

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

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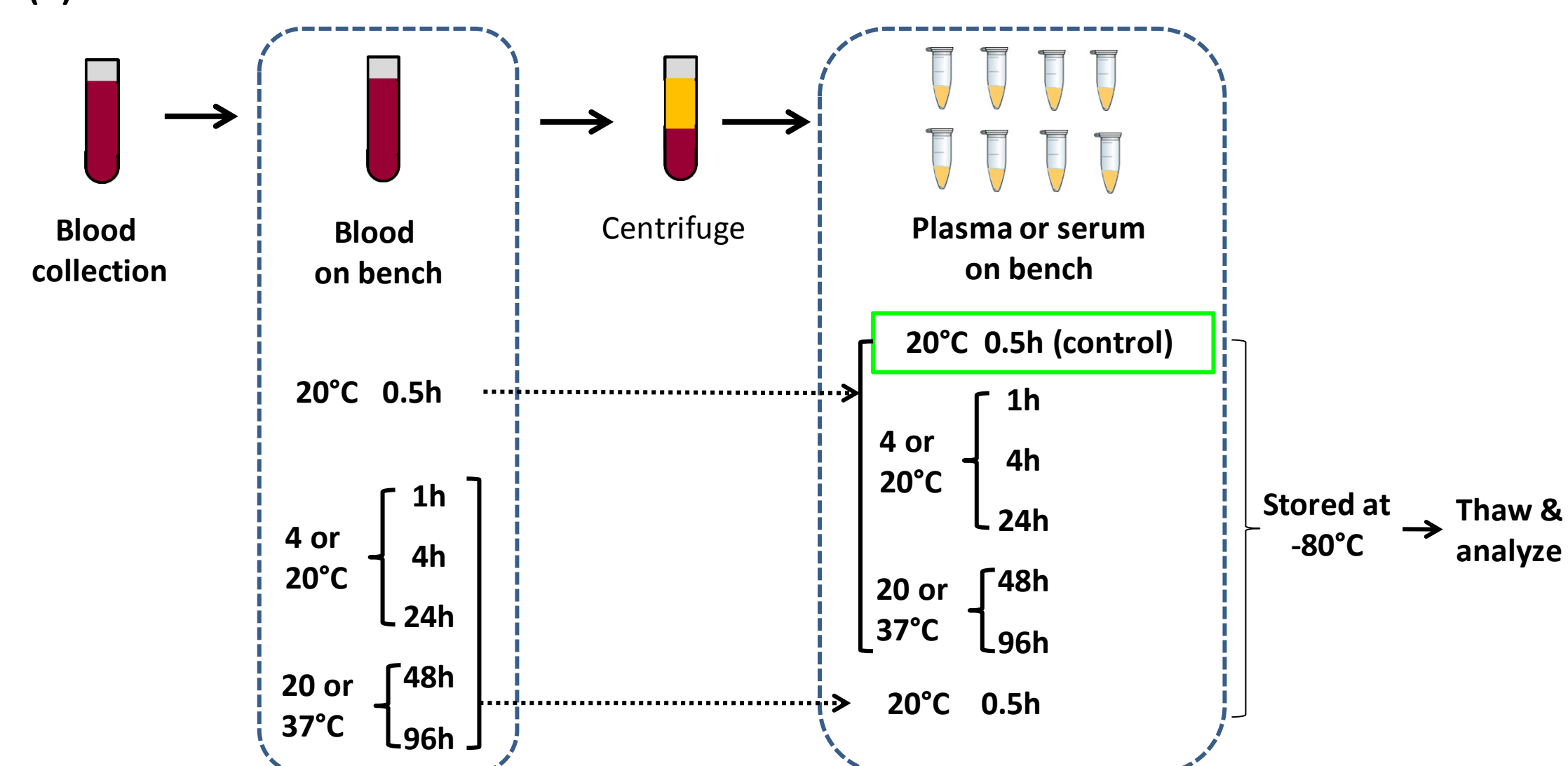
Introduction

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.

