BIOGNOSYS
NEXT GENERATION PROTEOMICS

TECHNICAL NOTE

Accurate and fast proteomics analysis of human plasma with PlasmaDive™ and SpectroDive™

In this technical note you will learn about:

- **Step-by-step set-up of parallel reaction monitoring (PRM) workflow with PlasmaDive™ and SpectroDive™ software**
- **Anticipated out-of-the-box results of accurately quantifying 100 proteins in human plasma in less than one working day**
- **Experimentally determined lower limits of quantification in human plasma for all 100 proteins featured in PlasmaDive™**

If you have any questions about the technical note or Biognosys' targeted proteomics platform please contact us at support@biognosys.com.

Parallel Reaction Monitoring (PRM)
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Introduction

Blood plasma and serum are the most common sample types in routine diagnostics as well as for biomarker discovery. Levels of blood proteins reflect the health status of single organs and the body as a whole. Changes in composition of proteins in the blood can be directly correlated to disease onset or therapy response.

Monitoring of blood proteins is today mainly performed by immunoassays. However, immunoassays have several limitations like specificity, low multiplexing ability, and its expensive development for new protein targets. These challenges can be overcome by using targeted proteomics, a mass spectrometry based method that allows absolute quantification of more than 100 proteins analyzed in high-throughput mode in thousands of samples.

Targeted proteomics currently relies on two main approaches: Multiple and Parallel Reaction Monitoring (MRM and PRM). MRM is a well-established method for targeted proteomics and is performed on triple quadrupole mass spectrometers, while its novel variant PRM (**Figure 1**) was recently introduced on the latest generation of high-resolution Orbitrap™ or Q-TOF mass spectrometric instruments.

Here we present a fast protocol to accurately quantify 100 proteins in human blood plasma using PRM on a Thermo Scientific™ Q Exactive™ HF platform and describe the easy step-by-step set-up using the Biognosys PlasmaDive™ multiplexed assay kit together with the Biognosys SpectroDive™ software for targeted proteomics analysis.

Methods

A pool of human blood plasma samples from healthy donors was processed according to the PlasmaDive™ manual. For lower limit-of-quantification (LLOQ) determination, samples were 10-fold serial diluted in 5 steps into plasma not spiked with reference (stable isotope standard or SIS) peptides. All samples were acquired on a ThermoScientific™ Q Exactive™ HF mass-spectrometer in PRM mode with a MS2 resolution of 30,000, a maximum fill-time of 55 ms and peptide elution over a 120 minute linear gradient with four technical replicates. The LLOQ was determined as the lowest dilution, where a precise quantitative value could be obtained.

The PRM method was set-up using SpectroDive™ 7, utilizing indexed retention-time (iRT) calibration and refinement in a single run (**Figure 2A,B**). With the information derived from this run, the optimal scheduling windows for all 200 target peptides were set with SpectroDive™ 7 (**Figure 2C**). Here scheduling windows of 12 minutes were used, to achieve a maximum of 80 concurrent precursor.

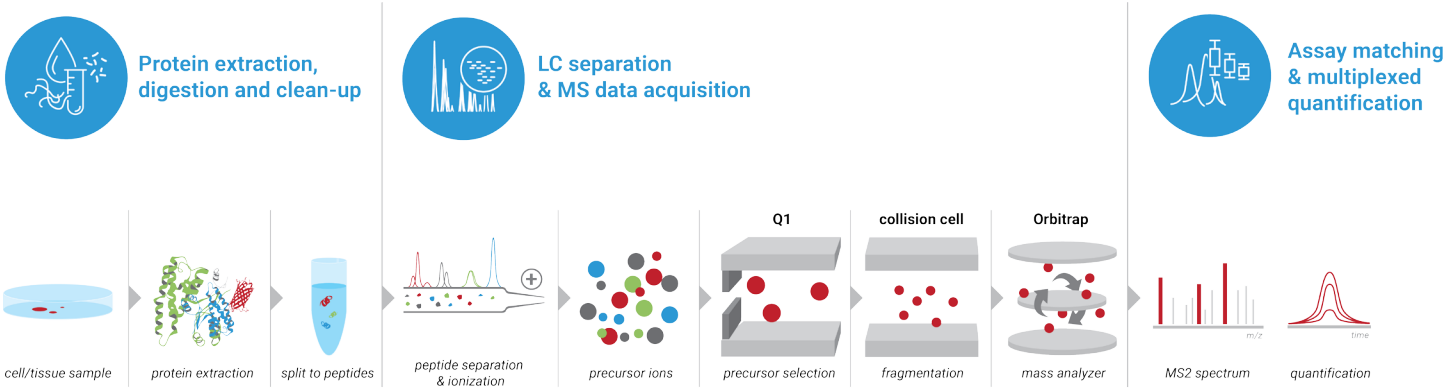

Figure 1. *Schematic representation of the parallel reaction monitoring (PRM) workflow.*

