Protein Plus BCT™: A Novel Blood Collection Tube for Stabilizing Plasma Proteins in Ambient Whole Blood Storage

Jing Li, Ph.D., Rachel Miller, Ph.D., Sama Mehta, Nick George, Ph.D., Jordan LaRue, Eunju Seong, Ph.D., Nursen Binbuga, M.S., and Karl Bisselou, Ph.D.

Streck Research and Development, La Vista, Nebraska

Introduction

Blood is an accessible biofluid containing important biomarkers like proteins, nucleic acids, and rare cells that can be used for diagnosis, prognosis, and prediction of therapeutic outcomes of diseases including cancer, cardiovascular diseases, neurological and inflammatory disorders. Most ongoing efforts to develop liquid biopsy assays focus on the detection of genomic or epigenomic variations in circulating cell-free DNA and transcriptomic changes in cell-free RNA. The circulating proteome can also provide invaluable insights into the clinical characteristics of patients, as it reflects real-time changes of disease progression and responses to treatments; however, the development of clinically validated assays utilizing circulating protein biomarkers may be more challenging than initially anticipated due to two major barriers that have significantly hindered research and assay development. First, the circulating proteome's large dynamic range (>10 log), complexity in structure and intrinsic instability have impeded the identification and quantification of low-abundance proteins or multiplexing proteins of different abundance. Second, many proteins of interest are also found in blood cells such as leukocytes and platelets. During blood collection and sample storage, *ex vivo* blood cell activation and lysis can occur, resulting in the release of blood cell-secreted proteins into the plasma that may mask the true abundance of the proteins of interest in the circulating blood.

Here, we show that Protein Plus BCT™ addresses this issue by maintaining draw-time plasma protein levels during ambient whole blood storage.

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

Methods

Blood Collection

Blood samples from self-proclaimed healthy donors were drawn into Protein Plus BCT, EDTA, and ACD-A tubes. All samples were stored at ambient temperature for up to 5 days. Plasma was isolated using a double-spin protocol (1800 ×g for 15 min and 2800 ×g for 15 min, both at room temperature) and frozen at -80 °C until use.

Platelet Activation Analysis by Flow Cytometry

Whole blood samples were stained with an antibody cocktail of CD61-PerCP (a platelet surface marker, BD® Biosciences) and CD62P-PE (an activated platelet surface marker, BioLegend®) and analyzed on a BD FACSCanto® II clinical flow cytometry system. Acquisition and analysis were performed by scatter gating (forward scatter [FSC] and side scatter [SSC]) followed by fluorescent gating. Activated platelets (%) were quantified by the CD61+CD62P+ platelet population of all gated platelets (FlowJo v10.7.2).

Analysis of Plasma Level of Protein Biomarkers by Immunoassay

Plasma levels of protein biomarkers were measured by i) Ella™ Simple Plex™ Assays (Bio-Techne®); ii) Luminex® xMAP® Technology Assays (Bio-Techne, samples tested by LuminexPLORE Lab (Diasorin, S.p.A.)); or iii) Olink® Proximity Extension Assay (Olink® Explore 384 Cardiometabolic panel, samples tested by Vanderbilt University Medical Center High-Throughput Biomarker Core Lab), following manufacturers' dilution and processing recommendations. Resultant data was analyzed by Streck.

Analysis of Plasma Proteome by Mass Spectrometry

Neat plasma samples were prepared for bottom-up analysis using the S-Trap™ 96-well plate (Protifi™) and analyzed on an Oribtrap™ Exploris™ 240 mass spectrometer (Thermo Scientific™). All raw spectra files were analyzed using MetaMorpheus (v0.0.320) and quantitative statistical analysis was performed using Perseus (v2.0.7.0). To achieve deep proteomic coverage, Streck, in collaboration with Seer, Inc., prepared samples using the Seer Proteograph XT™ workflow and analyzed them with an Orbitrap Astral™ mass spectrometer (Thermo Scientific) using data-independent acquisition (DIA). Data were analyzed using DIA-NN-1.8.1 and the Proteograph Analysis Suite (PAS).

