Impact of Human Blood Specimen Collection Processing, and Storage on Protein Integrity and Implications for Use in Clinical Research

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Introduction

Human plasma and serum proteins are a promising source of clinically relevant disease biomarkers. However, comprehensive guidelines for handling these specimens are lacking and their collection, manipulation and storage protocols are based mainly on accepted practices rather than careful comparative analysis and testing. We investigated the impact of collection tube types, incubation time and temperature before and after centrifugation, freeze/thaw cycles, and freezer storage time and temperature on protein integrity using proteomics approaches.

Methods

Plasma and serum were collected from volunteers under well controlled conditions, subjected to MARS-14 depletion, digested with trypsin and analyzed by LC-MS for discovery studies. Verification was performed using a highly multiplexed MRM assay after depletion and digestion.

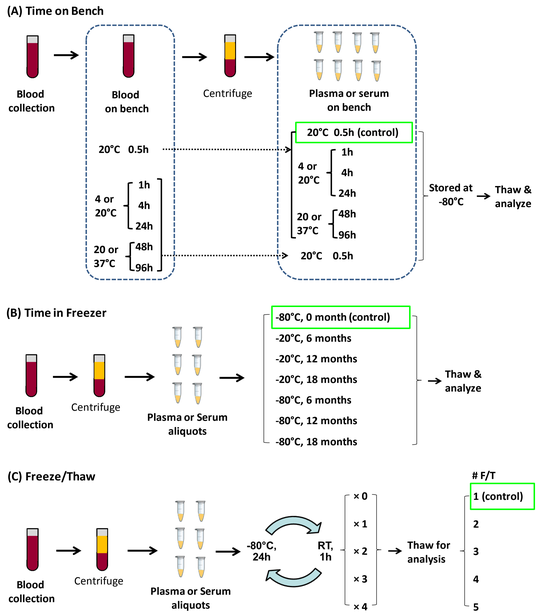

Figure 1. Design of sample handling and processing for (A) Time on Bench, (B) Time in Freezer, and (C) Freeze/Thaw studies.

Table 1. Recruited subjects and blood collection tube types for the study

Study	Sub-study	Cohorts				Blood Collection Tube Types				
		BC	BN	PC	PN	EDTA	EDTA+PI	P100	Serum	Heparin
Time on Bench	Discovery Study 1	80		45		o	o	o	o	o
	Discovery Study 2	3	3			o	o	o	o	o
	Discovery Study 3	10	10	10	10	o		o	o	o
	Verification	10	10	10	10	o		o	o	o
Time in Freezer	Discovery 0 month	10	10	10	10	o		o	o	o
	Discovery 6 months	10	10	10	10	o		o	o	o
	Discovery 12 months	10	10	10	10	o		o	o	o
	Discovery 18 months	10	10	10	10	o		o	o	o
	Verification 0 month		7		8	o			o	
	Verification 2 months		7		8	o			o	
	Verification 4 months		7		8	o			o	
	Verification 6 months		7		8	o			o	
Freeze/Thaw	Discovery	5		5		o	o	o	o	o
	Verification		5		5	o		o	o	o

Results (Time on Bench)

Verified blood or plasma incubation markers show the same trends between Discovery and Verification and nearly perfect separation between the control and incubated groups.

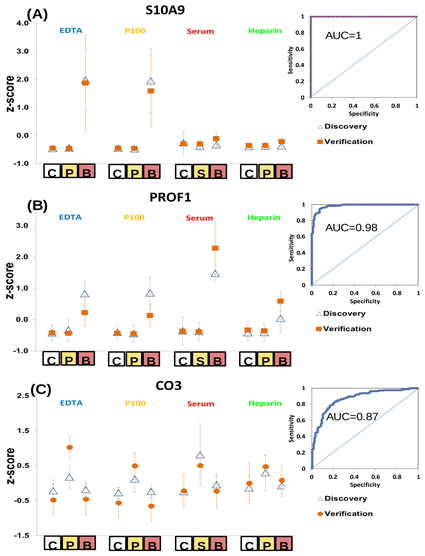

Figure 3. Averaged z-score (average of all peptide z-scores) per condition and tube type for (C) Protein S100-A9 (S10A9), (D) Profilin-1(PROF1) and (E) Complement C3 (CO3). The z-score is calculated for a peptide x as (abundance x – abundance mean) / abundance standard deviation from Discovery study (open triangle) and verification (solid square). Error bars are standard deviations of z-scores.