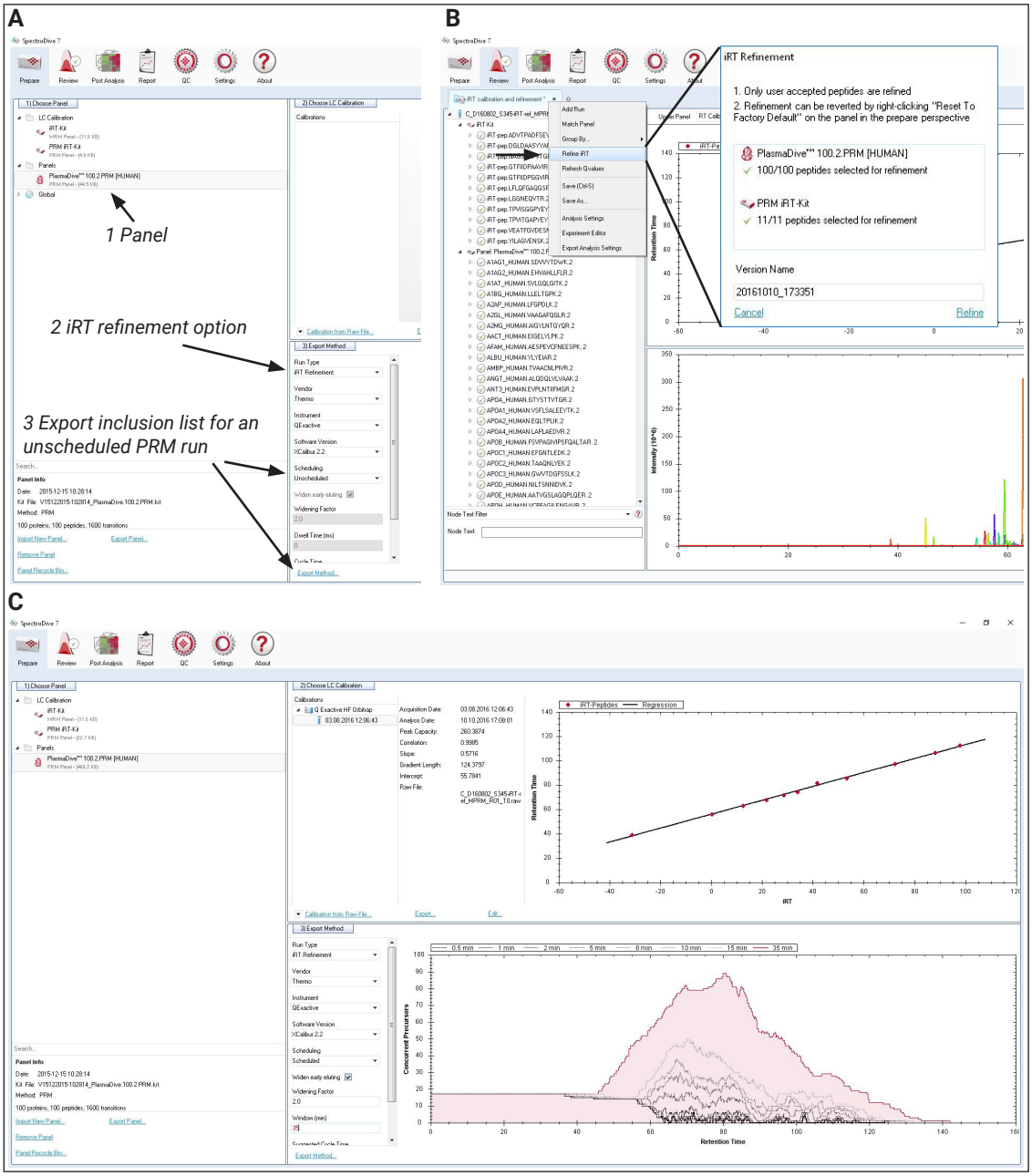

Figure 2. *Set-Up of SpectroDive 7 for PRM analysis with the PlasmaDive kit.* ***A*** *- For setting up the iRT calibration and refinement MS run, go to the Prepare perspective and select the PlsamaDive™ PRM panel (1) and the iRT refinement option (2). The scheduling option should be set to "unscheduled" and export the inclusion list via "export method" (3).* ***B*** *- Load the iRT calibration run and go to the Review perspective. Right click the iRT-Kit and the PlasmaDive™ Panel and choose "accept" (not shown; all peptides are now labeled with a green checkmark). Right click the experiment tab and choose "refine iRT". After clicking "refine" in the pop-up, the PlasmaDive™ panel is refined to your machine set-up.* ***C*** *- To set-up your analytical runs, go to the Prepare perspective. Choose PlasmaDive™ PRM as panel (1.), your previous MS run for LC calibration (2.) and set your parameters in the export method section (3.).*

Results

The easy and precise PRM scheduling workflow in SpectroDive™ resulted in a median absolute deviation of the measured retention time (RT) compared to the scheduled RT of less than 12 seconds (**Figure 3A**). In practical terms, this means that all 100 proteins which are covered in PlasmaDive™ could be measured within one minute of their scheduled RT on a 120 min gradient, corresponding to a maximum iRT off-set of 1.5 units. Consequently, every peptide could be precisely quantified with the PRM methodology, resulting in absolute concentrations for each protein spanning more than four orders of magnitude, with a median technical coefficient of variation (CV) of only 5 % (**Figure 3B**). To assess the LLOQ for the 100 proteins covered in PlasmaDive™ with PRM, we analyzed 10-fold dilutions, starting from samples processed according to the original protocol. Due to the nature of Orbitrap™ measurements, a true

background noise value without PlasmaDive™ spike-in cannot be determined, since these values are already zeroed directly in the instrument. Therefore we report the LLOQ to be below the last dilution, where we could still determine a precise quantitative value, for all PlasmaDive™ proteins in Table 1. The highest analytical depth is in the low 10s of ng/ml range and the median LLOQ is below 2 µg/ml (**Figure 4**).