Results

Analysis of circulating protein biomarkers is easily confounded by the release of contaminating blood cell-associated proteins during collection and processing. Therefore, careful consideration must be taken when selecting a blood collection tube. To demonstrate that blood samples collected into conventional serum or plasma blood collection tubes are susceptible to platelet and/or leukocyte activation during draw or storage, we measured draw-time plasma levels of blood cell-associated protein biomarkers (TGF-β1, MPO, or Granzyme B) in samples collected into EDTA, ACD-A or serum tubes. Samples collected into EDTA exhibit elevated plasma levels of TGF-β1 compared to those collected in ACD-A, suggesting that platelet activation and associated protein biomarker release occurs upon draw into EDTA, but not ACD-A (Figure 1A). Elevated levels of TGF-β1 and MPO are also present in samples collected into serum tubes, indicating that the post-draw clotting process in serum tubes leads to activation of both platelets and granulated leukocytes (Figure 1B). Further, while ACD-A limits draw-time platelet and leukocyte activation, its stabilization capacity is short-lived, as samples display increased plasma protein levels of TGF-β1 and Granzyme B within 24 hours of whole blood storage (Figure 1C).

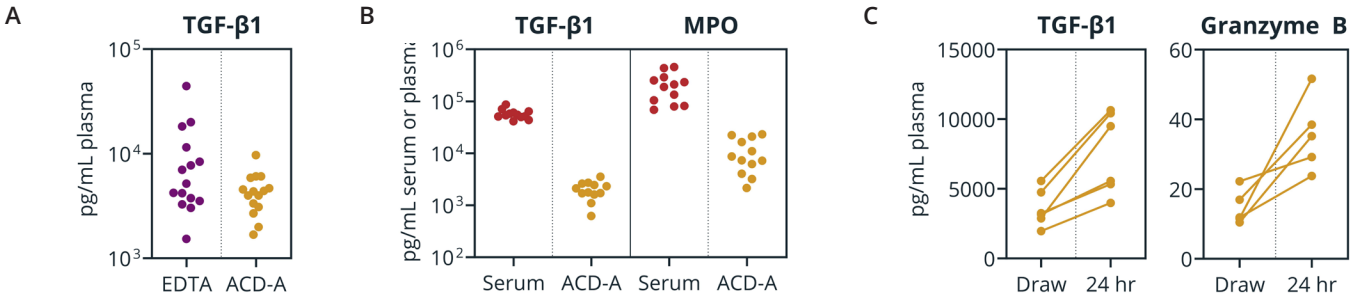

Figure 1: Conventional serum or plasma blood collection tubes do not meet the sample requirements for protein analysis. Whole blood was drawn into EDTA, ACD-A or serum tubes and protein levels of TGF-β1, MPO, or Granzyme B in isolated plasma or serum were analyzed at draw time (**A**, **B**) or after 24 hours of whole blood storage (**C**).

Recognizing the need for a specialized blood collection tube formulated to limit *ex vivo* hemolysis and platelet activation while also maintaining the draw-time concentrations of plasma derived proteins of interest during ambient storage, Streck developed Protein Plus BCT™. In contrast to the hemolysis that occurs following whole blood storage in EDTA, samples collected into Protein Plus BCT display no visible hemolysis at draw time or after 5 days of ambient temperature storage (Figure 2A). Flow cytometry analysis of whole blood samples immediately after the draw shows limited *ex vivo* platelet activation in samples collected into Protein Plus BCT and ACD-A but substantial platelet activation and large donor-to-donor variability in samples collected into EDTA (Figure 2B).

