PRG2009: Relative Protein Quantification in a Clinical Matrix

Identification Number: 32365

INTRODUCTION

The PRG has designed a study that explores the use of different approaches for determining quantitative differences for several target proteins in six samples of human plasma.

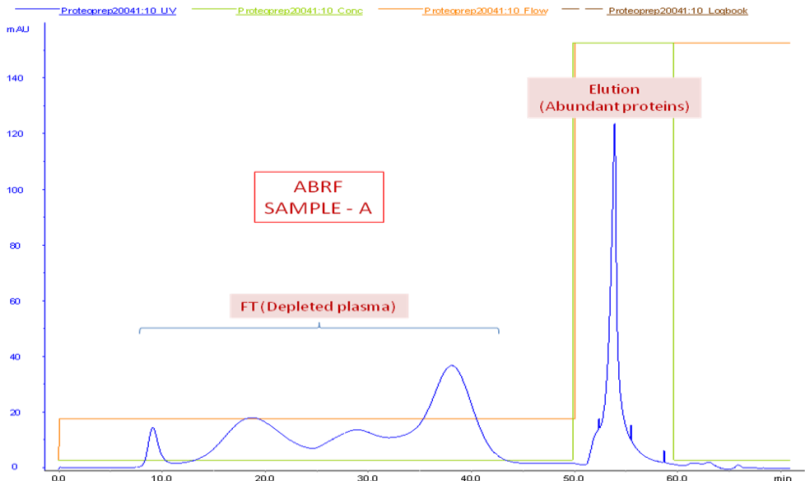
Study samples: 3 blind duplicate samples, which contain 40µl undepleted, freeze dried human plasma were provided. Each duplicate set of vials is spiked with different amounts of the following two human proteins and two rabbit proteins: Prostate specific antigen (human), Beta human Chorionic Gonadotropin, Glycogen phosphorylase A and B (rabbit). These proteins are spiked into plasma at a range of 2.5 fmol/µl to 1.25 pmol/µl

SAMPLE PROCESSING

Each undepleted human plasma sample was processed as follows:

1) Plasma was reconstituted in 80 µl PBS 1x, vortexed and filtered with a 0.22 µm Corning Spin-X (14,000 g, 5 min).

2) Twenty high-abundance proteins were depleted from 80 µl of each plasma sample using the ProteoPrep 20-LC-Column (Sigma Aldrich), installed in an ÄKTA-FPLC (GE Healthcare). In the picture below, the sample A chromatogram is shown as example.



3) The wash through was concentrated with an Amicon Ultra-4 YM3 filters (Millipore), at 4ºC, 4,000 g during 40-60 min.

4) The protein concentration of samples was determined with a NanoDrop.

Sample	Measure1	Measure2	Mean	Volumen	Prot. Conc (µg/µl)
A	0.21	0.27	0.240	107	25.68
B	0.43	0.4	0.415	77	31.96
C	0.44	0.43	0.435	130	56.55
D	0.23	0.26	0.245	110	26.95
E	0.38	0.39	0.385	85	32.73
F	0.25	0.23	0.240	123	29.52

5) Each depleted sample (25 µg) was precipitated with 100% acetone at –20 °C overnight. The pellet was dissolved in 20 µl 50% TFE (2,2,2-trifluoroethanol)/50 mM TEAB.

TRYPSIN DIGESTION, iTRAQ LABELLING AND PEPTIDE FRACTIONATION

1) Two different pools were considered in order to compare the 6 blind samples and identify each duplicate set of samples. In each labelling experiment a reference sample was included (sample C) to compare both pools. The table shows sample labelling design for each experiment.

POOL 1		POOL 2	
A	114	C	114
B	115	E	115
C	116	F	116
D	117		

2) Twenty-five micrograms of each depleted sample were reduced (4 µl TCEP 50 mM, 1h, 60ºC) and alkylated (4 µl MMTS 200 mM, 10 min, RT). The samples were then diluted 10-fold with 50 mM TEAB and digested with 1.25 µg Trypsin (Promega) ON at 37°C.

3) Protein digests were isotopically labelled for 1 hour at room temperature by adding one vial of iTRAQ reagent following the Manufacture Reference Guide. The labelling reaction was then stopped by the addition of 100 µl of milli-Q water, 30 min at RT.

4) Regarding to sample peptide complexity, pools were fractionated using 3100 OffGel Fractionator (Agilent). Twelve fractions were obtained from each pool using a 3-10 pH range and 12 cm strip

5) The samples were then mixed at equal ratios, dried and re-suspended in 1X OffGel Peptide Solution. The samples were focused using the recommended method for OffGel Peptides 12-wells fractionation with a maximum current of 50 µA. The focusing was stopped after total voltage reaches 50 kVh.

6) Peptide fractions were cleaned up with PepClean C18 spin columns (Pierce) following manufacturer's protocol.