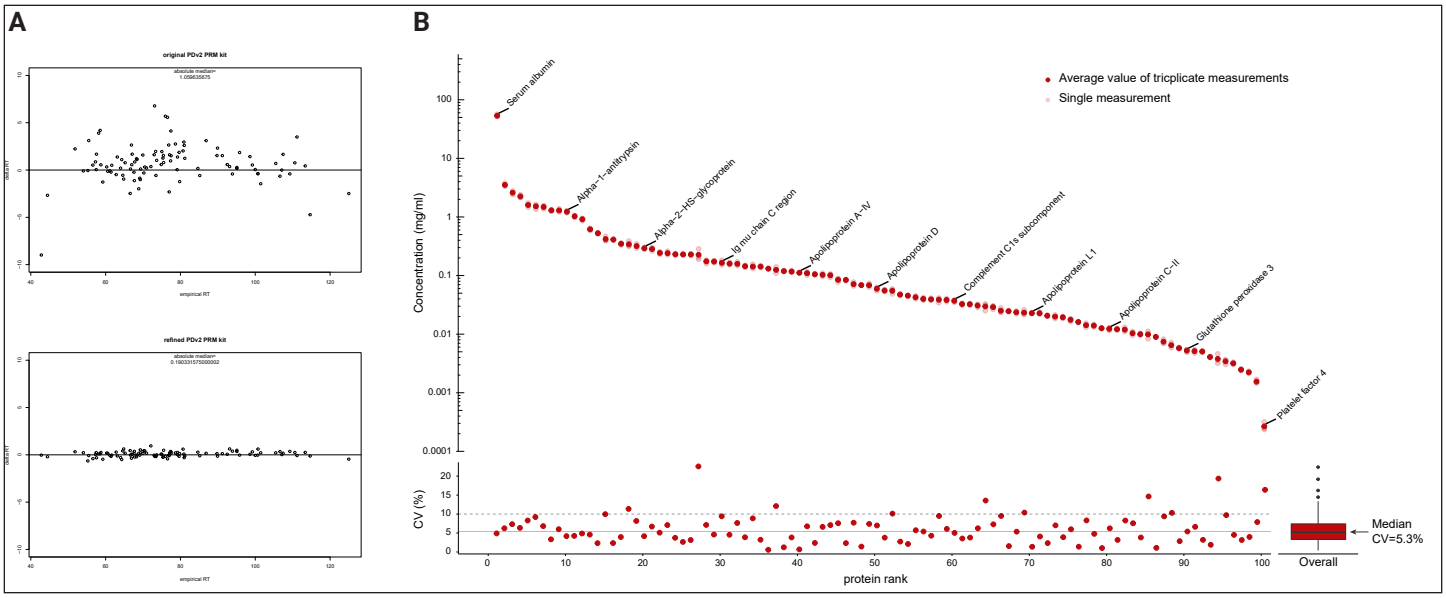

Figure 3. *Out-of-the-box results of the PlasmaDive™ with SpectroDive™ 7.* ***A*** *- By using iRT calibration and refinement the precursor scheduling becomes extremely accurate.* ***B*** *- Absolute amounts (mg/ml) in a pool of human plasma samples of all 100 proteins covered in PlasmaDive™.*

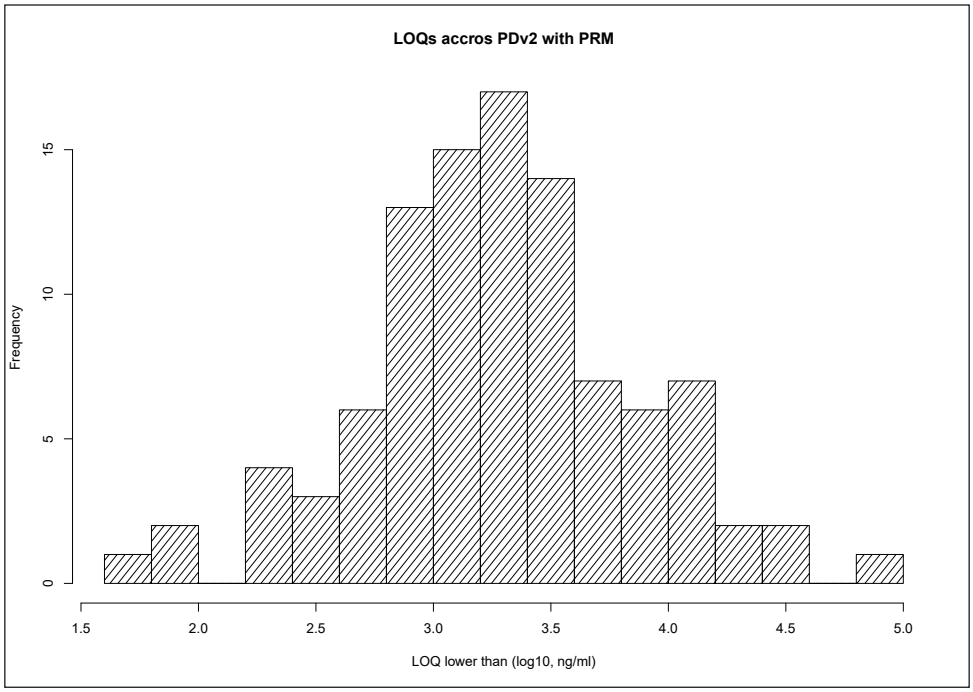

Figure 4. *Summary of LLOQs determined with PRM measurements for the entire range of proteins, which are analyzed with the PlasmaDive™ workflow.*

Conclusions

PlasmaDive™ in combination with SpectroDive™ allows for an easy set-up of a PRM workflow as well as obtaining accurate and sensitive results for blood plasma analysis. Results presented here of the out-of-the box workflow are achievable in less than one workday.

The advanced iRT refinement procedure described here is not strictly necessary when using PlasmaDive™, but to have the highest accuracy in RT prediction possible on any chromatographic setup, this option is available in SpectroDive™. Furthermore, an increase in sensitivity can be obtained by lowering the multiplexing from 100 proteins to a smaller subset.