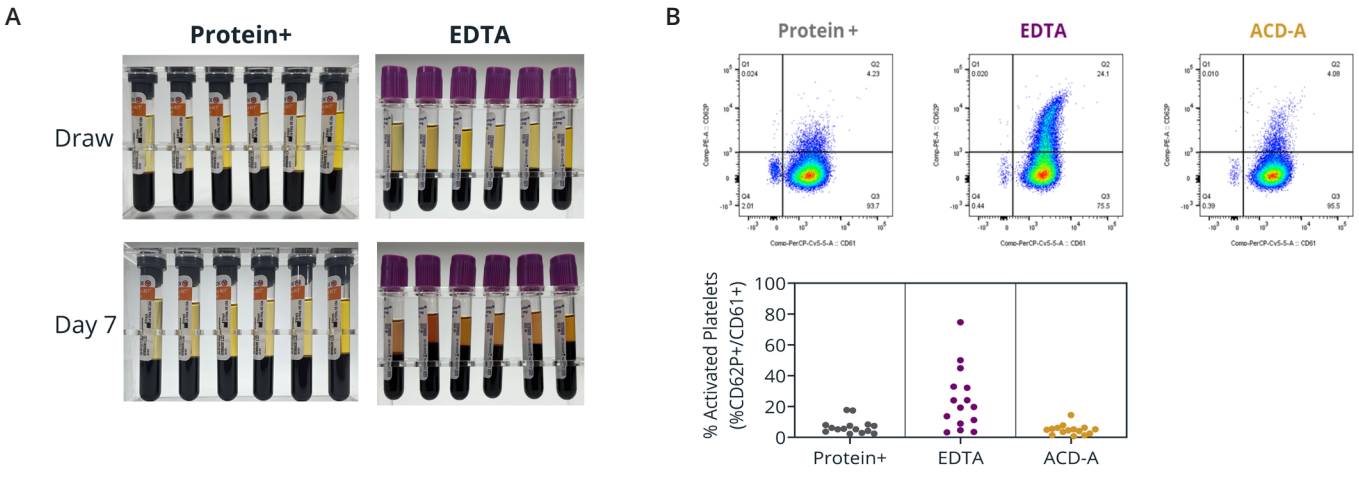

Figure 2: Protein Plus BCT limits *ex vivo* hemolysis and platelet activation. (**A**) *Ex vivo* hemolysis in blood samples drawn into Protein Plus BCT or EDTA at draw or after 5 days of ambient temperature storage. (**B**) Activated platelet populations (CD62P+/CD61+) in matched donor samples immediately following draw into Protein Plus BCT, EDTA or ACD-A (n=15).

To demonstrate that Protein Plus BCT maintains draw-time concentrations of plasma proteins of interest, we analyzed a total of 55 key plasma protein markers in samples collected into Protein Plus BCT, EDTA and ACD-A, at draw time or after 3 and 5 days of whole blood storage at ambient temperature. Samples collected into EDTA or ACD-A tubes exhibit a substantial increase in plasma protein levels after 3 and 5 days of ambient whole blood storage (Figure 3). In contrast, samples collected into Protein Plus BCT display draw-time plasma concentrations of these protein markers out to 5 days of whole blood storage (Figure 3).

