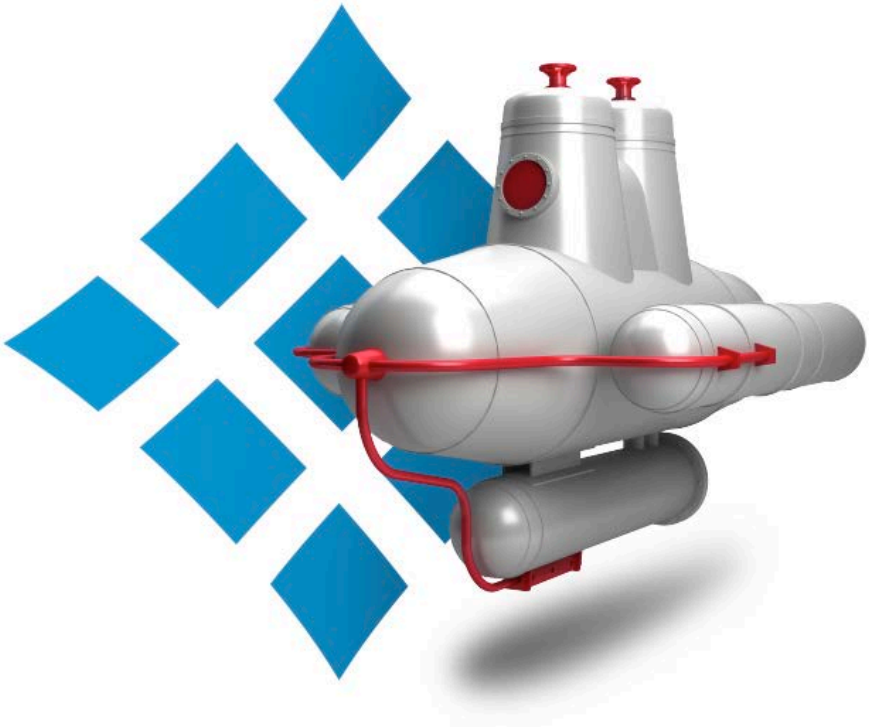

PlasmaDeepDive™

Multiplexed Assay Panel for depleted Human Plasma
MRM or PRM analysis

MANUAL

Table of Contents

PlasmaDeepDive™ Assay Panel Components

PlasmaDeepDive™ Kit	Part No: Ki-3006-48 or Ki-3006-96 Sufficient for analysis of 48 or 96 samples
Reference Peptide Mix	1x or 2x 1.1 ml glass vial, dark blue cap
Alkylation Solution	1x or 2x 10 ml tube, yellow cap
Reduction Stock Solution	1x or 2x 0.5 ml tube, orange cap
LC Solution	1x or 2x 10 ml tube, clear cap
10x Dilution Buffer	1x or 2x 10 ml tube, green cap
Denature Buffer	1x or 2x 10 ml tube, violet cap
Dissolution Buffer	1x or 2x 2.0 ml tube, light blue cap
96-well MACROSpin Plate	1x with 2x 96-well plate included
96-well Plate	3x 96-well plate packaged separately
96-well Plate Sealer	6x plate sealer
PlasmaDeepDive™ MRM and PRM Panel plug-ins	Available upon request at support@biognosys.com
PlasmaDeepDive™ Assay Panel Manual	Available at www.biognosys.com/shop/plasmadeepdive

Storage and Quality Control of the PlasmaDeepDive™ Kit

Immediately after receiving the kit store:

- Reference Peptide Mix at **-20°C**
- Reduction Stock Solution and Alkylation Solution at **+4°C** and **protected from light**
- All other components should be stored dry at room temperature (15–25°C)

In accordance with Biognosys' Quality Management System, each lot of the kit is tested against predetermined specifications to ensure consistent product quality.

Use Limitations

PlasmaDeepDive™ Assay Panel is intended for mass spectrometry proteomics applications and research use only. This product is not intended for the diagnostic, prevention, or treatment of a disease. All due care and attention should be exercised in the handling of the products.

Product Warranty and Satisfaction Guarantee

Biognosys guarantees the performance of the product when following the instructions and protocols described in this product manual. However, the user must determine the suitability of the product for its particular use. Should the product fail to perform satisfactorily due to any reason other than misuse, Biognosys will replace it free of charge. Biognosys reserves the right to change, alter, or modify any product to enhance its performance and design.

If you have questions about product specifications or performance, please contact us at support@biognosys.com. We also encourage you to contact us if you have any suggestions for improving product performance or for its use in new applications and techniques.

Technical Assistance

Our Technical Department is composed of experienced scientists with extensive practical and theoretical expertise in proteomic technologies and bioinformatics. If you have any questions or experience any difficulties with PlasmaDeepDive™ Assay Panel please do not hesitate to contact us at support@biognosys.com, call +41 44 738 20 40 or visit www.biognosys.com/shop/plasmadeepdive.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the material safety data sheet (MSDS) available online in convenient and compact PDF format at www.biognosys.com/shop/plasmadeepdive.

The following risk and safety phrases apply to components of PlasmaDeepDive™ Kit.