7) Fractions were concentrated by vacuum centrifugation prior to LCMALDI MS/MS analysis.

NANO REVERSE-PHASE LC-MALDI MS/MS ANALYSIS

Peptides were re-dissolved in 32 µl 0.1%TFA/2% ACN in water, but only 16 µl was injected. Peptides separation was performed on a TEMPO nanoHPLC system (Applied Biosystems) equipped with a ProteoPep C18 column (Dionex; 3-µm particles, 100 Å pore size and 75 µm I.D.), an autosampler and a SunCollect microfraction collector.

The mobile phase consisted of a gradient of solvents A (0.1% TFA; 2% ACN in water) and B (0.1% TFA; 80% ACN in water). Injection was performed with 96% solvent A. The peptides were separated with a linear gradient of solvent B from 0–5% in 5 min, followed by an increase until to 20% of solvent B in 10 min and to 55% in 45 min at a flow rate of 0.3µL/min. The column was washed and regenerated with 100% solvent B for 10 min and with 96% solvent A.

For MALDI MS/MS analysis, column effluent was mixed in a 1:4 ratio with MALDI matrix (3 mg/mL α-cyano-4- hydroxycinnamic acid in 0.1% TFA/70% ACN) (v/v) and deposed on an Opti-tof LC/MALDI plate (Applied Biosystems) at a frequency of one spot/15s.

MALDI plates were analyzed by MALDI-TOF/TOF 4800 Analyzer mass spectrometer (Applied Biosystems) in positive reflector ion mode. MS spectra from m/z 800–4000 were acquired for each spot using 1500 laser shots. The ten most intense peaks in each MS spectrum above an S/N threshold of 100 were selected for MS/MS.

The acquisition was focused on theoretical tryptic peptides masses corresponding to target proteins: BhCG, AhCG, PSA and Phos A/B.

DATA ANALYSIS

Peptides and proteins identification and comparative quantification were performed using the **Protein Pilot software** vs 2.0.1 (Applied Biosystems) with **Paragon Algorithm**.

MS/MS data was searched against the **Uniprot-SwissProt** database of protein sequences (Swiss-Prot, August 2008), using following parameters: sample type set as iTRAQ 4plex (peptide labelled), cystein alkylation with MMTS, 1 missed cleavage allowed in trypsin digestion and focus in biological modifications. Only proteins with a threshold >95% confidence were considered for protein identification, with at least one MS/MS spectra with confidence >80%. For quantification with iTRAQ isotope labelling, the corresponding Correction Factors were considered. Data was normalized for loading error by bias corrections.

RESULTS AND DISCUSSION

We have not achieved identify all the spike proteins in both pools. The results are resumed in the table below, containing additional information about sequence coverage and number of matched peptides (only labelled with iTRAQ reagent).

Spike Protein	**POOL 1 (Samples A, B, C, D)**		**POOL 2 (Samples C, E, F)**	
	Seq. Cover.	Num. Peptides	Seq. Cover.	Num. Peptides
Prostate specific antigen KLK3_HUMAN	**26.8%**	**2**	--	--
Beta h-Chorionic Gonadotropin CGB2_HUMAN	**40.5%**	**2**	**14.9%**	**1**
Glycogen phosphorylase A PYGM_HUMAN	--	--	--	--
Glycogen phosphorylase B PYGM_HUMAN	**51.9%**	**31**	**35.6%**	**13**

Related to these results, we have not identified Glycogen Phosphorylase A (rabbit) neither in pool 1 nor in pool 2, due to the only one difference between these two proteins is the phosphorylation site (Serine-15) in Glycogen Phosphorylase A isoform. In our opinion, MALDI mass spectrometer is not the properly technique to find and identify phosphorylated peptides. **So it is impossible to quantify the amount of Glycogen Phosphorylase A in samples.** **Prostate specific antigen is not considered in the quantification assay** since it is not identified in Pool 2.

Regarding to target proteins we can not conclude any information either duplicates detection or their relative quantification because individual quantification data of each protein show contradictory information about duplicates.
In the table below we show the ratios of different iTRAQ isotopes; we set sample C (reference sample) as denominator of ratios in the quantification and identification of duplicates.

Choriogonadotropin subunit beta	
A	2.4623
B	7.1738
C	1
D	0.6535
E	2.4189
F	5.3667

Glycogen phosphorylase muscle	
A	1.3915
B	1.0863
C	1
D	0.5156
E	0.9984
F	0.6182

Prostate specific antigen	
A	1.2381
B	1.8949
C	1
D	4.74
E	--
F	--

If we focus in beta Chorionic Gonadotropin ratios we could recognize duplicates: A=E; B=F; C=D. But if we observe the Glycogen phosphorylase (both A+B isoforms) ratios the previous hypothesis is not valid, because the detected duplicates (A=B; C=E; D=F) are not very solid in our opinion.