Application Note

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# Automated Plasma Proteomic Sample Preparation

## Using PreOmics iST Technologies and the Agilent Bravo Automated Liquid Handling Platform


### Authors

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## Abstract

Proteins within blood-based samples such as plasma and serum are frequently assayed in clinical research. While theoretically attractive due to the biological prevalence of proteins, studying the plasma proteome within blood-based samples has been extremely challenging due to a plethora of issues[1]. Chief among these issues is the time-consuming and intricate manual steps involved in sample preparation workflows. This makes the parallel processing of samples highly prone to errors, compromising the reproducibility needed for robust comparative analyses. These processing difficulties tend to be exacerbated as sample numbers increase, leading to unreliable results. However, large sample sizes are statistically indispensable to balance the thousands of variables (in this case, proteins and peptides) that are measured in a single, hypothesis-free MS run.

To overcome the previously mentioned issues, Geyer; *et al.* developed the rapid (two-hour) and robust "plasma proteome profiling" pipeline[2]. This protocol benefits from the synergy of the straightforward sample preparation workflow provided by the iST Kit from PreOmics coupled to high-throughput automation from the Agilent Bravo liquid handling platform. Unlike other sample preparation methods, this protocol accomplishes all stages of sample preparation (denaturation, reduction, alkylation, enzymatic digestion, peptide purification, and desalting) in a single reaction vial. By using the Bravo robotic platform, contamination is minimized, reducing systematic error that would be introduced by the user. By relying on these advantages, this single-run LC/MS/MS proteomic workflow enables reproducible quantitative analysis of hundreds of plasma proteomes in an automated fashion from 1 µL of material[2,3].

## Introduction

Proteins in the circulatory system mirror an individual's physiology. Currently, protein levels are generally determined using single-protein immunoassays, a procedure that is prone to several drawbacks[4]. High-throughput, hypothesis-free, and quantitative data using MS-based proteomics would be a highly desirable alternative, but represents unique challenges in it's own right. The most frequently cited issue of the high dynamic range of protein abundances within the proteome is balanced by the ever-improving specifications of mass spectrometers and scan modes[1,2]. However, the lack of robust, high-throughput proteomic workflows needed to identify and verify potential biomarkers in large cohorts has severely restrained the pre-analytical stage of the proteomic workflow.

To overcome this limitation, Geyer; *et al.* (2016)[2] introduced a rapid and robust "plasma proteome profiling" pipeline. Building on the recently described in-StageTip (iST) method[3] further streamlined the procedure for plasma samples specifically. Starting with only 1 µL of plasma from a single finger prick, all preparation steps (denaturation, reduction, alkylation, enzymatic digestion, peptide purification, and desalting) were performed in a single reaction vial: the iST Cartridge. By following the instructions of the iST Kit, the entire up-front sample processing took less than two hours and could readily be performed in a 96-well format on the Bravo liquid handling platform. Contrary to other sample preparation methods, this workflow does not require protein depletion, and enables quantitative analysis of hundreds of plasma proteomes from single 1 µL finger pricks with LC/MS/MS using 20-minute gradients.

This Application Note describes the two-step Agilent Bravo protocol for the plasma proteomic profiling sample preparation workflow applied to plasma samples from one female and one male subject. The plasma proteins were denatured, reduced, alkylated, and digested using the iST kit from PreOmics. Following preparation, the samples were subjected to mixed-phase peptide cleanup, then LC/MS/MS analysis. A total of 3,486 unique peptide sequences and 373 proteins were identified, with an average of 300 unique proteins quantified per run: the large majority being present in all of the 96 samples and only 2 % uniquely in single LC runs. Replicate analyses revealed the degree of reproducibility of the LC/MS workflow for plasma proteome characterization. A Pearson correlation coefficient of 0.98 was observed between complete workflow replicates.

## Experimental

### Materials

**Plasma samples:** Blood was taken by venous puncture to obtain 10 mL of blood. Following centrifugation at 2,000 × g for 20 minutes, plasma was harvested. With prior approval of the ethics committee of the Max Planck Society, blood was sampled from healthy donors, who provided written informed consent.