10X Dilution Buffer: Harmful if swallowed.

Alkylation Solution: Toxic if swallowed, may cause allergy or asthma symptoms or breathing difficulties if inhaled, may cause an allergic skin reaction, may cause long lasting harmful effects to aquatic life.

Dissolution Buffer: Highly flammable liquid and vapour, causes serious eye irritation.

Reduction Stock Solution: Harmful if swallowed, causes skin and eye irritation, may cause respiratory irritation.

Introduction: PlasmaDeepDive™ Assay Panel at a Glance

Blood is the most frequently used human biological sample in research and routine laboratory diagnostics. Protein levels in blood plasma reflect the health status of single organs as well as the body as a whole. Changes in blood protein levels can be directly correlated to a disease onset or therapy response and are often key indicators of a certain physiological or pathological condition. However, to evaluate the overall

status of the body simultaneous monitoring of levels of major blood proteins is required. PlasmaDeepDive™ Assay Panel is specifically designed for this task enabling quantification of up to 100 plasma proteins in a single sample with unbeatable specificity and precision. It sets a new standard for clinical research, biomarker screening and development of companion diagnostics.

The PlasmaDeepDive™ Assay Panel was designed for targeted proteomics approaches - Multiple Reaction Monitoring (MRM, also called SRM) or Parallel Reaction Monitoring (PRM) - which focus on quantifying predefined sets of proteins with high sensitivity and reproducibility. Biognosys is unique in its ability to perform scheduled highly multiplexed MRM and PRM measurements based on its innovative iRT concept and specifically developed signal processing software – SpectroDive™. Both the iRT and SpectroDive™ are integral parts of Biognosys' Assay Panels.

The PlasmaDeepDive™ Assay Panel requires as little as 50 µg of protein from depleted plasma samples and 48 hours to provide actionable clinically-relevant data. The panel is perfect for large-scale experiments requiring high-throughput.

PlasmaDeepDive™ Multiplexed Assay Panel optimally combines 100 peptide MRM or PRM assays in one scheduled method. Each peptide is representative of a human plasma protein (**Table 1**). Since the PlasmaDeepDive™ Assay Panel is designed for depleted plasma samples, 16 peptides corresponding to the typically depleted proteins are included into the panel to monitor the depletion quality (Group "Depleted Protein"). Furthermore, 9 housekeeping proteins (Group "QC Protein") are measured in the panel to allow for normalization and consequently higher reproducibility of the results. Other 75 proteins in the panel represent relevant targets for biomarker screening (Group "Target").

Table 1. List of proteins quantified with PlasmaDeepDive™ Assay Panel

UniPot ID	Protein Name	Group
P11021	78 kDa glucose-regulated protein	Target
Q15848	Adiponectin	Target
Q96KN2	Beta-Ala-His dipeptidase	Target
P01138	Beta-nerve growth factor	Target
P23560	Brain-derived neurotrophic factor	Target
P55290	Cadherin-13	Target
P33151	Cadherin-5 (CD144)	Target
Q96IY4	Carboxypeptidase B2	Target
Q9NQ79	Cartilage acidic protein 1	Target
P04637	Cellular tumor antigen p53	Target
P36222	Chitinase-3-like protein 1	Target
P26441	Ciliary neurotrophic factor	Target
P03951	Coagulation factor XI	Target
Q9NZP8	Complement C1r subcomponent-like protein	Target
P0C0L5	Complement C4-B	Target
P06850	Corticoliberin	Target
P01034	Cystatin-C	Target
Q01459	Di-N-acetylchitobiase	Target
P09172	Dopamine beta-hydroxylase	Target
Q12805	EGF-containing fibulin-like extracellular matrix protein 1	Target

P00533	Epidermal growth factor receptor	Target
P09104	Gamma-enolase	Target
P14136	Glial fibrillary acidic protein	Target
O43301	Heat shock 70 kDa protein 12A	Target
Q0VDF9	Heat shock 70 kDa protein 14	Target
P08107	Heat shock 70 kDa protein 1A/1B	Target
P34931	Heat shock 70 kDa protein 1-like	Target
P34932	Heat shock 70 kDa protein 4	Target
O95757	Heat shock 70 kDa protein 4L	Target
P17066	Heat shock 70 kDa protein 6	Target
P11142	Heat shock cognate 71 kDa protein	Target
Q04756	Hepatocyte growth factor activator	Target
P05019	Insulin-like growth factor I	Target
P01344	Insulin-like growth factor II	Target
P18065	Insulin-like growth factor-binding protein 2	Target
P17936	Insulin-like growth factor-binding protein 3	Target
P35858	Insulin-like growth factor-binding protein complex acid labile subunit	Target
Q06033	Inter-alpha-trypsin inhibitor heavy chain H3	Target
Q9NPH3	Interleukin-1 receptor accessory protein	Target
P22301	Interleukin-10	Target
O75874	Isocitrate dehydrogenase [NADP] cytoplasmic	Target
P41159	Leptin	Target
P18428	Lipopolysaccharide-binding protein	Target
P07195	L-lactate dehydrogenase B chain	Target
P14151	L-selectin (CD62L)	Target
P51884	Lumican	Target
P14780	Matrix metalloproteinase-9	Target
P01033	Metalloproteinase inhibitor 1	Target
P16455	Methylated-DNA--protein-cysteine methyltransferase	Target
P08571	Monocyte differentiation antigen CD14	Target
Q13201	Multimerin-1	Target
Q9UNW1	Multiple inositol polyphosphate phosphatase 1	Target
P20929	Nebulin	Target
P48681	Nestin	Target
P13591	Neural cell adhesion molecule 1 (CD56)	Target
O00533	Neural cell adhesion molecule L1-like protein	Target
P08949	Neuromedin-B	Target
O14786	Neuropilin-1 (CD304)	Target
P30990	Neurotensin/neuromedin N	Target
P60484	Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase PTEN	Target
P80108	Phosphatidylinositol-glycan-specific phospholipase D	Target
P02775	Platelet basic protein	Target
P07359	Platelet glycoprotein Ib alpha chain	Target
P40197	Platelet glycoprotein V (CD42d)	Target
P20742	Pregnancy zone protein	Target
P01303	Pro-neuropeptide Y	Target
P20366	Protachykinin-1	Target
Q9HD89	Resistin	Target