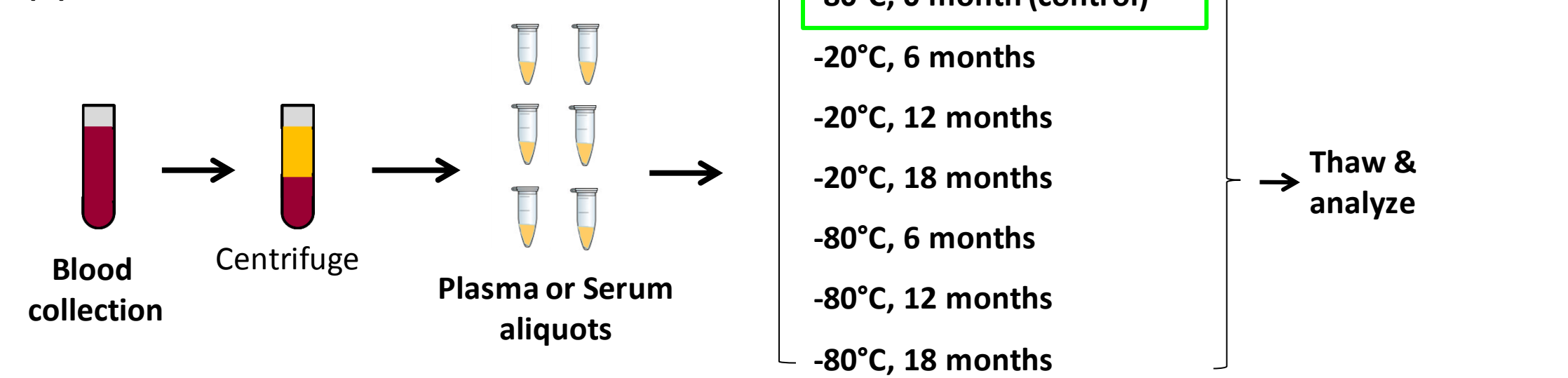
Methods

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.

(A) Time on Bench



(B) Time in Freezer



(C) Freeze/Thaw

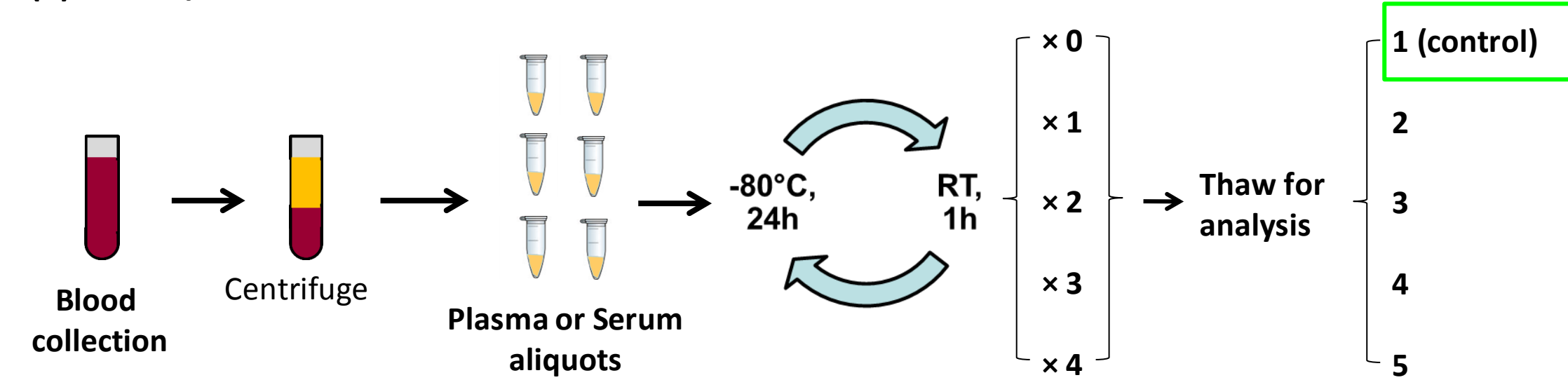


Figure 1. Design of sample handling and processing for (A) Time on Bench, (B) Time in Freezer, and (C) Freeze/Thaw studies.

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							
	Discovery Study 2	3	3							
	Discovery Study 3	10	10	10	10					
	Verification	10	10	10	10					
Time in Freezer	Discovery 0 month	10	10	10	10					
	Discovery 6 months	10	10	10	10					
	Discovery 12 months	10	10	10	10					
	Discovery 18 months	10	10	10	10					
Freeze/Thaw	Discovery	5	5							
	Verification	5	5							

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.

