Impact of Human Blood Specimen Collection, Manipulation, 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.

Table 1. Study design

Subjects	Breast cancer / healthy female Prostate cancer / healthy male					
Blood collection tube types	EDTA	EDTA+PI	P100	Serum	Heparin	
Time & temperature on bench	hours	0.5	1	4	24	48	96
		4 °C	20 °C			20 °C	37 °C
Freeze-thaw cycle	1 to 5 cycles					
Time in freezer	months	0	6	12	18	
		-20 °C	-80 °C			

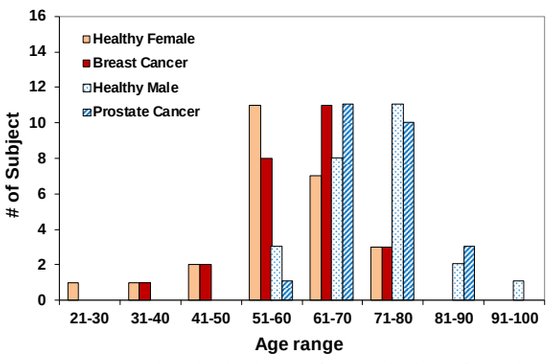

Figure 1. Age and gender distribution of recruited subjects for Study 2 and Verification

Table 2. Number of subjects and samples for Discovery study

Study	Subjects	Samples
1	125	320
Pilot	6	500
2	100	1600
Total	231	2420

Results (Time on Bench)

1. Comparison of two operators following the same SOP shows Op1 is late and more variable. Thus, recording processing data in real time is important to track pre-analytical variables.

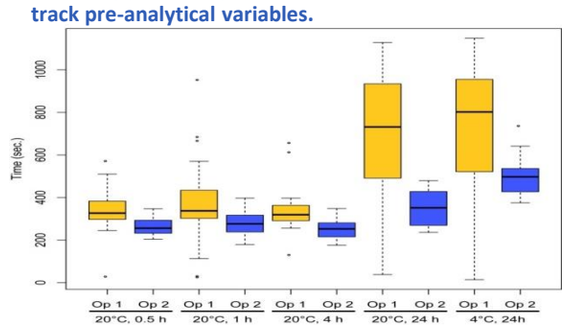

Figure 2. Box plot representation of processing time (post-centrifugation and prior to storage in freezer), grouped by tube ordering and two operators (Op1 and Op2).

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

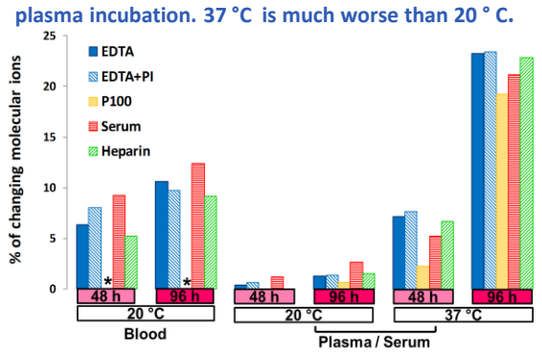

Figure 3. The percentage of changing molecular ions upon being subjected to different time and temperature son bench for EDTA, EDTA+PI, P100, Serum, and Heparin tubes. *: Blood incubation is not applicable in the P100 tube. Blood incubation samples at 37 °C was not available due to extensive hemolysis and thickening of samples.

3. A total of 26 proteins are verified as blood or plasma incubation markers

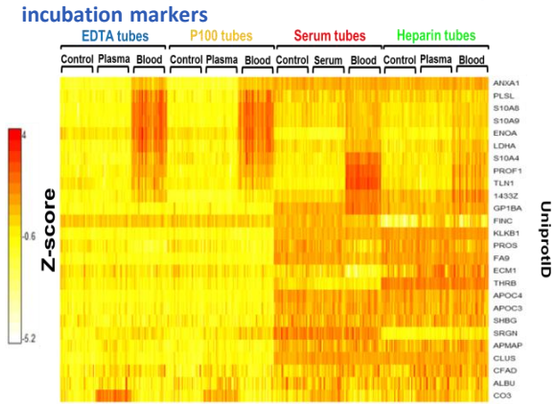

Figure 4. Heat map of 26 proteins verified by MRM assay. The y-axis z-score is calculated for a protein x as (abundance x – abundance mean) / abundance standard deviation. Control was incubated for 0.5 h before and after centrifugation, Blood or Plasma / Serum was incubated for 48 h before or after centrifugation.