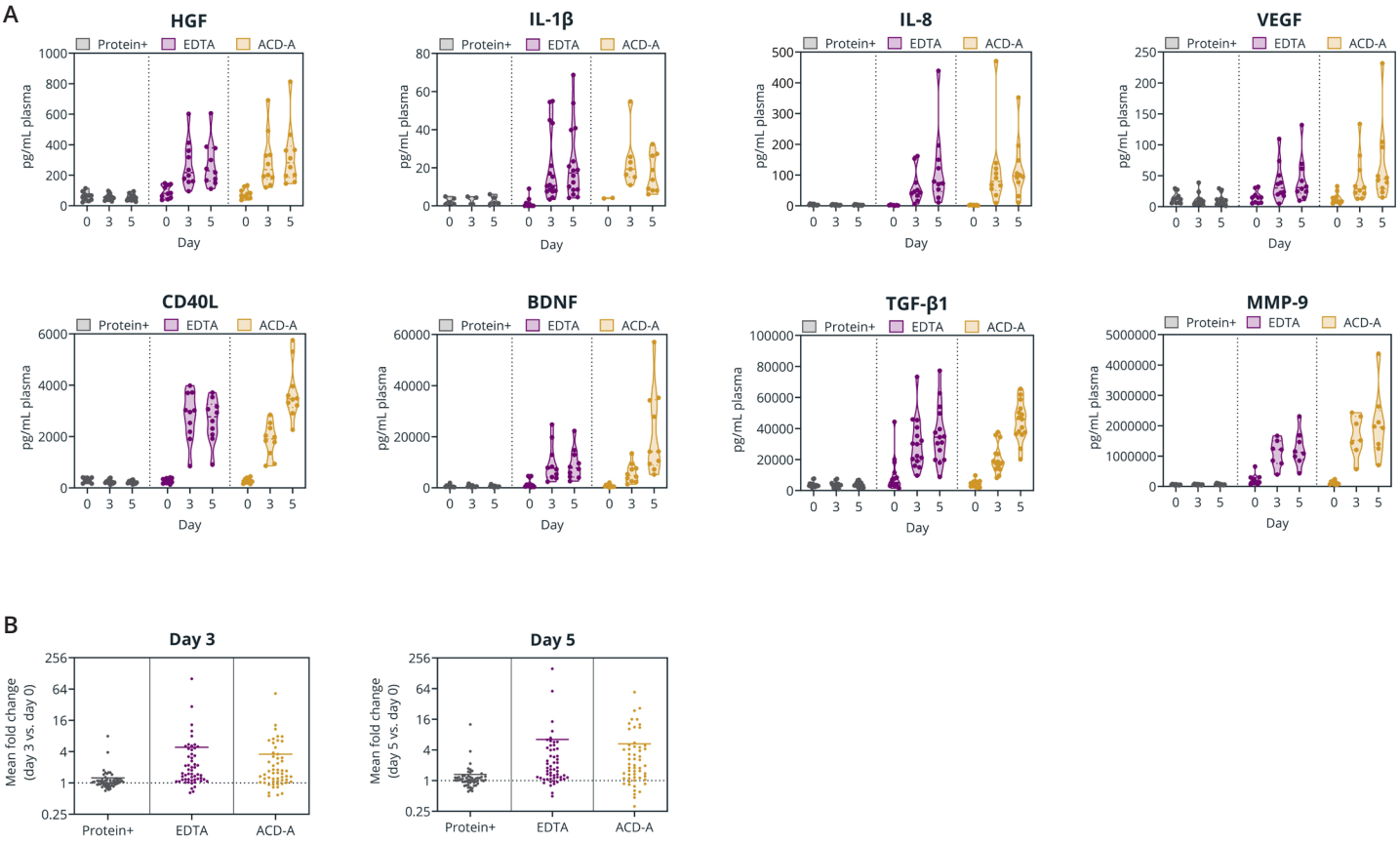

Figure 3: Analysis with Luminex® xMAP® and Ella™ Simple Plex™ immunoassays reveals that Protein Plus BCT maintains draw time concentrations of plasma proteins of interest. (**A**) Plasma levels of proteins of interest in samples collected into Protein Plus BCT, EDTA, or ACD-A at draw time or after 3 and 5 days of ambient temperature storage (representative biomarkers shown, n≥10). (**B**) Mean protein fold change relative to draw time abundance for 55 plasma protein markers of interest in samples collected into Protein Plus BCT, EDTA, or ACD-A after 3 and 5 days of ambient temperature storage (n=10).

The plasma protein stability conferred by the formulation in Protein Plus BCT can also be observed using Olink® Proximity Extension Assay (PEA) technology. Samples collected into Protein Plus BCT display minimal changes in plasma protein abundance from draw time to day 5 of storage relative to those collected into EDTA, which exhibit a marked change in abundance of several blood cell-related proteins (Figure 4A). When specific markers found abundant in platelets and/or white blood cells (IL-8 and S100P) are investigated, we observe a similar abundance between draw time and day 5 of storage for samples collected into Protein Plus BCT, but not EDTA or ACD-A (Figure 4B).