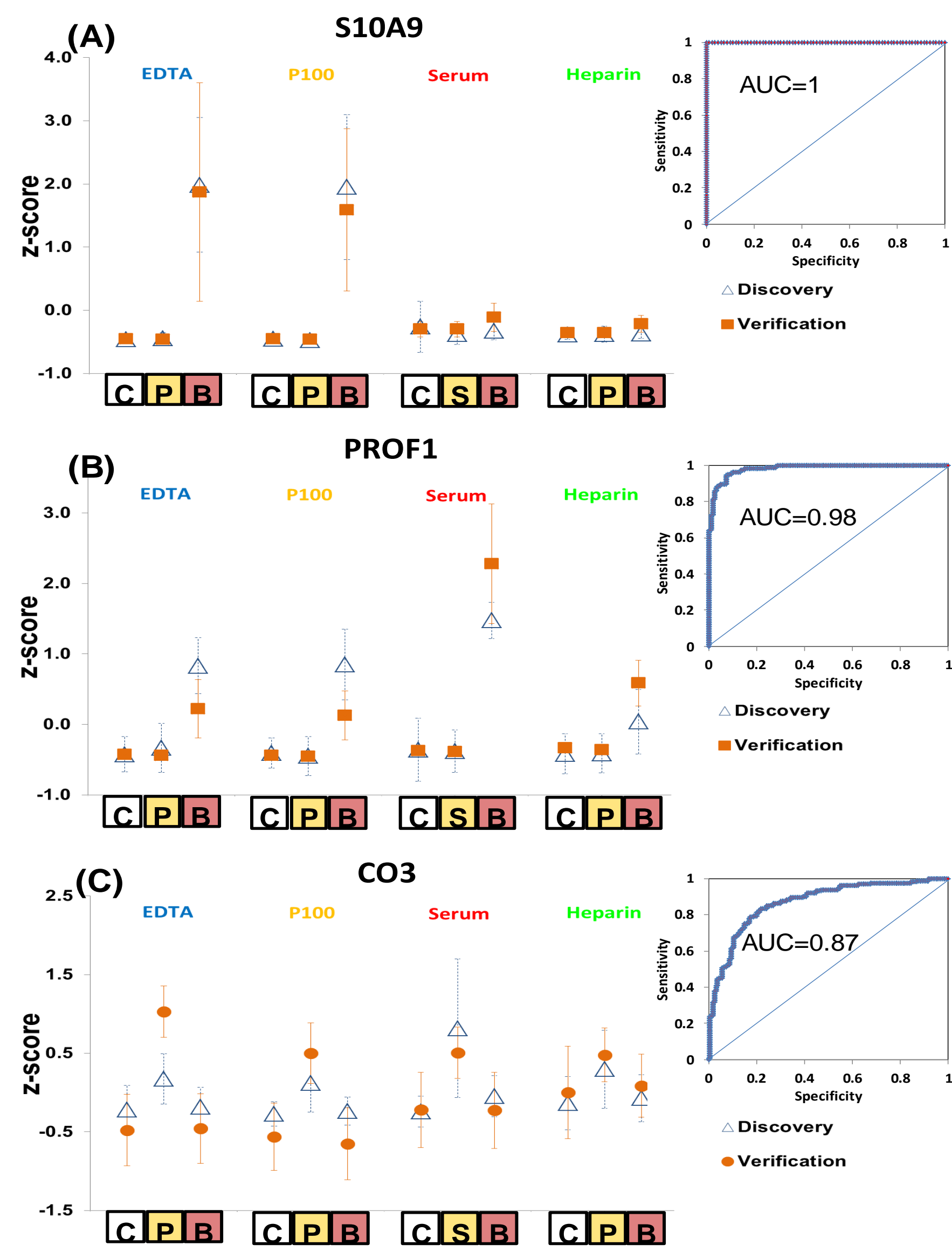


Figure 3. Averaged z-score (average of all peptide z-scores) per condition and tube type for (C) Protein S100-A9 (S10A9), (D) Profilin-1 (PROF1) and (E) Complement C3 (CO3). The z-score is calculated for a peptide x as (abundance x - abundance mean) / abundance standard deviation from Discovery study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