P02743	Serum amyloid P-component	Target
P38646	Stress-70 protein, mitochondrial	Target
Q16563	Synaptophysin-like protein 1	Target
P07996	Thrombospondin-1	Target
Q6EMK4	Vasorin	Target
P22891	Vitamin K-dependent protein Z	Target
P04275	von Willebrand factor	Target
P01009	Alpha-1-antitrypsin	Depleted Protein
P01023	Alpha-2-macroglobulin	Depleted Protein
P02647	Apolipoprotein A-I	Depleted Protein
P02652	Apolipoprotein A-II	Depleted Protein
P01024	Complement C3	Depleted Protein
P02671	Fibrinogen alpha chain	Depleted Protein
P02675	Fibrinogen beta chain	Depleted Protein
P02679	Fibrinogen gamma chain	Depleted Protein
P00738	Haptoglobin	Depleted Protein
P01876	Ig alpha-1 chain C region	Depleted Protein
P01857	Ig gamma-1 chain C region	Depleted Protein
P01859	Ig gamma-2 chain C region	Depleted Protein
P01871	Ig mu chain C region	Depleted Protein
P02787	Serotransferrin (Transferrin)	Depleted Protein
P02768	Serum albumin	Depleted Protein
P02766	Transthyretin	Depleted Protein
P04217	Alpha-1B-glycoprotein	QC protein
P00450	Ceruloplasmin	QC protein
P02790	Hemopexin	QC protein
P19823	Inter-alpha-trypsin inhibitor heavy chain H2	QC protein
Q14624	Inter-alpha-trypsin inhibitor heavy chain H4	QC protein
P01042	Kininogen-1	QC protein
P05155	Plasma protease C1 inhibitor	QC protein
P02774	Vitamin D-binding protein	QC protein
P04004	Vitronectin	QC protein

Important Notes before Start

Before starting with the sample preparation, read through the steps carefully and make sure all the required reagents and equipment are available.

Use minimally LC-grade solvents and water throughout the protocol to prepare buffers and solutions.

The kit components are sufficient for 48 or 96 depleted plasma samples.

The kit is designed to be used in 1 or 2 batches of 48 samples each. All reagents are supplied in two tubes / vials. All plates should be sealed after the first use and stored appropriately until the second usage. Note, that when starting processing the second batch wells of the 96-well plate matching the unused part of the MACROSpin plate should be used.

To process 96 samples as a single batch all provided reagents are needed. For all reagents the content should be combined from the both tubes / vials. Repeat steps 1-3 and 9 for both provided tubes and prepare 20 ml 1x Dilution Buffer in step 14 of Sample Preparation Procedure & LC-MRM/PRM Analysis.

Sample Requirements

Dried depleted human plasma with 50 µg total protein per sample.

Reserve one suitable sample for calibration run. If it is not possible to dedicate one sample for a calibration run, a separate LC-MS sample for calibration can be prepared following instructions in Section E of Sample Preparation Procedure & LC-MRM/PRM Analysis.

Buffer exchange after depletion might be necessary depending on the depletion system used. Otherwise residual crystals of salts and detergents may be forming after C18 clean-up.

For drying the samples after depletion with the required amount of total protein you might use one of the enclosed 96-well plates. If required, seal the plate after drying with a plastic film and store at -20°C.

SpectroDive™ Software and PlasmaDeepDive™ Panel Plug-in

The integral part of the PlasmaDeepDive™ Assay Panel is the SpectroDive™ software that enables easy MS method setup and automated signal processing of your LC-MRM/PRM measurements. SpectroDive™ supports all Biognosys' Assay Panels. For each panel corresponding software plug-ins are required.

To activate your SpectroDive™ license, which is bundled with the PlasmaDeepDive™ workflow kit, please contact support@biognosys.com. The downloading instructions and your personal **License Key** will be sent to your e-mail account within one working day. Also, the PlasmaDeepDive™ Panel plug-ins will be provided to you by the Biognosys team upon request to support@biognosys.com. You can also find all information about SpectroDive™ and full licensing models at www.biognosys.com/shop/spectrodive.

