Bottom-Up Plasma Preparation Methods with Protein Plus BCT™

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Introduction

Comprehensive and accurate characterization of the plasma proteome is often complicated by the vast dynamic range of circulating plasma proteins (>10 log). Further contributing to the challenges of accurate plasma proteome analysis are additional factors such as blood cell stability. In the time between blood collection and plasma isolation, red blood cells (RBCs), white blood cells (WBCs) and platelets can lyse and/or activate releasing their proteins into the plasma and altering the proteomic landscape from that which is native to the patient or donor. Protein Plus BCT is specially designed to minimize the degradation and activation of blood cells, therefore abating the release of their proteins to better maintain plasma concentrations of key protein biomarkers from draw time. Here we demonstrate that three representative bottom-up plasma preparation methods (in-solution digestion, S-Trap and SP3 (single-pot solid-phase-enhanced sample preparation)) are compatible with plasma isolated from Protein Plus BCT.

Protein Plus BCT is For Research Use Only. Not for use in diagnostic procedures.

Methods and Analysis

Blood Plasma Processing

Please refer to **Protein Plus BCT Instructions For Use (IFU)** on **streck.com** for more details.

Whole blood collected into EDTA and Protein Plus BCT was centrifuged at 1,800 xg for 15 minutes at room temperature following draw or after 3 and 5 days of ambient storage. The plasma fraction was carefully removed to not disturb the buffy coat and transferred into a 5 mL microcentrifuge tube then centrifuged at 2,800 xg for 15 minutes at room temperature. The resultant supernatant was removed carefully, aliquoted for proteomic analysis and frozen at -80 °C until use.

Bottom-Up Proteomic Sample Preparation

Aliquots of plasma isolated from both EDTA and Protein Plus BCT at each timepoint (draw time, day 3 and day 5) were prepared for proteomic analysis by three different methods (in-solution, S-Trap and SP3). In-solution digestion was completed using the **Bottom-Up Proteomic Sample Preparation Using In-Solution Digestion** protocol. For S-Trap sample preparation, an adapted workflow from the Protifi™ S-Trap™ was followed in which Tris was substituted for TEAB in the lysis buffer (See **Bottom-Up Proteomic Sample Preparation Using 96-well plate S-Trap** for a detailed protocol). SP3 was completed using the methods defined in the **Bottom-Up Proteomic Sample Preparation Using SP3** protocol.

Liquid Chromatography-Mass Spectrometry Analysis

Samples were analyzed using an LC-MS/MS system consisting of a Vanquish™ Neo HPLC coupled to an Orbitrap™ Exploris™ 240 mass spectrometer (ThermoFisher Scientific™). Peptides were loaded at a flow rate of 60 µL/min in buffer A (H_2O, 0.1% formic acid) onto an Easy-Spray™ PepMap™ Neo Column (2 µm C18, 75 µm x 150 mm). Peptides were eluted over 63.4 min at a flow rate of 350 nL/min with the following gradient, where buffers consist of A (H_2O, 0.1 % formic acid) and B (80% acetonitrile, 0.1% formic acid): time 0.1 min, 4% buffer B; time 0.7 min, 4.5% buffer B; time 1 min, 5% buffer B; time 38 min, 20% buffer B; time 56.9 min, 35% buffer B; time 57.4 min, 55% buffer B; time 57.5 min, 99% buffer B; time 63.4 min, 99% buffer B.

The nanospray source voltage was set to 1500 V. Full-mass profile scans were performed in the Orbitrap between 375 and 1,500 m/z at a resolution of 60,000 with a normalized AGC target of 300%. The MS1 scans were followed by MS/MS HCD scans in the Orbitrap of the highest intensity parent ions in a 3-second cycle time at a 30% relative collision energy and resolution of 15,000, with a 2 m/z isolation window. Charge states 2-6 were included, and dynamic exclusion was enabled with a repeat count of one over a 50-second duration and 10-ppm exclusion width. The AGC target was set to “standard”, and the maximum inject time was set to “auto.”

Data Analysis

Spectral files were analyzed with the search software program MetaMorpheus (version 0.0.320) against the human UniProt protein database. All default search settings were utilized for Calibration, GPTMD, and Search tasks. Match between runs and normalization were enabled for quantification.