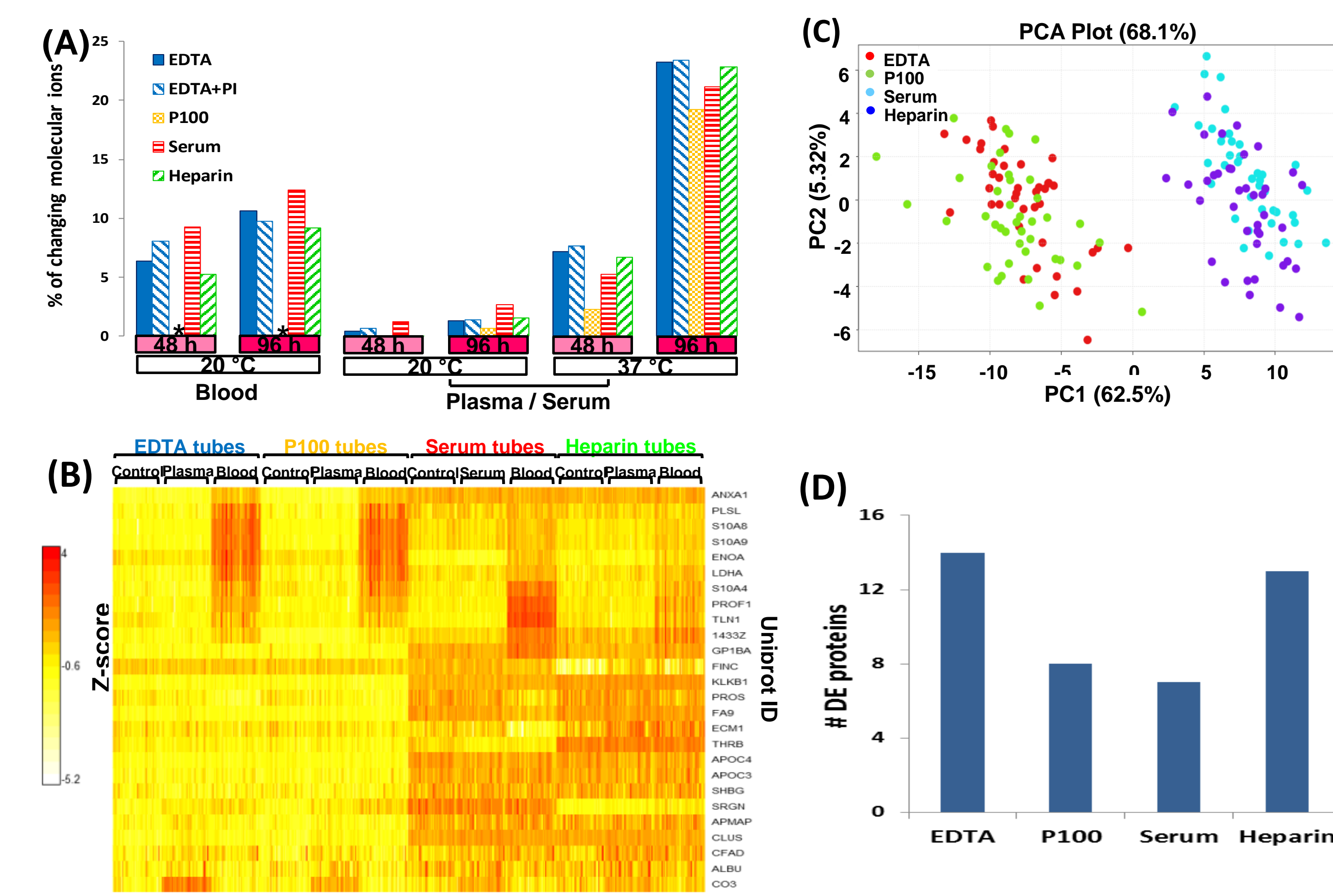


Figure 4. (A) The percentage of changing molecular ions (versus Control) from Discovery Pilot Study upon blood and plasma incubation for 48 and 96 h at 20 °C. A molecular ion was called differentially expressed or changing given adjusted $p < 0.05$ and fold change ≥ 2 . * Blood incubation was not performed 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.