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

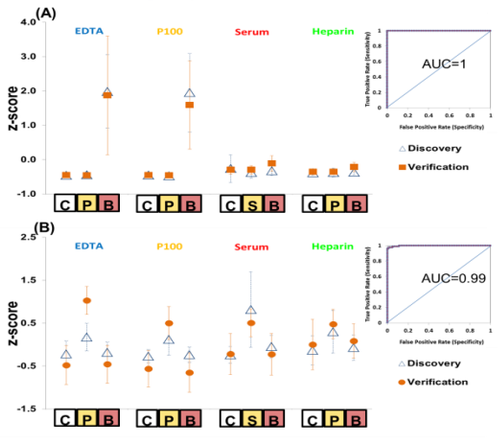

Figure 5. Averaged z-score (average of all peptide z-scores) per condition and tube type for (A) Protein S100-A9, and (B) Complement C3. Error bars are standard deviations of z-scores. EDTA, P100, Serum, and Heparin tubes are shown for all subjects (20 Normal and 20 Cancer subjects). The legends for x-axis are as followed. C: Control (pre-spin 0.5 h / post-spin 0.5 h), P or S: Plasma or Serum incubation (pre-spin 0.5h / post-spin 48 h), and B: Blood incubation (pre-spin 48 h / post-spin 0.5 h) at 20 °C. Nearly perfect AUCs are obtained from verification data upon analysis of ROC curves shown (panel right).

Results (Time in Freezer)

5. 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.

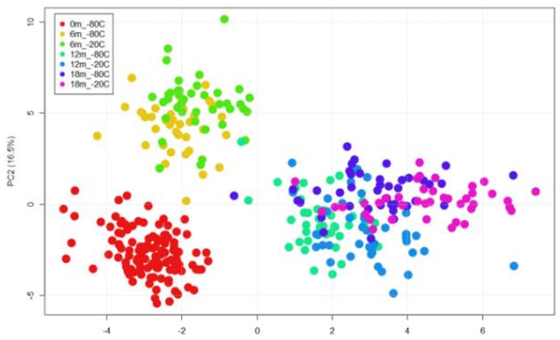

Figure 6. Principal Component Analysis (PCA) of all identified proteins in EDTA tubes over 6, 12, and 18 months of storage at -20 °C and -80 °C. The samples are from 40 subjects (10 healthy female, 10 breast cancer patients, 10 healthy male, and 10 prostate cancer patients).

6. An example protein showing degradation over time when stored in the freezer in Discovery and Verification.

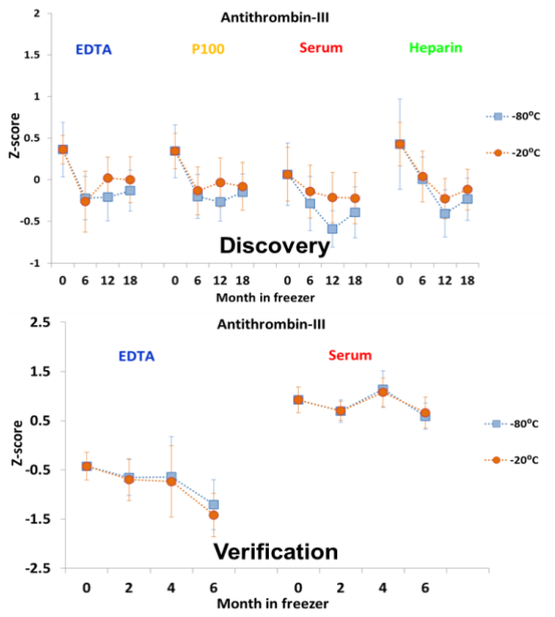

Figure 7. Averaged z-score (average of all peptide z-scores) per condition and tube type for Antithrombin-III. (Top) Discovery Study where EDTA, P100, Serum, and Heparin tubes are shown for all subjects (20 Normal and 20 Cancer subjects) for 0 month at -80°C and 6, 12, or 18 month at -80°C or -20°C. (Bottom) Verification where EDTA and Serum tubes are shown for all subjects (7 female and 8 male healthy subjects) for 0 month at -80°C and 2, 4, or 6 month at -80°C or -20°C.

7. An example protein released during cell lysis, shows greater impact at -20 °C vs. -80 °C, and is verified as a marker for freezer storage.