Table 1. *A list of proteins included in the PlasmaDive™ multiplexed panel and their resective LOQs expressed as µg per ml of plasma.*

Protein Description	Protein Accession	LOQ (µg/ml)
Alpha-1-acid glycoprotein 1	P02763	1.566
Alpha-1-acid glycoprotein 2	P19652	4.363
Alpha-1-antitrypsin	P01009	14.716
Alpha-1B-glycoprotein	P04217	0.679
Alpha-2-antiplasmin	P08697	1.171
Leucine-rich alpha-2-glycoprotein	P02750	1.022
Alpha-2-macroglobulin	P01023	1.270
Alpha-1-antichymotrypsin	P01011	26.716
Afamin	P43652	2.262
Serum albumin	P02768	1.111
Protein AMBP	P02760	0.167
Angiotensinogen	P01019	6.464
Antithrombin-III	P01008	2.750
Apolipoprotein(a)	P08519	5.312
Apolipoprotein A-I	P02647	1.665
Apolipoprotein A-II	P02652	0.923
Apolipoprotein A-IV	P06727	1.938
Apolipoprotein B-100	P04114	30.388
Apolipoprotein C-I	P02654	1.065
Apolipoprotein C-II	P02655	0.078
Apolipoprotein C-III	P02656	0.855
Apolipoprotein D	P05090	0.233
Apolipoprotein E	P02649	0.236
Beta-2-glycoprotein 1	P02749	3.322
Apolipoprotein L1	O14791	9.389
Apolipoprotein M	O95445	0.294
Biotinidase	P43251	23.918