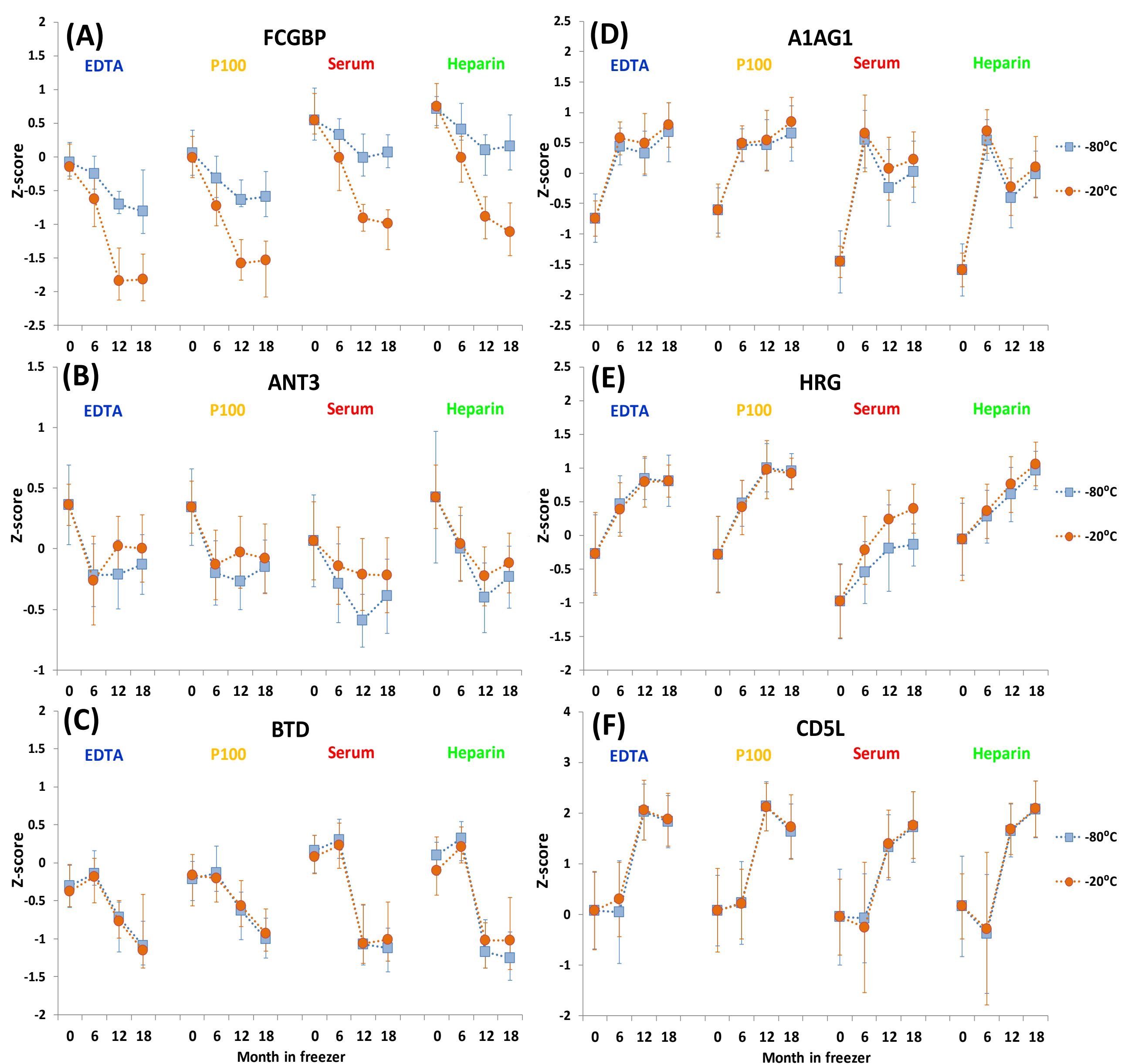


Figure 7. Averaged z-score (average of all peptide z-scores) per condition and tube type for (A) IgGfC-binding protein (FCGBP), (B) Antithrombin-III (ANT3), (C) Biotinidase (BTD), (D) Alpha-1-acid glycoprotein 1 (A1AG1), (E) Histone-rich glycoprotein (HRG), and (F) CD5 antigen-like (CD5L). Error bars 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