SpectroDive™ can be installed and run on a notebook or desktop computer. The minimal system requirements are: **Windows 7 or higher, 4GB RAM, 50GB free hard disc space, software .NET 4.5.**

SpectroDive™ Installation

- Close all running programs
- Open the SpectroDive™ installer (administrator rights are required) and follow the wizard instructions

- Carefully read through the license agreement
- Choose the location for installation
- Check settings and proceed with installation
- A confirmation message will appear after successful installation

SpectroDive™ Activation

When running SpectroDive™ for the first time you will be prompted to activate the software. For activation use your personal License Key from the e-mail with the downloading instructions.

An internet connection is required for the on-line SpectroDive™ activation. If your computer is not connected to the internet or the activation is blocked by your firewall, please follow the SpectroDive™ instructions for the off-line activation.

PlasmaDeepDive™ Panel Plug-in Import (MRM or PRM)

- In SpectroDive™ select the 'Prepare' perspective by clicking on the corresponding icon on the top.
- Import the **.kit** file for PlasmaDeepDive™ by clicking on 'Import New Panel' in the low part of '1) Choose Panel' section on the left.
- The panel "PlasmaDeepDive™ [HUMAN]" or "PlasmaDeepDive.PRM™ [HUMAN]" is now available in the 'Panel' folder.

Additionally Required Laboratory Equipment and Consumables

Multichannel (5 µl – 200 µl) and single channel pipettes (0.5 µl – 1000 µl) with corresponding tips
Liquid trays for multichannel pipettes
Glass syringe (100 µl)
pH paper (recommended universal pH indicator paper pH 1-10 with colour scale) or pH meter with small combined glass electrode
Vortex mixer
15 ml plastic tubes
Benchtop centrifuge
Thermomixer at +37°C compatible with 96-well plates
Centrifuge with plate rotor and cooling option (+4°C)
Vacuum centrifuge with plate rotor
LC-MS vials or autosampler compatible with 96-well plate

Additionally Required Reagents, Solvents and Solutions

Sequencing grade modified trypsin, stock solution at 0.4 µg/µl (recommended Promega Product Catalog#V5113)
20% (v/v) TFA solution, prepare at least 2 ml for 48 samples (Note 1, Note 2)
Methanol, at least 10 ml for 48 samples
Water
Acetonitrile
C18 Cleaning Solution, prepare at least 15 ml for 48 samples (Note 1) ♦ 80% (v/v) Acetonitrile ♦ 0.1% (v/v) TFA (Note 2, Note 3) ♦ In water
C18 Washing Solution, prepare at least 60 ml for 48 samples (Note 1) ♦ 1% (v/v) Acetonitrile ♦ 0.1% (v/v) TFA (Note 2, Note 3) ♦ In water
C18 Elution Solution, prepare at least 20 ml for 48 samples (Note 1) ♦ 50% (v/v) Acetonitrile ♦ 0.1% (v/v) TFA (Note 2, Note 3) ♦ In water

Note 1
Solution can be stored at room temperature for up to one year.

Note 2
Use a glass syringe to pipette strong acidic solutions like concentrated TFA.

Note 3
Use 20% (v/v) TFA solution.

Sample Preparation Procedure & LC-MRM/PRM Analysis

A. Denaturation, reduction and alkylation

1. Dissolve 10x Dilution Buffer with water to a total volume of 5 ml (Note 4), vortex until solubilized.
2. Dissolve Reduction Stock Solution with 250 µl water, vortex, briefly spin down.
3. Prepare Denature Buffer:
   3.1. Add 500 µl of 10x Dilution Buffer to the Denature Buffer tube. Keep 10x Dilution Buffer in fridge until further usage in step 14.
   3.2. Add 25 µl of Reduction Stock Solution to the Denature Buffer tube.
   3.3. Fill up Denature Buffer with 2.3 ml of water (Note 4, Note 5).

Note 4 To solubilize the provided solid components, dissolve first in a small amount of the stated solvent and fill-up to final volume carefully.

Note 5
Warm up the tube to help solubilizing the reagent by holding the tube in warm (<40°C) tap water.

Note 6
If depletion buffer

4. Add 80 µl Denature Buffer to each dried depleted plasma sample (50 µg protein/sample).
5. Gently shake the plate or vials on thermomixer for 1 min at room temperature (Note 6).
6. Briefly spin down to collect all liquid at the bottom of the wells.
7. Incubate the plate or vials at +37°C, 600 rpm on thermomixer for 30 min (Note 6).
8. If not already in a 96-well plate, transfer samples to a 96-well plate (Note 7, Note 8).
9. Let the samples cool to room temperature while preparing the Alkylation Solution:
   9.1. Fill up Alkylation Solution with water to 3 ml (Note 4), vortex, briefly spin down (Note 9).
10. Using a multichannel pipette, add 15 µl/well of Alkylation Solution.
11. Gently shake the plate on thermomixer for 1 min at room temperature.
12. Briefly spin down to collect all liquid at the bottom of the wells.
13. Incubate the plate at room temperature **in the dark** for 30 min.

wasn`t exchanged residual salts and detergent crystals may form.

