAK | Afamin | DADPDTFFAK |
| Alpha-1-acid glycoprotein 1 | NWGLSVYADKPETTK | Alpha-1-acid glycoprotein 1 | NWGLSVYADKPETTK |
| Alpha-1-antichymotrypsin | EIGELYLPK | Alpha-1-antichymotrypsin | EIGELYLPK |
| Alpha-2-antiplasmin | LGNQEPGGQTALK | Alpha-1-antitrypsin | LSITGTYDLK |
| Alpha-2-macroglobulin | AIGYLNTGYQR | Alpha-2-antiplasmin | LGNQEPGGQTALK |
| Apolipoprotein A-I | ATEHLSTLSEK | Alpha-2-macroglobulin | AIGYLNTGYQR |
| Apolipoprotein A-IV | LGEVNTYAGDLQK | Antithrombin-III | DDLYVSDAFHK |
| Apolipoprotein B-100 | FPEVDVLTK | Apolipoprotein A-I | ATEHLSTLSEK |
| Apolipoprotein E | LGPLVEQGR | Apolipoprotein A-IV | LGEVNTYAGDLQK |
| Attractin | SVNNVVVR | Apolipoprotein B-100 | FPEVDVLTK |
| Beta-2-glycoprotein 1 | ATVVYQGER | Apolipoprotein E | LGPLVEQGR |
| Biotinidase | SHLIIAQVAK | Attractin | SVNNVVVR |
| Carbonic anhydrase 1 | VLDALQAIK | Beta-2-glycoprotein 1 | ATVVYQGER |
| CD5 antigen-like | LVGGLHR | Carbonic anhydrase 1 | VLDALQAIK |
| Cholinesterase | YLTLNTESTR | CD5 antigen-like | LVGGLHR |
| Clusterin | ELDESLQVAER | Clusterin | ELDESLQVAER |
| Coagulation factor XII | EQPPSLTR | Complement C1r subcomponent | GLTLHLK |
| Complement C1r subcomponent | GLTLHLK | Complement C3 | TGLQEVEVK |
| Complement C3 | TGLQEVEVK | Complement component C9 | LSPIYNLVPVK |
| Complement component C9 | LSPIYNLVPVK | Complement factor B | EELLPAQDIK |
| Complement factor B | EELLPAQDIK | Fibrinogen gamma chain | YEASILTHDSSIR |
| Fibulin-1 | TGYYFDGISR | Fibulin-1 | TGYYFDGISR |
| Hemoglobin subunit alpha | VGAHAGEYGAEALER | Haptoglobin | DIAPTLTLYVGK |
| Hemopexin | NFPSPVDAAFR | Hemoglobin subunit alpha | VGAHAGEYGAEALER |
| Heparin cofactor 2 | TLEAQLTPR | Hemopexin | NFPSPVDAAFR |
| Hyaluronan-binding protein 2 | VVLGDQDLK | Heparin cofactor 2 | TLEAQLTPR |
| Inter-alpha-trypsin inhibitor heavy chain H2 | SLAPTAAAK | Hyaluronan-binding protein 2 | VVLGDQDLK |
| Kininogen-1 | TVGSDTFYSFK | Inter-alpha-trypsin inhibitor heavy chain H2 | SLAPTAAAK |
| Pigment epithelium-derived factor | LQSLFDSPDFSK | Kininogen-1 | TVGSDTFYSFK |
| Plasma protease C1 inhibitor | FQPTLLTLPR | Pigment epithelium-derived factor | LQSLFDSPDFSK |
| Plasminogen | LFLEPTR | Plasminogen | LFLEPTR |
| Prothrombin | ELLESYIDGR | Serotransferrin | DGAGDVAFVK |
| Serotransferrin | DGAGDVAFVK | Serum albumin | LVNEVTEFAK |
| Vitronectin | FEDGVLDPDYPR | Vitronectin | FEDGVLDPDYPR |