Short Term Scientific Mission (STSM) Scientific Report

Title: SRM training program for method implementation in the Proteomics Department of the Institute of Cellular Biology and Pathology “N. Simionescu” – the Home Institute: Institute of Cellular Biology and Pathology "Nicolae Simionescu" (Bucharest, Romania)

Action number: CA16113 - CliniMARK: ‘good biomarker practice’ to increase the number of clinically validated biomarkers.

Applicant: Cerveanu-Hogas Aurel from the Institute of Cellular Biology and Pathology “Nicolae Simionescu”, Department of Proteomics - coordinated by Dr. Felicia Antohe;

Start / End date: July 09 2019 –August 06 2019;

Host Details

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The present application was oriented towards a training program. The applicant acquired practical experience in using the TSQ Vantage triple-quadrupole system and the ensuing data analysis in targeting and validating potential biomarkers, with SRM methodology (Selected Reaction Monitoring). Practical experiments, method design and refinement were done using the Host Institution’s samples. The expertise which was gained during this STSM helps consolidate the Home Institution’s proteomics platform for future studies concerning biomarker targeting, verification and validation. The applicant was trained to accurately perform the following critical aspects of SRM methodology: daily basic

maintenance for the LC-MS, Quality controls, Sample preparation using in-gel digestion, SRM method development and refinement for analysis using Skyline software.

The **sample preparation** workflow was conducted on human plasma, which was divided into 5 replicates (X1, X2, X3, X4, X5).

- The samples were first concentrated in 4% SDS-PAGE for 5 minutes at 100 V, followed by Coomasie Blue staining. The gel was washed with ultrapure water and each band containing the total sample proteins was sliced out;
- In the next step the gel pieces were washed using different concentrations (70% and 50% and 0%) of ammonium bicarbonate (AB), in acetonitrile solutions for Coomasie stain removal;
- Protein carbamidomethylation was next performed for the prevention of di-sulfide bridge formation using a reducing solution of dithiothreitol in AB (100mM, 30 min, 55-60ºC) and an alkylating buffer of iodoacetamide in AB (100mM, 30 min, at room temperature);
- Protein enzymatic digestion was performed with mass spectrometry grade trypsin (enzyme/substrate ratio =1:100, 37ºC) to obtain the peptides of interest, which were afterwards extracted from the gel using a solution containing 75% acetonitrile and 5% formic acid;
- Solid phase extraction was employed for salt removal from the peptide mixtures, which would hinder the peptide ionization during electrospray. The process involved C18 flow-through columns, which were sequentially conditioned, equilibrated and loaded with the peptide mixtures.
- The eluted peptides (in 50% acetonitrile, 0.1% formic acid) were vacuum dried and resuspended in 0.1% formic acid.
- The peptide samples were chromatographically separated (3%-90% acetonitrile gradient) and the mass spectra were acquired in SRM mode.

Skyline (v4.2.0) bioinformatic workflow comprised initially the *in-silico* digestion of a background proteome from *UniProt* website and import of a transition library from *PeptideAtlas*. Several crucial processing steps continued thereafter, such as:

- We added two fasta files of the proteins of interest (S100A8 and S100A9) together with the associated modifications which were done in Skyline software (of the synthetic homologues);
- We picked the 7 most intense “y” ions for each peptide;
- To predict the retention times (RT) at which the peptides elute we created a RT Calibration Curve using standards with specific RT;
- Afterwards, we injected a solution containing a concentration of 10 ng/µL synthetic peptides;
- Next, in Skyline we picked the 5 best transitions for each of the peptides;
- The Collision Energy (CE) optimization assumes a number of multiple injections in the MS in which we determine the optimal CE for the transitions of each of the peptides, followed by endogenous peptides upload in Skyline;
- To quantify the proteotypic peptides we needed to find the optimal concentration of synthetic peptides that must be added to our samples;
- We spiked different concentrations of synthetic peptides into our pooled sample to check which concentration is optimal (also used to create the calibration curve);
- The ideal concentration of synthetic peptides would have to be equal to the concentration of the endogenous peptides from our samples. After different concentration measurements we established that the 0.05 ng/µL fits our purpose.
- We analyzed the samples with the MS and picked the 3 best transitions;
- Using the ratio between the total areas of the synthetic peptides and the endogenous ones we calculated the amount of each endogenous peptide;
- This method was now optimised for the 4 selected peptides regardless of sample type.

The low abundant peptide “GNFHAVYR”, contained in the S100A8 protein was successfully quantified in the analysed sample with an average of 13.26 fmol/µg of total protein. Although the quantification of the other 3 proposed peptides has not succeeded, the applicant and the Host Institution specialized personnel agreed on a sample preparation protocol to improve the sensitivity of the method based on enrichment of the peptides or depletion of the high abundant proteins in the plasma samples. Repeatability and variance measurements of individual and pooled samples were performed to detect technical challenges of the two types of analyses.

Individual samples	**GNFHAVYR**	**Final Conc. of peptide (fmol/ug of total protein)**	**Final Conc. of peptide (mg/dL of total plasma**	**Average concentration (fmol/ug of total protein) for individual samples**	**Standard Deviation**
	X1	**10.81**	**0.052**	**13.17**	**2.87**
	X2	**17.52**	**0.084**		
	X3	**14.64**	**0.07**		
	X4	**11.99**	**0.057**		
	X5	**10.91**	**0.052**		
Pooled sample	**GNFHAVYR**	**Final Conc. of peptide (fmol/ug of total protein)**	**Final Conc. of peptide (mg/dL of total plasma)**	**Average concentration (fmol/ug of total protein) for pooled samples**	**Standard Deviation**
	Replicate pool 1	**10.81**	**0.052**	**13.82**	**2.59**
	Replicate pool 2	**17.52**	**0.084**		
	Replicate pool 3	**14.64**	**0.07**		
	Replicate pool 4	**11.99**	**0.057**		
	Replicate pool 5	**14.15**	**0.068**		

Table 1: Concentration measurements of individual vs pooled samples and variance measurements for individual vs pooled samples

As seen in the table above, a slight variation between the X1-X5 samples was observed, that is acceptable nevertheless for the first analyses.

The standard deviations are small, demonstrating the feasibility of the analysis whether the samples are measured individually or pooled together.