Protein Description	Protein Accession	LOQ (µg/ml)
Complement C1q subcomponent subunit A	P02745	11.98
Complement C1q subcomponent subunit B	P02746	1.130
Complement C1q subcomponent subunit C	P02747	0.043
Complement C1r subcomponent	P00736	2.848
Complement C1s subcomponent	P09871	0.639
C4b-binding protein alpha chain	P04003	0.447
Corticosteroid-binding globulin	P08185	2.004
CD5 antigen-like	O43866	0.450
Ceruloplasmin	P00450	0.903
Complement factor B	P00751	3.116
Complement factor H	P08603	13.508
Complement factor I	P05156	1.365
Cholinesterase	P06276	0.205
Clusterin	P10909	0.368
Complement C2	P06681	3.680
Complement C3	P01024	7.444
Complement C4-A	P0C0L4	18.591
Complement C5	P01031	9.195
Complement component C8 alpha chain	P07357	5.993
Complement component C9	P02748	1.760
Platelet basic protein	P02775	1.525
Coagulation factor XIII A chain	P00488	0.475

Protein Description	Protein Accession	LOQ (µg/ml)
Coagulation factor XIII B chain	P05160	69.413
Coagulation factor X	P00742	0.326
Coagulation factor IX	P00740	1.067
Fibulin-1	P23142	3.227
Alpha-2-HS-glycoprotein	P02765	1.634
Fetuin-B	Q9UGM5	0.533
Fibrinogen alpha chain	P02671	3.973
Fibrinogen gamma chain	P02679	0.979
Fibronectin	P02751	2.560
Gelsolin	P06396	0.726
Glutathione peroxidase 3	P22352	0.873
Hemoglobin subunit beta	P68871	0.073
Hemoglobin subunit delta	P02042	4.327
Hemopexin	P02790	1.419
Heparin cofactor 2	P05546	1.423
Haptoglobin	P00738	2.810
Haptoglobin-related protein	P00739	1.935
Histidine-rich glycoprotein	P04196	7.466
Plasma protease C1 inhibitor	P05155	1.194
Ig alpha-1 chain C region	P01876	0.902
Ig alpha-2 chain C region	P01877	4.596
Ig gamma-1 chain C region	P01857	0.951
Ig gamma-2 chain C region	P01859	1.208
Ig gamma-3 chain C region	P01860	1.916
Ig mu chain C region	P01871	1.886

Protein Description	Protein Accession	LOQ (µg/ml)
Plasma serine protease inhibitor	P05154	4.418
Inter-alpha-trypsin inhibitor heavy chain H1	P19827	2.853
Inter-alpha-trypsin inhibitor heavy chain H2	P19823	12.786
Inter-alpha-trypsin inhibitor heavy chain H4	Q14624	1.922
Kallistatin	P29622	3.407
Plasma kallikrein	P03952	6.996
Kininogen-1	P01042	5.125
Pigment epithelium-derived factor	P36955	1.680
N-acetylmuramoyl-L-alanine amidase	Q96PD5	2.102
Platelet factor 4	P02776	0.930
Plasminogen	P00747	13.376
Serum paraoxonase/arylesterase 1	P27169	1.675
Proteoglycan 4	Q92954	13.486
Retinol-binding protein 4	P02753	2.765
Serum amyloid A-4 protein	P35542	0.687
Selenoprotein P	P49908	2.114
Sex hormone-binding globulin	P04278	0.940
Tetranectin	P05452	1.329
Thyroxine-binding globulin	P05543	2.325
Prothrombin	P00734	14.768
Serotransferrin	P02787	3.144
Transthyretin	P02766	0.542
Vitamin D-binding protein	P02774	2.021
Vitronectin	P04004	2.193
von Willebrand factor	P04275	2.920
Zinc-alpha-2-glycoprotein	P25311	0.437