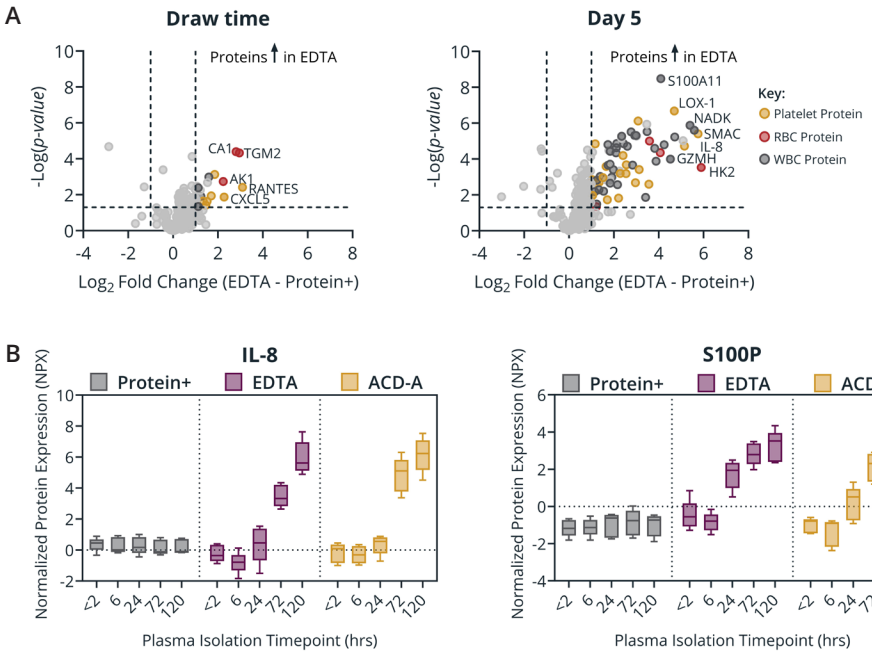

Figure 4: Protein Plus BCT is compatible with Olink PEA technology. (**A**) Differential analysis of plasma protein abundances from the Olink Explore Cardiometabolic Panel in samples collected into EDTA or Protein Plus BCT at draw time and after 5 days of whole blood storage at ambient temperature (n=5). Markers with fold change < -1 or > 1 and a –log(*p-value*) > 1.3 represent proteins that are statistically significantly changing in abundance between the two BCTs. Proteins with a positive $\log_2$ fold change are more abundant in EDTA than Protein Plus BCT. Colored markers denote changing proteins known to come from platelets (gold), RBCs (red), or WBCs (dark grey). (**B**) Abundance of representative proteins (IL-8 and S100P) during storage in Protein Plus BCT, EDTA or ACD-A.

To illustrate that plasma isolated from Protein Plus BCT is also compatible with mass spectrometry-based proteomic analysis methods, neat plasma from samples collected and stored in Protein Plus BCT or EDTA for 3 and 5 days of ambient temperature storage was analyzed using bottom-up proteomics and LC-MS/MS. Differential analysis shows that samples collected into EDTA exhibit significant changes in plasma protein abundance after 3 and 5 days of ambient temperature storage compared to those collected into Protein Plus BCT (Figure 5).

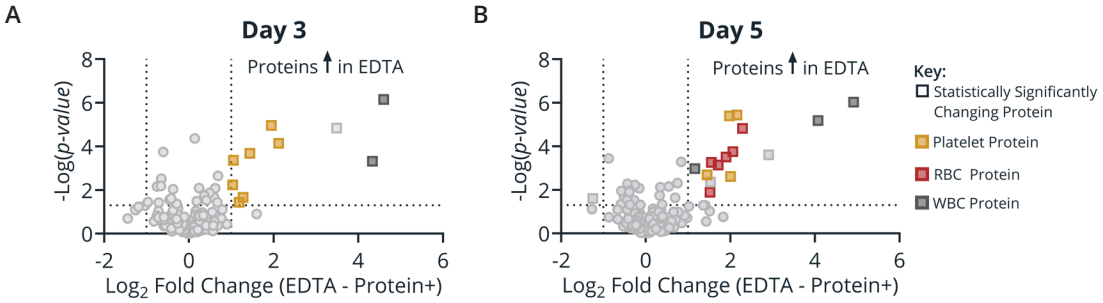

Figure 5: Mass spectrometry analysis indicates that Protein Plus BCT provides increased proteome stability. Differential LC-MS/MS analysis of neat plasma protein abundances between EDTA and Protein Plus BCT after 3 (**A**) or 5 (**B**) days of whole blood storage at ambient temperature (n=6). Square markers represent proteins statistically significantly changing in abundance between the two BCTs ($\log_2$ fold-change >1 and -log(*p-value*) >1.3). Proteins with a positive $\log_2$ fold change are more abundant in EDTA than Protein Plus BCT. Colored markers are for changing proteins known to come from platelets (gold), RBCs (red), or WBCs (dark grey).