### Workflow

**iST sample preparation—lysis, protein denaturation, reduction, alkylation and digestion:** The iST sample preparation protocol provides the user with the option of processing 1 to 96 samples in 96-well plates, simultaneously. For this experiment, 96 plasma samples were processed, corresponding to 48 × 1 µL aliquots from each of the two individuals. Following the protocol, the Bravo sequentially draws reagents from the appropriate source plates, adds reagent to the samples, and mixes the solution in the sample plate following the inputs provided in the application interface.

### Preparation of reagents

1. Dissolve plasma sample in water in a ratio of 1:10 (v:v); concentration of ~ 5 µg/µL
2. Add 10 µL of sample to rows 1–12 to an MTP → SAMPLE
3. Add 120 µL of lyse to rows 1–6 to an MTP → LYSE
4. Add 210 µL of resuspend to each tube of digest, and shake (RT; 500 rpm; 10 minutes)
5. Add 120 µL of digest to rows 1–6 to an MTP → DIGEST
6. Add 120 µL of stop to rows 1–12 to an MTP → STOP

Figure 1 shows the correct placement of the reagents on the Agilent Bravo platform.

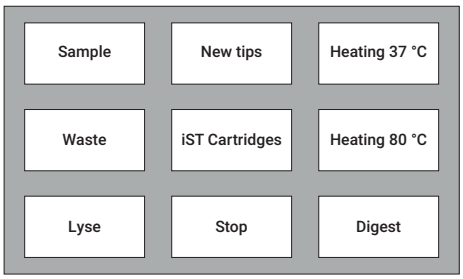


**Figure 1.** The plate configuration for protocol 1: lysis, protein denaturation, reduction, alkylation, and digestion.

## Agilent Bravo System workflow

- Start the robot and incubator
- 80 °C on position 6
- 37 °C on position 3
- Place plates as shown in Figure 1
- Start the protocol
  - Lyse pipetted to iST Cartridges
  - Sample pipetted to iST Cartridges
  - Close iST Cartridges
  - iST Cartridges are transferred to position 6
  - Heating of sample in iST Cartridges at 80 °C for 10 minutes
- Cooling down for five minutes
- Centrifuge iST Cartridges up to 400 g
- Place iST Cartridges at position 5
- Remove the lid
- Restart the protocol
  - Pipette digest to iST Cartridges
  - iST Cartridges transferred to position 3
  - Close iST Cartridges
  - Tryptic digest iST Cartridges at 37 °C for 60 minutes
- Centrifuge iST Cartridges up to 400 g
- Place iST Cartridges on position 5
- Restart the protocol
  - Pipette stop to iST Cartridges
- Place iST Cartridges in adapter onto waste
- Centrifuge iST Cartridges: 3,800 rcf; three minutes

Close the peptide-containing tube or cartridge (use a cap for bottom). Peptides can be frozen at –20 °C. Storage of peptides should not exceed two weeks at –20 °C. For extended storage, finish the protocol and store at –80 °C.

**Table 1.** On-deck sample and reagents.

| iST Sample preparation—protein denaturation, reduction, alkylation, and digestion | |
|---|---|
| **Deck location and reagent** | **Labware type** |
| 1 - Plasma sample, 5 µg/µL in water | twin.tec PCR Plate 96 LoBind (Eppendorf p/n 0030129504) |
| 2 - New tips | 384ST 70 µL Tips, Sterile, (p/n 19133-112) |
| 3 - Heating shaker | Teleshake Heated Shaking Station (G5498B#009) |
| 4 - Waste | |
| 5 - iST Cartridges | Adapter plate (PreOmics; P.O.00027) |
| 6 - Heating | Peltier Thermal Station (G5498B#035) |
| 7 - Lyse | twin.tec PCR Plate 96 LoBind (Eppendorf p/n 0030129504) |
| 8 - Stop | twin.tec PCR Plate 96 LoBind (Eppendorf p/n 0030129504) |
| 9 - Digest | twin.tec PCR Plate 96 LoBind (Eppendorf p/n 0030129504) |