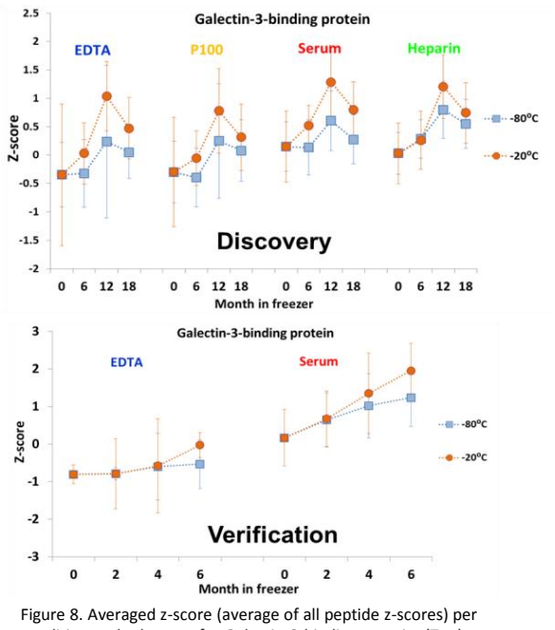

Figure 8. Averaged z-score (average of all peptide z-scores) per condition and tube type for Galectin-3-binding protein. (Top) Discovery Study where EDTA, P100, Serum, and Heparin tubes are shown for all subjects (20 Normal and 20 Cancer subjects) for 0 month at -80°C and 6, 12, or 18 month at -80°C or -20°C. (Bottom) Verification where EDTA and Serum tubes are shown for all subjects (7 female and 8 male healthy subjects) for 0 month at -80°C and 2, 4, or 6 month at -80°C or -20°C.

Results (Freeze/thaw)

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

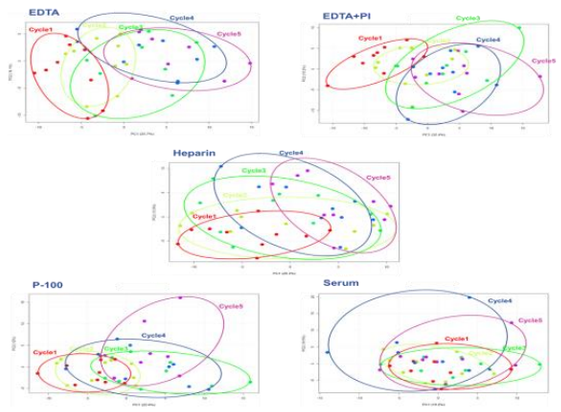

Figure 9. Principal Component Analysis (PCA) of molecular ions tracked over 5 freeze-thaw cycles comparing 5 tube types (EDTA; EDTA + PI; Heparin; P-100 and Serum) from Freeze/Thaw Cycle Study. Red cycle 1; light green, cycle 2; green cycle 3; blue, cycle 4; purple, cycle 5.

9. An example protein showing degradation over F/T cycles 1 through 5 only in Heparin tubes.

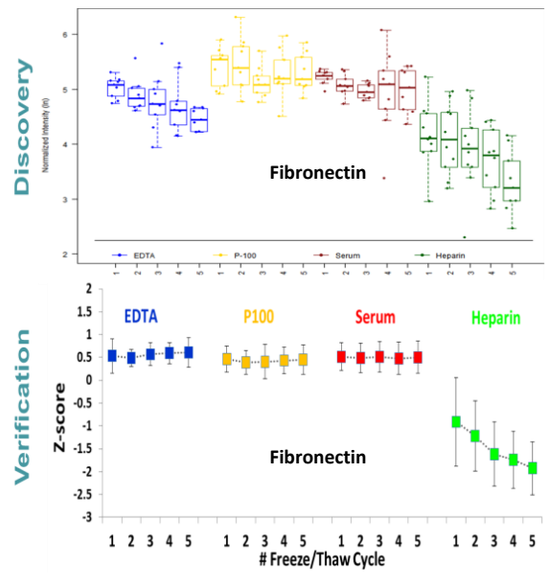

Figure 10. (Top) Box plot representation of Fibronectin from Discovery and (Bottom) averaged z-score (average of all peptide z-scores) from Verification per tube type. Error bars in the Bottom figure are standard deviation of z-scores. EDTA, P100, Serum, and Heparin tubes are shown for all subjects for freeze/thaw cycle 1 to 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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