Note 7
If only 48 samples are processed at a time, seal the unused wells.

Note 8
Randomize sample location on plate.

Note 9
Light sensitive, prepare shortly before usage and keep in dark.

B. Dilution

14. Prepare 1x Dilution Buffer by mixing in a separate tube 1 ml of 10x Dilution Buffer and 9 ml of water.
15. Using a multichannel pipette, add 114 µl/well of 1x Dilution Buffer to a **new** 96-well plate (Note 7).
16. Using a multichannel pipette, add 38 µl/well of denatured depleted plasma sample (from Section A, step 13).

Optional: you might freeze remaining sample in the plate for a repeated analysis.

17. Check pH to be 8–9 in a few wells by pipetting 0.5 µl onto a pH paper or by using a pH-electrode (Note 10).
    17.1. If pH is below 8, adjust it in **all** wells using 5 µl of 10x Dilution Buffer.
    17.2. Check again in a few wells, repeat steps 17.1 and 17.2 if necessary.

Note 10
Avoid sample cross-contamination by using fresh tips or cleaning the electrode with water for every sample.

C. Digestion using endoprotease trypsin

18. Thaw trypsin (0.4 µg/µl) and spin down briefly.
19. Add 1 µl/well of trypsin to the 96-well plate from Section B using a single channel pipette.
20. Gently shake the plate on thermomixer for 1 min at room temperature.

21. Briefly spin down to collect all liquid at the bottom of the wells.
22. Incubate the plate at +37°C, 600 rpm on thermomixer for 3 hours.
23. Acidify samples by adding 25 µl/well of 20% (v/v) TFA solution using a multichannel pipette (Note 11).
24. Check pH to be below 2 in a few wells by pipetting 0.5 µl onto a pH paper or by using a pH-electrode.
    24.1. If pH is above 2, adjust it in **all** wells adding 5 µl of 20% (v/v) TFA solution.
    24.2. Check again in a few wells, repeat steps 24.1 and 24.2 if necessary.
25. Gently shake the plate on thermomixer for 1 min at room temperature.
26. Briefly spin down to collect all liquid at the bottom of the wells.

Note 11
Foaming is possible; add 20% (v/v) TFA solution slowly.

Optional: if convenient store samples at -20°C until C18 clean-up.

D. Sample clean-up using 96-well MACROSpin plates

Spin plate preparation

27. Place 96-well MACROSpin Plate on top of a **new** 96-well plate.
28. Remove the aluminium protection from the wells that will be used.
29. Add 200 µl/well of methanol to each well, centrifuge at 100 x *g* for 1 min, and discard flow-through.
30. Using a multichannel pipette, add 200 µl/well of C18 Cleaning Solution, centrifuge at 100 x *g* for 1 min, and discard flow-through.
31. Using a multichannel pipette, add 200 µl/well of C18 Washing Solution, centrifuge at 400 x *g* for 1 min, and discard flow-through.
32. Repeat step 31 two more times.

Sample loading to spin plate

33. Centrifuge (thawed) 96-well plate from Section C with your samples at 1000 x *g* for 1 min.
34. Place 96-well MACROSpin Plate on a **new** 96-well plate, keep the old plate for later steps (36 to 39).
35. Load samples (supernatant) on 96-well MACROSpin Plate using a multichannel pipette, centrifuge at 400 x *g* for 1 min, **do not discard flow-through** (Note 12).
36. Place spin plate on the 96-well plate previously used during the spin plate preparation (steps 27 to 32).
37. Load flow-through from step 35 on 96-well MACROSpin Plate using a multichannel pipette, centrifuge at 400 x *g* for 1 min and then discard the flow-through (Note 12).

Note 12
You may increase the centrifugation force up to 1'000 x g in case of low flow rates through the MACROSpin plate.

38. Add 200 µl/well of C18 Washing Solution using a multichannel pipette, centrifuge at 400 x *g* for 1 min and discard the flow-through (Note 12).
39. Repeat step 38 two more times.
    If applicable, seal the unused wells after step completion and store the plate upon further usage (Note 7).

Elution and sample preparation

40. Place 96-well MACROSpin Plate on a **new** 96-well plate.
41. Add 170 µl/well of C18 Elution Buffer using a multichannel pipette, centrifuge at 400 x *g* for 1 min, keep flow-through in 96-well plate.
42. Repeat step 41 once again, collecting all eluates in the same collection plate (Note 7).

Optional: if necessary store 96-well plate with samples at -20°C until drying.

43. Dry down the combined eluates using a vacuum centrifuge (Note 13, Note 14).

Note 13
Transfer eluates to Eppendorf tubes, if necessary.

Note 14
Dried samples can be stored at -20°C until usage.