**Table 2.** Analytical instrumentation.

| iST Kit 96x (P.O.000027) for bottom-up proteomic sample preparation | |
|---|---|
| iST Cartridges | Cartridges for 1 to 100 µg protein starting material |
| ADAPTER Plate | Enables placing cartridges in the 96-well MTP plate. |
| WASTE Plate | Deep well plate for collecting waste after washes. |
| MTP plate | LoBind plate for collecting peptides after elution. |
| **Automation** | |
| Core automation platform | Bravo Liquid Handling Platform for Standalone Use (G5523A) |
| Agilent Pipetting Head | 96ST Disposable Tip Head (G5498B#041) |
| Agilent Bravo accessories | Bravo with Gripper Standalone (G5523A#003) |
| Additional accessories | Liquid Handling Accessories (G5498B) |
| Additional accessories | Alignment Station (G5498B#028) |
| Additional accessories | MTC Controller from Inheco (G5498B#015) |
| Additional accessories | Teleshake Heated Shaking Station (G5498B#009) |
| Additional accessories | Slot/Shaker Card (G5498B#019) |
| Additional accessories | CPAC Ultraflat without Controller (G5498B#021) |
| Additional accessories | Custom Nest for CPAC (G5498B#017) |
| Additional accessories | Peltier Thermal Station (G5498B#035) |
| Additional accessories | Bravo risers, 146mm (G5498B#055) |
| Additional accessories | Adapter Plate for INHECO CPAC NUNC (G5498B#012) |
| Additional accessories | V_P PCR Plate Insert VP741I6A (G5498B#013) |
| **LC/MS/MS** | |
| LC system | EASY- nLC 1000 ultra-high-pressure system (Thermo Fisher Scientific) |
| Nano-electrospray ion source | Nano-electrospray ion source (Thermo Fisher Scientific) |
| MS setup | Q Exactive HF Orbitrap (Thermo Fisher Scientific) |
| LC column | 40 cm HPLC-columns |

### iST sample preparation—Peptide cleanup

The tryptic peptides were washed with organic and aqueous washing solution to remove hydrophobic and hydrophilic contaminants such as lipids and salts.

### Preparation of reagents

1. Add 120 µL of Wash 1 on rows 1–12 (twin.tec PCR Plate 96 LoBind; Eppendorf)
2. Add 120 µL of Wash 2 on rows 1–12 (twin.tec PCR Plate 96 LoBind; Eppendorf)
3. Add 120 µL Elute on rows 1–12 (twin.tec PCR Plate 96 LoBind; Eppendorf)
4. Add 120 µL LC Load on rows 1–6 to a twin.tec PCR Plate 96 LoBind; Eppendorf)

Figure 2 shows the correct placement of the reagents on the Bravo platform.

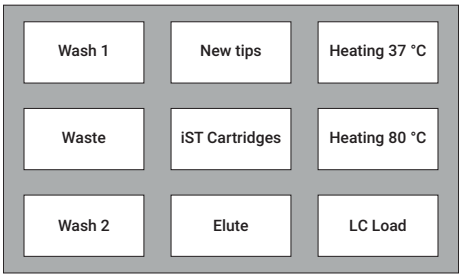


**Figure 2.** The plate configuration (also laid out in Table 1) for step 2—peptide cleanup.

### Workflow on the Bravo System

- Start the robot
- Place iST Cartridges on position 5
- Start the protocol
  - Wash 1 pipetted to iST Cartridges
- Centrifuge iST Cartridges: 3,800 rcf; three minutes
- Place iST Cartridges on position 5
- Restart the protocol
  - WASH 2 pipetted to iST Cartridges
- Centrifuge iST Cartridges: 3,800 rcf; three minutes
- Place iST Cartridges on MTP plate
- Place iST Cartridges on position 5
- Restart the protocol
  - Elute pipetted to iST Cartridges
- Centrifuge iST Cartridges: 3,800 rcf; three minutes
- Speed-Vac ELUTE in MTP plate to completely dryness at 45 °C
- Place MTP plate on position 5
  - LC Load pipetted to MTP plate
  - Sonicate or shake (2,000 rpm) MTP plate to completely resuspend peptides