C: Control
P: Plasma incubation
S: Serum incubation
B: Blood incubation

Blood incubation induces more changes than does plasma incubation. 37 °C is much worse than 20 ° C.

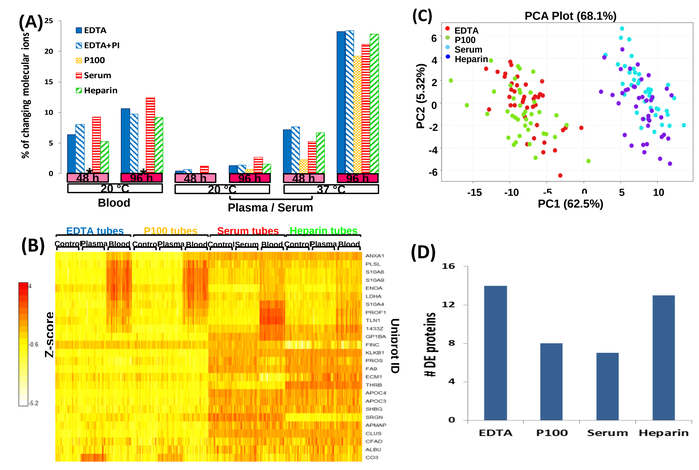

Figure 4. (A) The percentage of changing molecular ions (versus Control) from Discovery Pilot Study upon blood and plasma incubation for 48 and 96 h at 20 °C. A molecular ion was called differentially expressed or changing given adjusted p < 0.05 and fold change >= 2. * Blood incubation was not performed in the P100 tube due to a limit in the amount of blood allowed to be drawn as per the PAMF IRB. (B) Principal Component Analysis (PCA) of all detected proteins from Verification Study comparing EDTA (red dots), P100 (green dots), Serum (aqua dots), and Heparin tubes (blue dots) at the Control condition for all 40 subjects. (C) Heat map (from Verification data) of 26 proteins verified by MRM assay on samples from 40 subjects. (D) Number of differentially expressed proteins on plasma/serum incubation compared to Control.

Results (Time in Freezer)

Changes occur after 6 months of plasma/serum storage at -80 °C, with even more changes observed at -20 °C, implying -80°C is insufficient. Data separates by time more than by temperature implying that at both temperatures, changes continue over time.

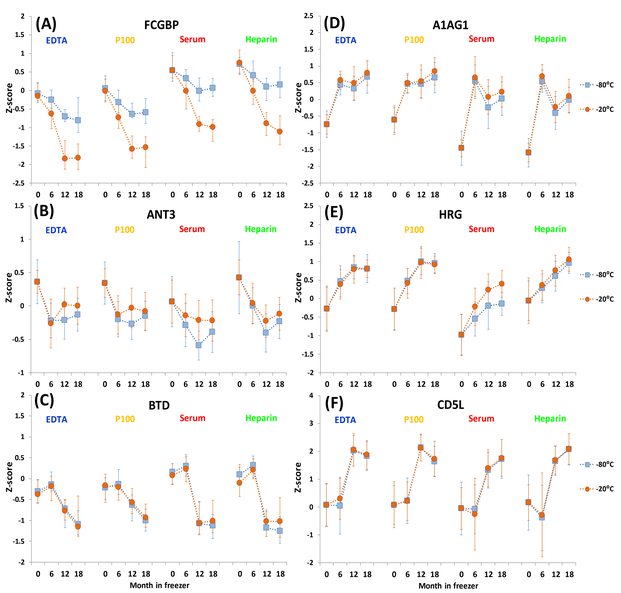

Figure 7. Averaged z-score (average of all peptide z-scores) per condition and tube type for (A) IgGFc-binding protein (FCGBP), (B) Antithrombin-III (ANT3), (C) Biotinidase (BTD), (D) Alpha-1-acid glycoprotein 1(A1AG1), (E) Histine-rich glycoprotein (HRG), and (F) CD5 antigen-like (CD5L). Error bars are standard deviation of z-scores. EDTA, P100, Serum, and Heparin tubes are shown for 0 month at -80°C and 6, 12, or 18 month at -80°C or -20°C