Results

Protein Plus BCT™ Provides Protein and Peptide Identifications Comparable with Plasma Isolated from EDTA for All Sample Preparation Methods

To determine whether plasma isolated from Protein Plus BCT can be used with common sample preparation methods, bottom-up proteomic analysis was performed on plasma isolated from Protein Plus BCT and prepared using in-solution digestion, S-Trap or SP3. Because plasma isolated from EDTA is widely utilized for proteomics and is known to be compatible with these sample preparation methods, EDTA plasma digests served as a reference for the Protein Plus compatibility experiments. Plasma isolated from Protein Plus BCT produced a comparable number of protein and peptide identifications at 1% FDR (false discovery rate) to the EDTA reference for all the evaluated methods. The percent difference between the total number of protein or peptide identifications from EDTA and Protein Plus plasma was less than 2.5% or on average 1%, respectively, for all methods (Figure 1 A-C, peptide data not shown). When comparing protein identifications, the Jaccard similarity index between Protein Plus BCT and EDTA plasma samples was greater than 0.90 at any plasma isolation timepoint (Figure 1 D-F). The Jaccard similarity index for peptide-level comparisons was slightly lower, with an average value of 0.85 across all comparisons (data not shown). Because peptide identifications inherently have more variability than proteins, for which presence is inferred by the identification of one or more peptides, this lower Jaccard index value for peptide identifications was expected. Overall consistency observed between Protein Plus BCT and EDTA plasma digests indicates that all evaluated methods (in-solution, S-Trap and SP3) are compatible with the plasma isolated from Protein Plus BCT.

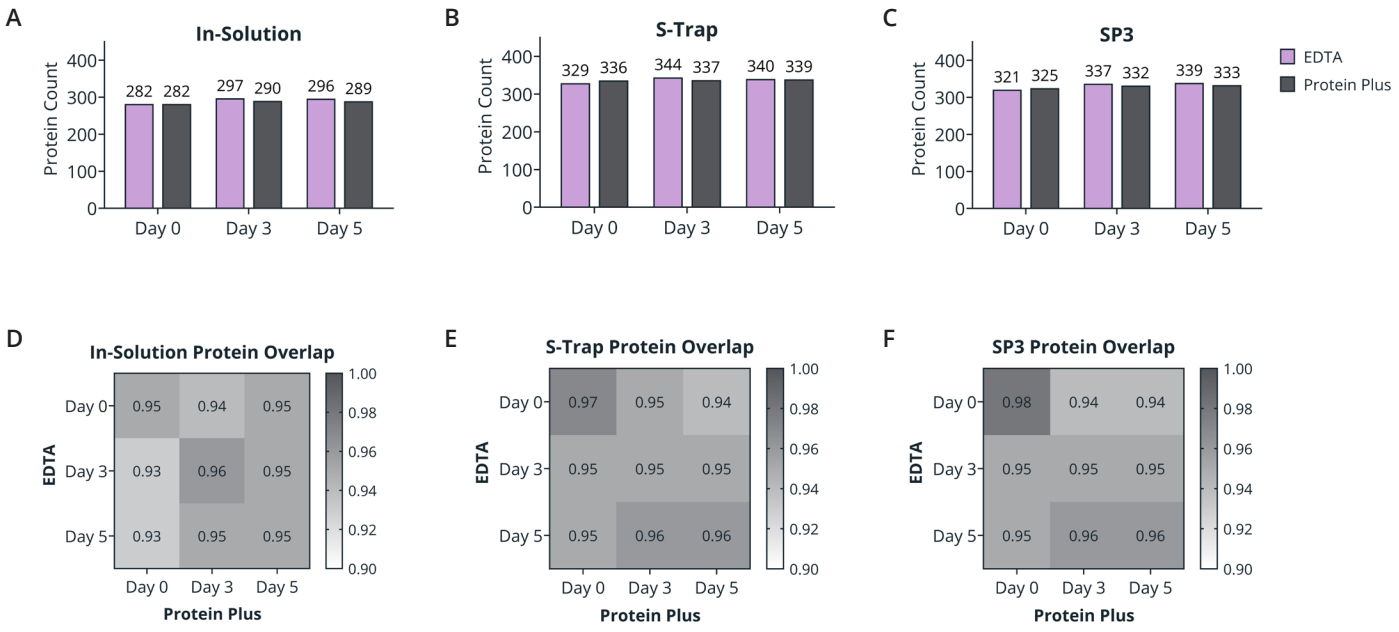

Figure 1: Plasma isolated from Protein Plus BCT provides consistent protein identifications relative to EDTA for all sample preparation methods evaluated. Total number (**A-C**) and Jaccard similarity index (**D-F**) of protein identifications at 1% FDR in plasma from samples collected into Protein Plus BCT and EDTA isolated at draw time (Day 0) or after 3 and 5 days of ambient temperature storage and prepared using in-solution digestion (**A, D**), S-Trap (**B, E**) or SP3 (**C, F**). (n= 5).