### LC/MS/MS analyses

Samples were measured using LC/MS instrumentation consisting of an EASY-nLC 1000 ultra-high-pressure system (Thermo Fisher Scientific) coupled with a nano-electrospray ion source (Thermo Fisher Scientific) to a Q Exactive HF Orbitrap (Thermo Fisher Scientific). Purified peptides were separated on 40-cm HPLC columns (75 µm id; in-house packed into the tip with ReproSil-Pur C18-AQ 1.9 µm resin; Dr. Maisch).

Peptides were eluted with a linear 15-minute gradient of 10–50 % buffer B (0.1 % formic acid, 60 % acetonitrile v/v), followed by a 5-minute 98 % wash at a flow rate of 450 nL/min. Column temperature was kept at 60 °C.

### Data analysis

MS raw files were analyzed by MaxQuant software version 1.5.2.10[5], and peptide lists were searched against the human Uniprot FASTA database (version June 2014) and a common contaminants database by the Andromeda search engine[6] with cysteine carbamidomethylation as a fixed modification and N-terminal acetylation and methionine oxidations as variable modifications.

**Table 3.** On-deck sample and reagents.

| iST sample preparation –Protein denaturation, reduction, alkylation and digestion | |
|---|---|
| Deck location and reagent | Labware type |
| 1 - WASH 1 | twin.tec PCR Plate 96 LoBind (Eppendorf p/n 0030129504) |
| 2 - New Tips | 384ST 70µL Tips, Sterile, (p/n 19133-112) |
| 3 - Heating shaker | Teleshake Heated Shaking Station (G5498B#009) |
| 4 - Waste | |
| 5 - iST Cartridges | Adapter plate (PreOmics; P.O.00027) |
| 6 - Heating | Peltier Thermal Station (G5498B#035) |
| 7 - Wash 2 | twin.tec PCR Plate 96 LoBind (Eppendorf p/n 0030129504) |
| 8 - Elute | twin.tec PCR Plate 96 LoBind (Eppendorf p/n 0030129504) |
| 9 - LC-Load | twin.tec PCR Plate 96 LoBind (Eppendorf p/n 0030129504) |

The false discovery rate was set to 0.01 for both proteins and peptides with a minimum length of seven amino acids, and was determined by searching a reverse database. Enzyme specificity was set as C-terminal to arginine and lysine, using trypsin as the protease, and a maximum of two missed cleavages were allowed in the database search. Peptide identification was performed with an allowed initial precursor mass deviation up to 7 ppm, and an allowed fragment mass deviation of 20 ppm. Label-free quantitation (LFQ) was performed with a minimum ratio count of 1[7].

## Results and discussion

This Application Note presents an easily applicable plasma proteomic workflow that benefits from the synergy of the iST sample preparation method developed by PreOmics and high-throughput automation afforded by the Bravo system. Using this method, prospective users can analyze large cohorts of plasma proteomic samples in a rapid, robust, and highly reproducible manner.

Building on the recently described in-StageTip (iST) method[3] (PreOmics GmbH, Germany), we established a parallel automated protocol on the Bravo system. Starting with 1 µL of plasma, all preparation steps were performed in a single reaction vial: the iST Cartridge. Following the iST Kit instructions, sufficient protein digestion had already occurred after one hour.

Peptides were purified with an organic as well as an aqueous washing step to remove contaminants such as lipids and salts. The entire up-front procedure took less than two hours and can readily be performed in a 96-well format on the Bravo, resulting in pure peptides ready for MS analysis.

To assess this automated protocol, 96 plasma samples (corresponding to 48 samples each from a female and male individual) were processed and analyzed. We used MaxQuant for quantitative label-free analysis of the LC/MS/MS data[7]. We calculated the Pearson correlation coefficients for the entire workflow replicates within each individual, resulting in mean correlation values of the quantified protein signals of 0.98 (Figure 3).