In an effort to expand the depth of coverage for our LC-MS-based analysis, proteome stability in samples collected and stored in Protein Plus BCT and EDTA was examined using the Seer Proteograph XT™ platform before mass spectrometry analysis. From draw time to day 3 of ambient temperature storage, peptide and protein identifications in plasma isolated from EDTA markedly increase (137% and 68%, respectively), whereas peptide and protein identifications in plasma isolated from Protein Plus BCT only increase marginally during the same amount of time (13% and 7% respectively) (Figure 6A). Further, samples collected into EDTA display an increase of 567% more proteins after 3 days of storage than those collected into Protein Plus BCT.

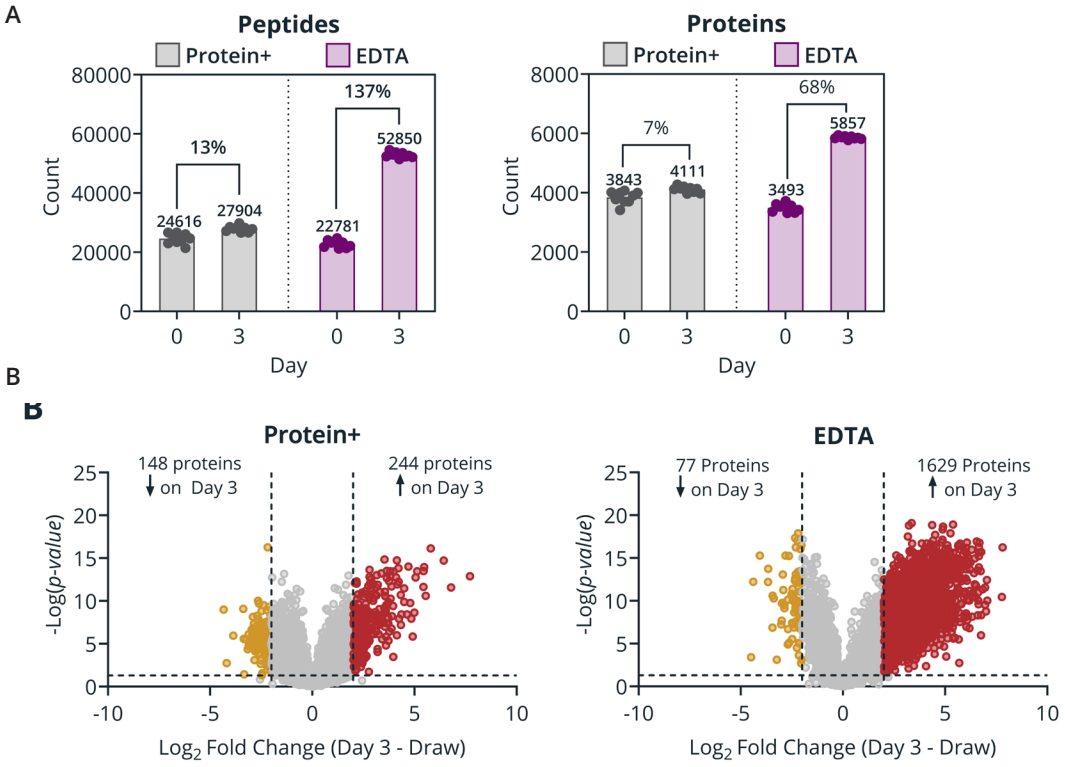

Figure 6: Protein Plus BCT maintains proteome stability to the expanded depth of coverage enabled by the Seer Proteograph XT platform. (**A**) Overall protein and peptide counts in plasma isolated from Protein Plus BCT or EDTA at draw time and after 3 days of ambient temperature storage (n=3). (**B**) Differential analysis of protein abundance between plasma isolated at draw time or after 3 days of whole blood storage for EDTA (left) and Protein Plus BCT (right). Red markers: $\log_2$ fold change >2, -log(*p-value*)> 1.3.

Conclusion

We demonstrate that Protein Plus BCT maintains draw-time plasma protein levels during extended whole blood storage at room temperature by limiting *ex vivo* platelet activation, hemolysis, and blood cell degradation. Plasma isolated from this tube is compatible with both immunoassay platforms and mass spectrometry-based proteomic workflows. Protein Plus BCT ensures sample integrity and minimizes preanalytical variables, providing cancer researchers and clinical assay developers greater flexibility in sample handling and improved confidence in downstream plasma protein analysis.