E. LC-MS sample preparation & LC settings

44. Dissolve dried samples (step 43) in 22.5 µl/well of LC Solution by pipetting up and down with a multichannel pipette.
45. Gently shake the plate on thermomixer for 1 min at room temperature.
46. Centrifuge the dissolved samples at 4°C and 1000 x *g* for 20 min.
47. Transfer 6 µl of sample supernatants to LC-MS vials or to an autosampler-compatible 96-well plate. Store remaining samples at -20°C.
48. Prepare Reference Peptide Mix by adding to the glass vial 20 µl of Dissolution Buffer; vortex briefly.
49. Add 100 µl of LC Solution to the Reference Peptide Mix.
50. Vortex the Reference Peptide Mix, sonicate for 5 min if possible.
51. Add 2 µl/sample of Reference Peptide Mix to each LC-MS vial using a single channel pipette.
52. Inject 3 µl/sample for all LC-MS measurements. Please consider the recommended LC settings below.

If you have included a Calibration Sample in your 96-well plate continue with Section F. Otherwise follow the steps 53-55.

53. Prepare a Calibration Sample by pooling into a separate LC-MS vial of 1 µl from 9 randomly selected samples from step 44.
54. Add 3 µl of Reference Peptide Mix to the Calibration Sample.

55. Inject 3 µl of the calibration sample for LC-MS measurements. Please consider the recommended LC settings below, depending on your acquisition method:

Recommended nano-flow LC settings for LC-MRM:

Time, min	Solvent A 1% AcN / 0.1% FA / H_2O	Solvent B 3% H_2O / 0.1% FA / AcN
0	100%	0%
30	65%	35%
32	0%	100%
40	0%	100%
Flow rate	0.3 µl/min	
Sample loading	3µl/injection (approx. 2µg protein/injection)	

Recommended nano-flow LC settings for LC-PRM:

Time, min	Solvent A 1% AcN / 0.1% FA / H_2O	Solvent B 3% H_2O / 0.1% FA / AcN
0	100%	0%
120	65%	35%
122	0%	100%
130	0%	100%
Flow rate	0.3 µl/min	
Sample loading	3µl/injection (approx. 2µg protein/injection)	

If you are acquiring your samples in PRM mode, continue at step 73.

F. LC-MRM retention time (RT) calibration and scheduled measurement of PlasmaDeepDive™ Assay Panel

LC-MS retention time calibration

56. Open the "Prepare" perspective in SpectroDive™ by clicking on the corresponding icon top left.
57. In section "1) Choose panel" select iRT-Kit from the folder "LC calibration".
58. In section "3) Export Method" select your instrument and software version, set Dwell Time to 10-20 ms.
59. Export the method (transition file) as comma-separated file by clicking on 'Export Method …' and save it to the computer connected to your LC-MS system.
60. Import the method (transition file) to your unscheduled LC-MRM (also called SRM) instrument method.
61. Measure your calibration sample from step 55 in unscheduled MRM mode on your standard LC setup.
62. Transfer the calibration run file (.RAW or .WIFF) to the computer running SpectroDive™.
63. Open the 'Review' perspective in SpectroDive™ by clicking on the corresponding icon on the top.

64. To load the calibration run file click on 'Load raw data…' and select the run file, click 'Start'.
65. Verify the calibration linearity and the intensities of iRT peptides in the calibration run.

Scheduled measurement of PlasmaDeepDive™ panel

66. Change to 'Prepare' perspective by clicking on the corresponding icon on the top to recalibrate the PlasmaDeepDive™ panel for the current LC setup.
67. Select the panel 'PlasmaDeepDive™ [HUMAN]' in the '1) Choose Panel' section
68. Select the calibration run used for calibration in step 65 in the '2) Choose LC calibration' section. The most recently loaded run is shown on top of the table, however, you may select from other previously loaded runs.
69. Adjust the instrument and method settings in '3) Export Method' section (Note 15).
    69.1. Change to a scheduled method on the pull-down menu 'Scheduling'.
    69.2. Adjust the retention time window in minutes in the field 'Window (min)' in order to not to exceed a maximum number of concurrent transitions (e.g. assuming a constant cycle time of 2.5 s, one should not exceed maximal 250 concurrent transitions since this may lead to a dwell time of lower than 10 ms).
70. Export the method (transition file) by clicking on 'Export Method …' and save it to a location of your choice.
71. Attach the transition file to the schedule instrument method of choice and start the data acquisition of your samples from step 52.
    71.1. Make sure that you use the exact same LC setup and gradient as used for the calibration.
    71.2. Make sure that you use constant cycle time.
72. Measure all samples in your experiment using the same scheduled instrument method (Note 16).
    72.1. Continue with data analysis at step 90.

Note 15
The plot in section '3) Export Method' shows the number of concurrent transitions or precursors (i.e. the number of transitions or precursors the instrument has to cycle through at a certain time in the gradient).

Note 16
In case of major system changes (e.g. new column) a new calibration run has to be performed and a new scheduled instrument method has to be generated according to the Section 0.