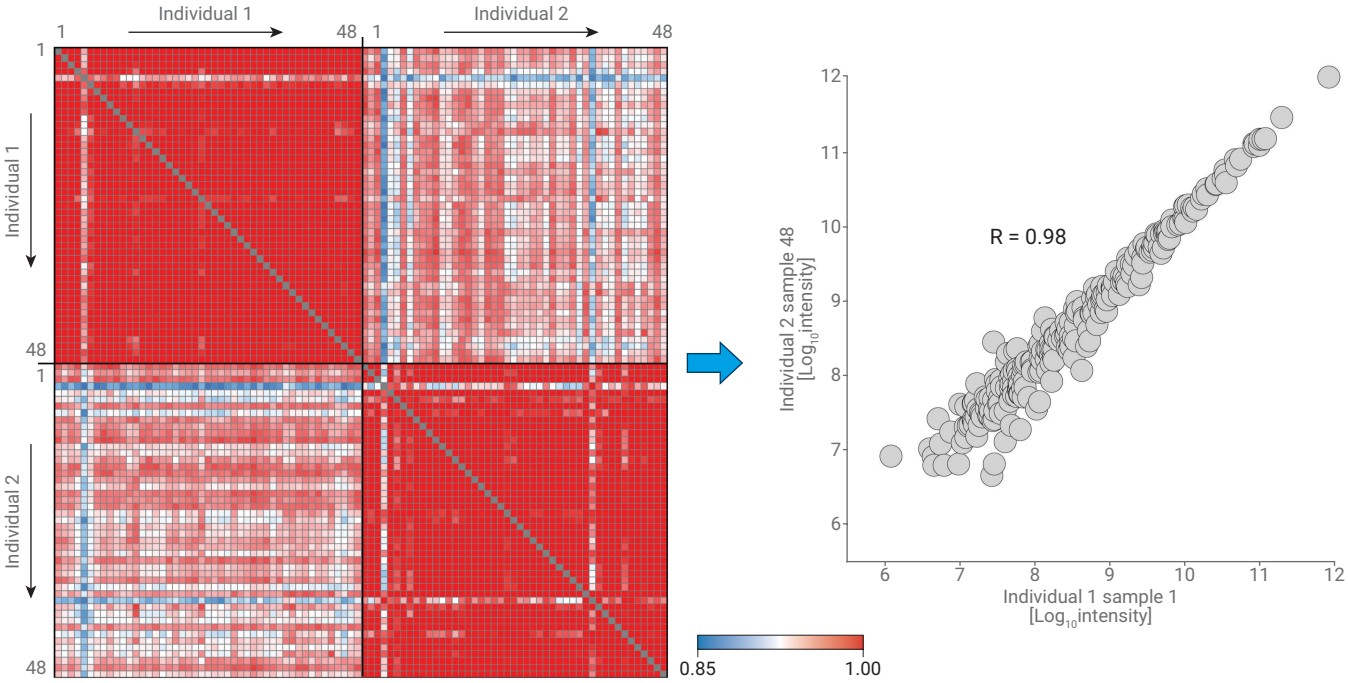


**Figure 3.** Color-coded Pearson correlation coefficients for the binary comparison of 96 technical workflow replicates. Pearson correlation coefficients of up to 0.98 demonstrate high reproducibility.

To emphasize the excellent reproducibility of technical replicates, we overlaid the total ion chromatograms (TICs) generated for three replicates from one of the samples (Figure 4). Importantly, this excellent level of reproducibility was achieved after going through the complete plasma proteomic sample preparation workflow involving in-solution digestion and peptide purification.

The abundance range of proteins within the plasma proteome spanned almost six orders of magnitude (Figure 5), and consisted of 300 quantified proteins per average run. Within these 300 proteins, a diverse range of classic plasma protein functionalities were represented. These included proteins from the lipid homeostasis system such as apolipoproteins A1 and B, proteins involved in inflammatory responses such as complement factor C5, and transport proteins such as retinol-binding protein or the insulin sensitivity parameter adiponectin (ADIPOQ).