Stabilization Benefits Provided by the Protein Plus Formulation are Preserved Across All Sample Preparation Methods

Protein Plus BCT is formulated to limit lysis and/or activation of blood cells (RBCs, WBCs and platelets) during prolonged whole blood storage at ambient temperature, thereby minimizing proteome alterations that can occur when immediate plasma isolation is not feasible. To verify that Protein Plus BCT maintains sample integrity during ambient temperature storage, protein intensities from plasma isolated from samples collected into Protein Plus BCT at draw time and after 5 days of ambient temperature storage and prepared using in-solution digestion, S-trap or SP3 were compared. When the $\log_{10}$ transformed intensity values for each protein at draw time and day 5 were plotted, all sample preparation methods displayed R^2 values greater than 0.95 and slopes of at least 0.98 (Figure 2). These data suggest that Protein Plus BCT minimizes protein abundance changes as plasma isolation is delayed regardless of the sample preparation method used.

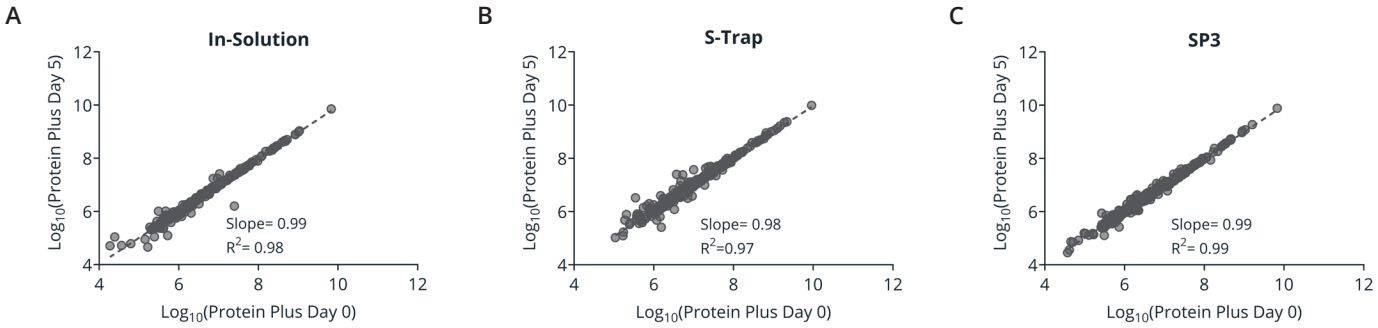

Figure 2: Minimization of protein abundance variation over prolonged whole blood storage enabled by Protein Plus BCT™ is independent of sample preparation method. Log_{10} transformed MS1 LFQ intensities of proteins identified from plasma isolated samples collected into Protein Plus BCT at draw time (Day 0) and after 5 days of whole blood storage and prepped using in-solution (**A**), S-Trap (**B**) and SP3 (**C**) sample preparation methods.

To highlight the impact of blood cell stabilization on proteomic results, changes in abundance of Platelet Factor 4 (PF4/CXCL4), a key biomarker for platelet activation released from platelet alpha granules, were investigated (Figure 3). The average log_2 fold change for CXCL4 for plasma from samples collected into EDTA and isolated after 5 days of ambient storage relative to draw-time isolation was greater than 2 for all methods, which corresponds to a more than 4-fold increase in abundance (Figure 3). In contrast, an average log_2 fold change of less than 1 was observed for plasma from samples collected into Protein Plus BCT and isolated after 5 days of ambient storage versus at draw time regardless of preparation method (Figure 3). These data suggest that Protein Plus BCT minimizes the release of platelet-associated protein contents into the plasma, maintaining native proteome abundances. For additional biomarker data, please see **Protein Plus BCT™: A Novel Blood Collection Tube for Stabilizing Plasma Proteins of Interest in Ambient Whole Blood Storage** on streck.com.