G. LC-PRM retention time (RT) calibration and scheduled measurement of PlasmaDeepDive™ Assay Panel

LC-MS retention time calibration

73. Open the "Prepare" perspective in SpectroDive™ by clicking on the corresponding icon top left.

74. In section “1) Choose panel” select “PRM iRT-Kit” from the folder “LC calibration”.
75. In section “3) Export Method” select your instrument and software version
76. Export the method (inclusion list) as comma-separated file by clicking on ‘Export Method …’ and save it to the computer connected to your LC-MS system.
77. Import the inclusion list to your unscheduled PRM instrument method.
78. Measure your calibration sample in unscheduled PRM mode on your standard LC setup.
79. Transfer the calibration run file (.RAW or .WIFF) to the computer running SpectroDive™.
80. Open the ‘Review’ perspective in SpectroDive™ by clicking on the corresponding icon on the top.
81. To load the calibration run file click on ‘Load raw data…’ and select the run file, click ‘Start’.
82. Verify the calibration linearity and the intensities of iRT peptides in the calibration run.

Scheduled measurement of PlasmaDeepDive™ Assay Panel

83. Change to ‘Prepare’ perspective by clicking on the corresponding icon on the top to recalibrate the PlasmaDeepDive™ panel for the current LC setup.
84. Select the panel ‘PlasmaDeepDive™.PRM’ in the ‘1) Choose Panel’ section
85. Select the calibration run used for calibration in step 65 in the ‘2) Choose LC calibration’ section. The most recently loaded run is shown on top of the table, however, you may select from other previously loaded runs.
86. Adjust the instrument and method settings in ‘3) Export Method’ section (Note 15).
    86.1. Change to a scheduled method on the pull-down menu ‘Scheduling’.
    86.2. Adjust the retention time window in minutes in the field ‘Window (min)’ in order to not to exceed a maximum number of 80 concurrent precursors

87. Export the method (inclusion list) by clicking on 'Export Method …' and save it to a location of your choice.
88. Import the inclusion list into the PRM instrument method of choice (Note 17) and start the data acquisition of your samples from step 52.
    88.1. Make sure that you use the exact same LC setup and gradient as used for the calibration.
89. Measure all samples in your experiment using the same scheduled instrument method (Note 16).

Note 17
We recommend to use a resolution of 17.5k (Orbitrap) or 30k (Orbitrap HF) which corresponds to a max fill time of 55ms per precursor, due to the high level of multiplexing of the assay panel.

H. PlasmaDeepDive™ Assay Panel data analysis

90. After the panel measurements are done, transfer the raw files to the computer running SpectroDive™.
91. Change to the 'Review' perspective and click on 'Load Raw Data…' directly or first on the 'plus' sign on top to generate a new experiment tab.

Optional: define a name for the experiment in the field 'Name'.

92. Load run files (.RAW or .WIFF) and click 'Start'.
93. An experiment tab will open. In the left panel the LC-MS runs are shown as nodes in a tree structure. In the status bar you can see the progress of loading the mass spectrometer raw data. If you click on the LC-MS run node on the left you will see two charts on the right. On top in the "LC Gradient" plot you can see the retention times of the iRT peptides indicating the linearity of your LC-gradient. In the lower plot you can see the summed up ion traces for all the peptides that were measured.
94. You may make a comment to each LC-MS run e.g. assigning a sample ID or general remarks by right clicking on the LC-MS run name in the left panel and selecting 'Comment (C)'.
95. By clicking on the LC-MS run node, the panels measured in this run will appear.
96. By clicking on a panel node the peptide nodes will appear.
97. When you click on a peptide node two graphical panels on the right will appear.
    97.1. The top right panel displays all the transitions of this peptide as well as the peak boundaries and the Qvalue. The Qvalue indicates the confidence of the endogenous (light) peptide identification (lower Qvalue indicates higher confidence) (Note 18).

Note 18
SpectroDive™ by default qualifies a protein as

97.2. The bottom right panel shows the sum of all transitions together with the peak boundaries as well as quantitative information about this peptide.

97.3. You can zoom in the plots and perform various actions by right clicking on the plot. Right clicking also enables you to set the scale back to default after zooming.

97.4. You can select from various plots to be displayed in the pull-down menus on top for 'Upper panel' and 'Lower panel' e.g. to 'Alignment view' in order to review one peptide across all runs.

98. Review the peptide signals (User Verification).

98.1. When a peptide is selected, you can manually accept or reject it. Use the menu that appears by right clicking on the peptide node or toggle using the keyboard hotkeys "A" and "R". If no action is performed, the peptides remain in the analysis as unchecked (no user verification).

98.2. To manually change peak integration, readjust the green integration boundaries (green box). Drag and drop the border to the correct location. The status of the peak will change to "User Manually Integrated".

98.3. To return to the SpectroDive™ initial integration, right click on the respective peptide node and select 'Remove User Peak'.

98.4. You may make a comment to each peptide by right clicking on the peptide node and selecting 'Comment (C) (Note 19).

99. Peptide node includes the transition group node. Transition group nodes are used to group transitions according to isotopic labelling. If you select the transition group node on the left you will again see two graphical panels on the right. The top panel shows the transitions of this transition group in different colours. The bottom panel displays information about the peak. You can see quantitative as well as chromatographic information.

100. One level below you can find the transition node. If transition node is selected you will see again two graphical panels on the left that show the information specific to this transition.