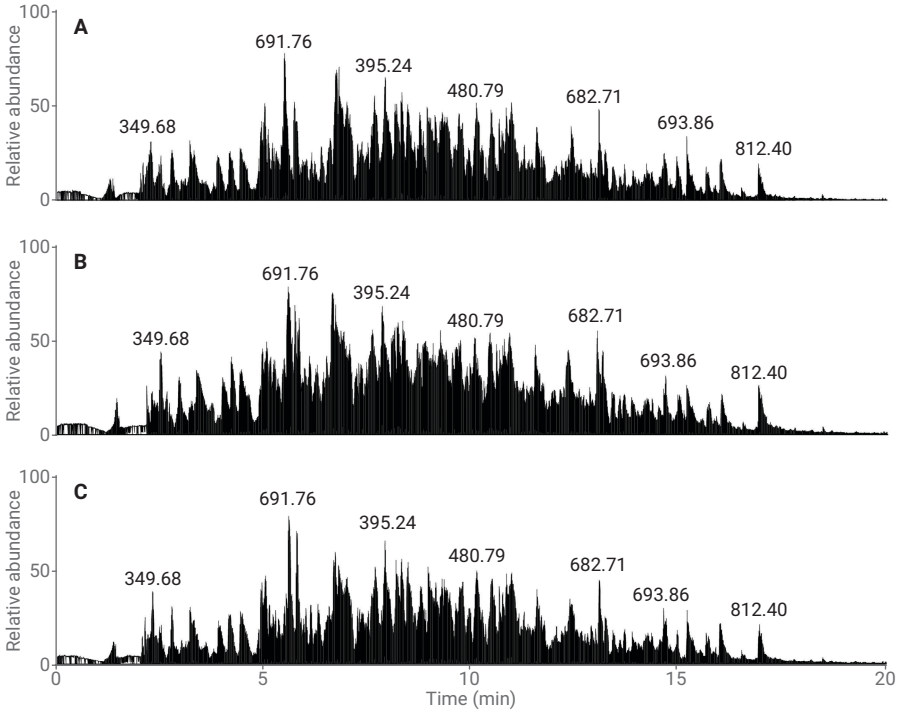


**Figure 4.** Direct comparison of three TICs of different technical replicates of the individual sample. The numbers above the peaks indicate the *m/z*.