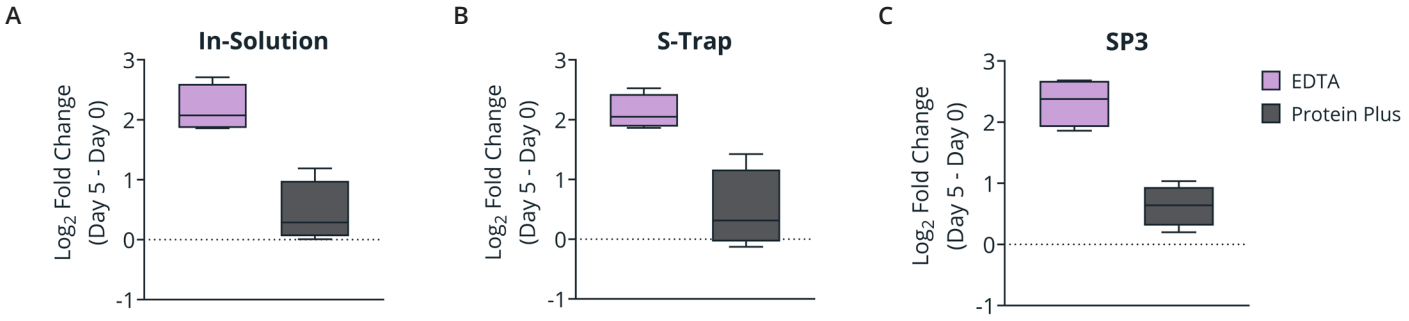

Figure 3: Improved maintenance of draw-time levels of platelet activation marker CXCL4 in Protein Plus BCT relative to EDTA is observed across all proteomics preparation methods. Log_2 fold change of Platelet Factor 4 (CXCL4) abundance in plasma from samples collected into EDTA or Protein Plus BCT and prepped using in-solution (**A**), S-Trap (**B**) and SP3 (**C**) sample preparation methods after 5 days of ambient temperature whole blood storage relative to those prepared at draw time (Day 0). For additional biomarker data, please see **Protein Plus BCT: A Novel Blood Collection Tube for Stabilizing Plasma Proteins of Interest in Ambient Whole Blood Storage** on streck.com.

Conclusion

These data demonstrate that Protein Plus BCT is compatible with several proteomic sample preparation approaches including the in-solution digestion, S-Trap and SP3 methods. The workflows provided in this technical note allow for successful bottom-up proteomic sample preparation from plasma isolated from blood collected into Protein Plus BCT.

Bottom-Up Proteomic Sample Preparation Using In-Solution Digestion

1. Combine 1 µL of plasma with 20 µL of freshly prepared denaturation buffer (50 mM ammonium bicarbonate (pH 8.0), 10 mM TCEP, 5% SDC) in a microcentrifuge tube.
2. Incubate samples for 10 min at 60 °C.
3. Add 5 µL of freshly prepared alkylation solution (100 mM iodoacetamide)
   a. **Iodoacetamide is light sensitive. Store in the dark until use.**
4. Incubate samples at room temperature in the dark for 60 min.
5. Add 170 µL of 50 mM ammonium bicarbonate (pH 8.0) to dilute SDC.
6. Add trypsin dissolved in 50 mM ammonium bicarbonate (pH 8.0) to a 1:10 enzyme-to-protein ratio.
7. Incubate digestion at 47 °C for 2.5 hours.
8. Add 10 µL of 20% formic acid and incubate for 30 min at room temperature to halt tryptic digestion and precipitate SDC from solution.
9. Centrifuge samples at 15,000 xg for 10 min.
10. Transfer the peptide supernatant to a new microcentrifuge tube.
11. Dry down the peptide solution under nitrogen or using a vacuum centrifuge. Store dried peptides at -80 °C until LC-MS analysis.
12. Reconstitute peptides in 95:5 H_2O:ACN, 0.1% formic acid prior to LC-MS analysis.

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Bottom-Up Proteomic Sample Preparation Using 96-well plate S-Trap™