101. You can use multiple filtering options at the bottom of the left panel to select specific nodes of the tree (e.g. in Qvalue filter when 'Value' is set ⩽ 0.01, only the peptides with Qvalues ⩽0.01 are displayed).

identified when Qvalue is ⩽0.01.

Note 19
This is not equal to the comments that can be made to each run.

Generation of report

102. Switch to the 'Report' perspective.

103. Choose 'PlasmaDeepDiveReport' schema on the left.

104. Export the report to a tab separated table file (.xls file). This file format can be further edited with e.g. Microsoft Excel.

Report Columns Description

The SpectroDive™ report is run based. Each row in the report corresponds to a run.

The following columns are included into the report:

Column name	Description
Experiment Name	The name of experiment that have been specified while loading the data. If the user hasn't specified anything, SpectroDive™ automatically assigns a default name.
Run Name	The name(s) of run file(s) without the .RAW or .WIFF extension.
Run Comment	Comment made while reviewing the run in step 94 (optional).
Protein Id	The UniProt accession number of the protein.
Protein Name	The UniProt identifier of the protein.
Is Identified	TRUE or FALSE, states whether the protein has been identified with confidence (Qvalue ≤0.01) in the respective sample.
Absolute Amount [fmol]	Absolute amount of the protein in the injected sample. The protein amount in the original sample has to be re-calculated.
Qvalue	Qvalue assigned by SpectroDive™ (see step 97.1).
User Verified	Specifies whether the user has accepted, rejected or not checked the protein manually in the respective sample (see step 98.1).
User Integrated	TRUE or FALSE, states whether the peptide peak has been manually integrated by the user.
Protein Comment	Comment made while reviewing the peptide in step 98.4.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving issues that may arise.

Issue	Possible Cause	Recommended Solution
Crystals appear in samples after adding Denature Buffer	Salts from depletion have not solubilized completely in Denature Buffer	This should not influence the end results. However, try not to transfer crystals into next steps.
LC column is clogged during sample analysis	Solid particles were not completely removed from the samples during the centrifugation (step 46)	Transfer final samples to single vials and repeat a cold centrifugation (4°C) as described in step 46.
The first iRT peptide behaves irregularly	LC gradient starts with > 5% AcN	Ensure you use the LC settings as recommended in Section E. Because of the high peptide hydrophilicity slight irregularities of the first iRT peptide behaviour still can be observed. This usually does not influence the quality of calibration.
iRT calibration is not linear	Non-linear gradient was used	Use linear gradient and LC settings as recommended in the Section E.
	A minor issue in chromatographic system if there are only 1 or 2 outliers from linearity	Check manually if iRT peaks are correctly integrated and if the peak shapes are satisfactory. If yes, exclude outliers from calibration.
	A major defect in chromatographic system if iRT calibration is not linear through the whole range	Please contact the technical support of the LC manufacturer.

Issue	Possible Cause	Recommended Solution
No signal on LC-MS when analyzing samples	Problem with sample preparation	Prepare and analyze a control sample with Reference Peptide Mix diluted in LC Buffer (keep the dilution rate as in the Section E of the protocol). If you see a signal on LC-MS your initial samples were not prepared correctly.
	Problem with the LC-MS system	If you don't see any signal on your LC-MS after analyzing the control sample from above please contact the technical support of your LC-MS vendor.
SpectroDive™ cannot load runs from my Thermo Scientific instrument	The Thermo API might not be properly registered	Contact Thermo Scientific to download the newest version of the msFileReader API. Run the installer in administrator mode.
An error "iRT calibration failed" appears when loading my runs into SpectroDive™	The Reference Peptide Mix was not spiked into samples before LC-MS analysis	Check the raw files in the XCalibur software for iRT peptides and if they are not visible re-analyze the samples by spiking-in the Reference Peptide Mix according to the protocol. Further details on the iRT peptides can be found at www.biognosys.com/shop/irt-kit
	The raw files are corrupted	Transfer the raw files from the instrument again.
SpectroDive™ did not analyze my runs, I only see grey run names in the analysis tree	The Reference Peptide Mix was not spiked into samples as per protocol before LC-MS analysis	Spike the Reference Peptide Mix in the samples (Section E) and repeat the analysis.

Issue	Possible Cause	Recommended Solution
Only iRT peptides are displayed in SpectroDive™ analysis tree	The corresponding Panel Plug-in is not correct or not installed	Install the correct Panel Plug-in for the kit you are using.
	Analytical run was performed with incomplete transition list (e.g. without transitions for panel peptides)	Check if the transition list contains panel peptides. If not, do the calibration according to the protocol (Section 0) and re-analyze the samples with the corrected MS-method.
Exported report is empty or does not correspond to the description in the manual	A correct SpectroDive™ Panel Plug-in is missing	Install a correct Panel Plug-in for the kit you are using.
	The incorrect report schema was chosen in step 103 when generating the report	Choose the correct report schema and export the report again.
Some of the protein quantities in my report are shown as "Not Detectable"	Low quality of the analytical run (unsatisfactory LC-MS performance)	Control performance of your LC-MS system and repeat the sample analysis.
	The protein concentrations are below the limit of quantification	Detectability of target proteins may vary between samples as some of the proteins are only detectable in certain medical conditions.

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