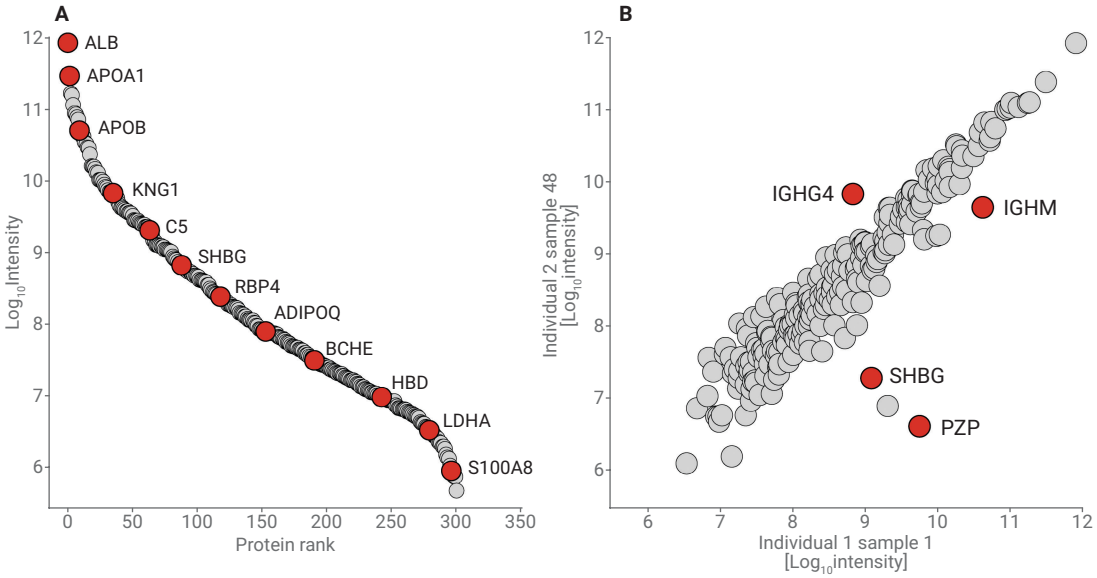


**Figure 5.** A) Distribution of proteins quantified in the 20-minute gradients of the 96 workflow replicates. B) Direct comparison of the two plasma samples to visualize the magnitude of IGHG4, IGHM, SHBG, and PZP in the background of the other quantified plasma proteins.

This label-free workflow is well suited to capture the natural and pathological variation of protein levels between individuals[2]. For example, the direct comparison of protein quantification levels between two samples (one female and one male), shows that the pregnancy zone protein (PZP) and sex hormone-binding globulin (SHBG) are of high absolute abundance in the plasma proteome in the female sample (Figure 5). The main functional role of SHBG is binding estrogen, whereas PZP traps proteases. Immunoglobulins are membrane-bound or secreted glycoproteins produced by B lymphocytes. The immunoglobulins IGHG4 and IGHM are of different abundance between the two samples, demonstrating the high inter-individual variation of protein expression level.

## Conclusion

The PreOmics iST Kit can seamlessly be used on the Bravo system without the need for protocol adaptation. Using this combination of methods, the user is equipped with a robust, sensitive, and rapid technique for the identification of plasma proteins, ultimately resulting in LC/MS/MS-grade peptides in only two hours of total sample processing time. Due to the automation afforded by the Bravo Liquid Handling Platform, results from the whole workflow replicates were also highly reproducible. The reproducibility of the workflow was sufficient to elucidate significant differences between the two plasma samples. We conclude that this degree of reproducibility is more than adequate to discover novel biologically relevant changes within the plasma proteome, especially in longitudinal studies, as has been shown in another study by Geyer; *et al.* (2017)[1]. This Application Note demonstrates these features by performing a sample preparation of 48 replicates of 1 µL of plasma from one female and one male individual, resulting in a total of 96 samples processed.

On average, 300 proteins were covered within the plasma proteome per run, including classic functions of the plasma proteome that can be used to stratify samples according to biologically relevant parameters. By taking advantage of the combination of high throughput and robustness afforded by merging the two technologies into one workflow, the preparation of hundreds or even thousands of plasma samples has become feasible. We believe that this synergy will be indispensable for large-scale clinical research applications.

## References


1. Geyer, P. E.; *et al.* Revisiting biomarker discovery by plasma proteomics. *Molecular Systems Biology* **2017**, *13(9)*, 942.
2. Geyer, P. E.; *et al.* Plasma Proteome Profiling to Assess Human Health and Disease. *Cell Systems* **2016**, *2(3)*, 185–195. http://doi.org/10.1016/j.cels.2016.02.015
3. Kulak, N. A.; *et al.* Minimal, encapsulated proteomic-sample processing applied to copy-number estimation in eukaryotic cells. *Nature Methods* **2014**. http://doi.org/10.1038/nmeth.2834
4. Anderson, L. Six decades searching for meaning in the proteome. *Journal of Proteomics* **2014**, *107*, 24–30. http://doi.org/10.1016/j.jprot.2014.03.005.
5. Cox, J.; Mann, M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nature Biotechnology* **2008**, *26(12)*, 1367–1372. http://doi.org/10.1038/nbt.1511
6. Cox, J.; *et al.* Andromeda: a peptide search engine integrated into the MaxQuant environment. *J. Proteome Res*. **2011**, *10(4)*, 1794–1805. http://doi.org/10.1021/pr101065j
7. Cox, J.; *et al.* Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. *Molecular & Cellular Proteomics* **2014**, *13(9)*, 2513-26.


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