1. Combine 1 µL of plasma with 20 µL of 2X Lysis Buffer (10% SDS and 100 mM Tris (pH 8.5)) in a microcentrifuge tube.
2. Add 19 µL of water (18MΩ or LC-MS grade).
3. Add 2 µL of freshly prepared reduction solution (120 mM TCEP).
4. Incubate samples for 15 min at 55 °C.
5. Add 2 µL of freshly prepared alkylation solution (500 mM iodoacetamide).
   a. **Iodoacetamide is light sensitive. Store in the dark until use.**
6. Incubate samples at room temperature in the dark for 30 min.
7. Add 6 µL of 12% phosphoric acid.
   a. **Check the pH of the sample to ensure it is below pH 1.0 before proceeding. Add more phosphoric acid if necessary.**
8. Add 350 µL of Binding/Wash Buffer (100 mM TEAB (pH 7.5 – pH adjusted with phosphoric acid), 90% methanol).
   a. At this point the solution may become cloudy if protein concentration is sufficiently high.
9. Vortex samples for 10 sec.
10. Place S-Trap plate over a 96-deep well plate.
11. Add samples to the wells of the S-Trap plate.
12. Centrifuge the S-Trap and receiver plates at 1,500 xg for 2 min to load the proteins onto the S-Trap column material.
    a. Ensure all the sample volume has passed through the trap, spin for additional time if required.
13. Add 200 µL of Binding/Wash Buffer in each sample well to wash the trapped proteins.
14. Centrifuge the S-Trap and receiver plates at 1,500 xg for 2 min.
    a. Ensure all the wash volume has passed through the trap, spin for additional time if required. Empty the receiver plate into a waste container as needed.
15. Repeat steps 13 and 14 three times.
16. Transfer the S-Trap plate to a fresh 96-deep well receiver plate.
17. Add trypsin dissolved in 50 mM TEAB (pH 8.5) to a 1:10 enzyme-to-protein ratio.
18. Add additional 50 mM TEAB (pH 8.5) until the total volume in each sample well is 125 µL.
19. Loosely cover the plate with the provided S-Trap plate cover.
20. Incubate digestion at 47 °C for 2.5 hours.
21. Add 80 µL of 50 mM TEAB (pH 8.5) to each sample well.
22. Centrifuge S-Trap and receiver plate at 1,500 xg for 2 min **saving all flow through containing eluted peptides.**
23. Add 80 µL of 0.2% formic acid to each sample well.
24. Centrifuge S-Trap and receiver plate at 1,500 xg for 2 min **saving all flow through containing eluted peptides.**
25. Add 80 µL of 50% ACN, 0.2% formic acid to each sample well.
26. Centrifuge S-Trap and receiver plate at 1,500 xg for 2 min **saving all flow through containing eluted peptides.**
27. Combine all eluted flow through (if necessary) and dry down the eluted peptides under nitrogen or using a vacuum centrifuge. Store dried peptides at -80 °C until LC-MS analysis.
28. Reconstitute peptides in 95:5 H_2O:ACN, 0.1% formic acid prior to LC-MS analysis.

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Bottom-Up Proteomic Sample Preparation Using SP3

1. Combine 1 µL of plasma with 76 µL of freshly prepared denaturation buffer (50 mM Tris (pH 8.5), 10 mM TCEP, 5% SDS) in a microcentrifuge tube.
2. Incubate samples for 10 min at 60 °C.
3. Add 3 µL of freshly prepared alkylation solution (500 mM iodoacetamide).
   a. **Iodoacetamide is light sensitive. Store in the dark until use.**
4. Incubate samples at room temperature in the dark for 60 min.
5. Prepare magnetic beads (Sera-Mag SpeedBeads Carboxyl hydrophilic and hydrophobic, Fisher Scientific 09-981-121/09-981-123)
   a. Combine 500 µg of each bead type per sample into a fresh tube.
   b. Place the tube with the bead slurry onto a magnetic rack and remove the stock supernatant.
   c. Wash the beads with 1 mL of water (18MΩ or LC-MS grade).
   d. Place the tube with the bead slurry onto a magnetic rack and remove the wash.
   e. Reconstitute the beads in water (18MΩ or LC-MS grade) to a final concentration of 50 µg/µL.
6. Add 20 µL of beads to each sample, pipetting gently up and down to mix completely.
7. Add 100 µL of 100% ethanol to force protein aggregation on the bead surface.
8. Shake the protein bead mixture at 1,000 rpm for 5 min at room temperature.
9. Place the tubes on the magnetic rack and wait for bead migration (at least 1 min).
10. Remove supernatant.
11. Add 500 µL of 80% ethanol to wash the proteins bound to the magnetic beads. Pipette the mixture gently 3-4 times.
12. Place the tubes on the magnetic rack and wait for bead migration (at least 1 min).
13. Remove supernatant.
14. Repeat steps 11-13 twice.
    a. On the last removal of wash supernatant leave no more than 5 µL of solution to minimize the risk of contaminant carryover.
15. Add trypsin dissolved in 50 mM ammonium bicarbonate (pH 8.0) to obtain a 1:10 enzyme-to-protein ratio.
16. Add additional 50 mM ammonium bicarbonate (pH 8.0) until the total volume in each sample well is 100 µL.
17. Digest samples on the thermomixer at 47 °C for 2.5 hours at 1,000 rpm so that the bead slurry is in constant motion.
18. Centrifuge samples at 20,000 xg for 1 min following digestion.
19. Place the sample tubes on the magnetic rack and wait for bead migration (at least 1 min).
20. Remove peptide supernatant (~ 100 µL) to a new microcentrifuge tube.
21. Dry down the peptide solution under nitrogen or using a vacuum centrifuge. Store dried peptides at -80 °C until LC-MS analysis.
22. Reconstitute peptides in 95:5 H_2O:ACN, 0.1% formic acid prior to LC-MS analysis.

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