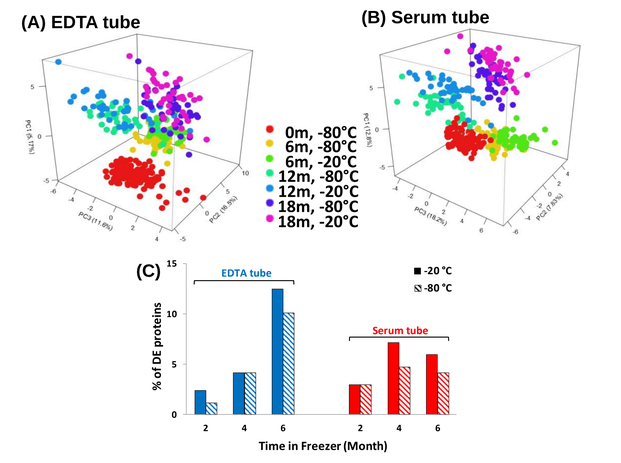

Figure 7. Principal Component Analysis of proteins in EDTA (G) and Serum (H) tubes over 6, 12, and 18 months storage at -20 °C and -80 °C (discovery). (I) Percentage of differentially expressed proteins seen in verification study.

Results (Freeze/thaw)

Incremental changes occur over Freeze/Thaw cycles; P100 and Serum tubes are more protective.

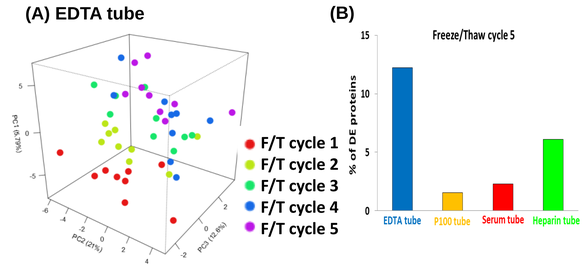

Figure 9. (A) Principal Component Analysis (PCA) of proteins in EDTA tubes against freeze/thaw cycle (discovery study). (B) Percentage of differentially expressed proteins seen in freeze/thaw cycle verification study

Summary

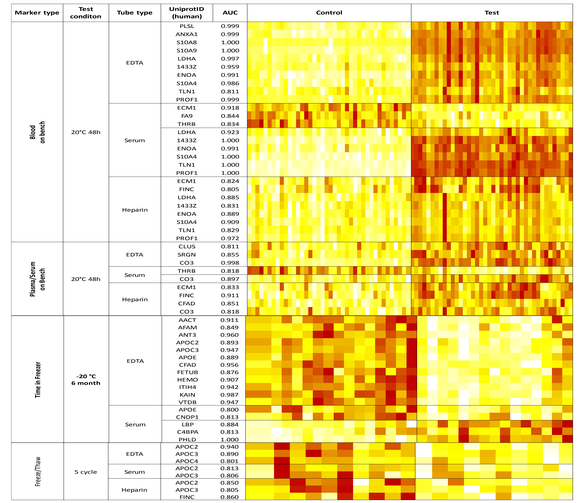

Figure 10. List of verified candidate classifiers (AUC > 0.8) for sample integrity. AUCs are calculated for each protein by comparing control and test condition as follows. Blood on Bench: control (20°C 0.5 h) vs 20°C 48 h, Plasma/Serum on Bench: control (20°C 0.5h) vs 20°C 48h, Time in Freezer: control (-80°C 0 m) vs -20°C 6 m, Freeze/Thaw: control (cycle 1) vs cycle 5

Conclusions

- Sample preparation Operator characteristics should be recorded.
- Do not leave blood on bench; 37 °C is much worse than 20 °C.
- Do not leave serum/plasma for >24h at 20 °C or at 37 °C.
- Changes occur after 6 months at -80 °C, more changes at -20°C.
- Damage due to number of F/T cycles is incremental.
- P100 and Serum tubes outperform when left on bench and in F/T.
- Majority of discovered markers are successfully verified in independent samples. Single markers with >0.98 AUC in ROC analysis are obtained. A sample integrity panel will be validated.

Acknowledgements

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