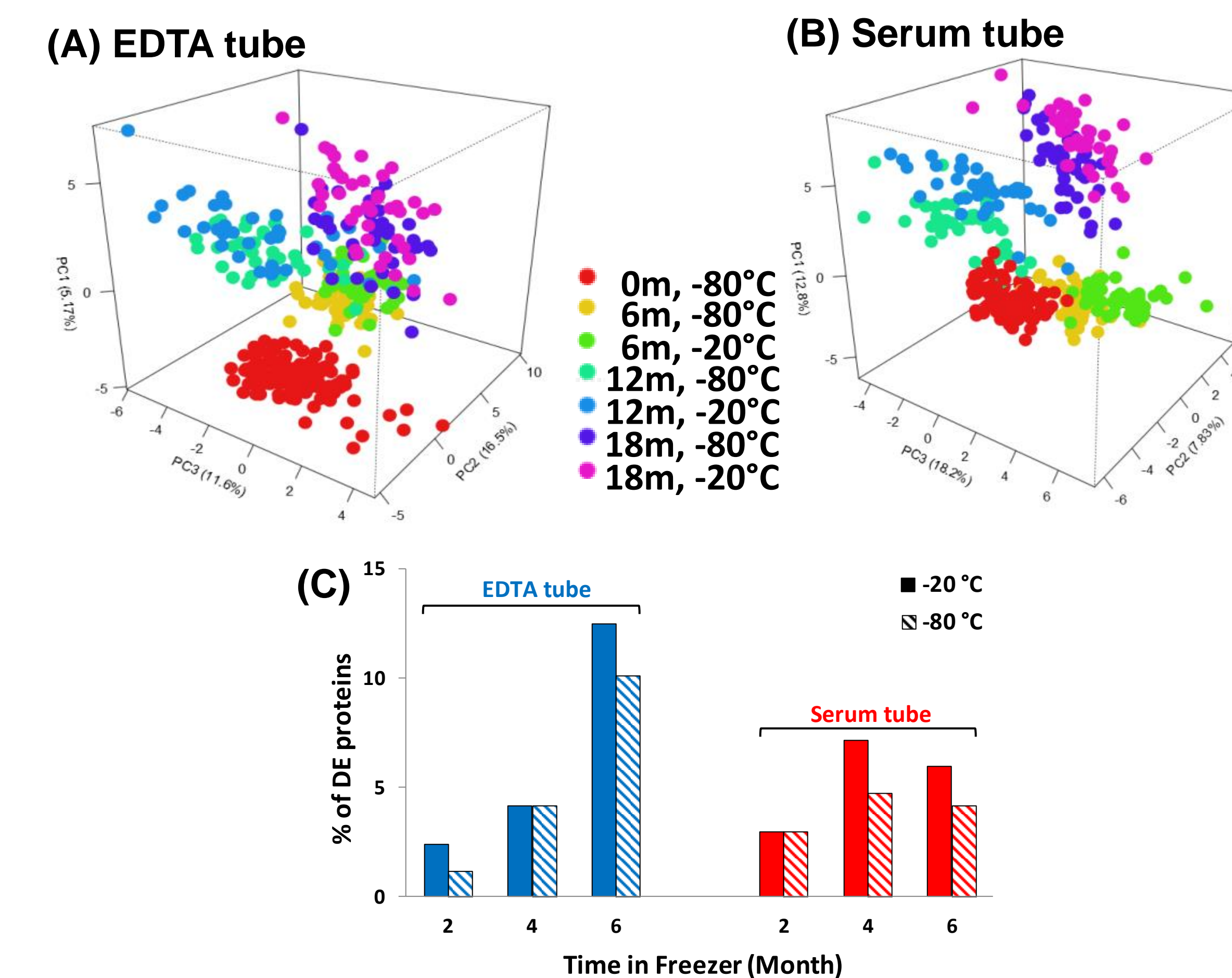


Figure 7. Principal Component Analysis of proteins in EDTA (G) and Serum (H) tubes over 6, 12, and 18 months storage at -20 °C and -80 °C (discovery). (I) Percentage of differentially expressed proteins seen in verification study.

Results (Freeze/thaw)

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

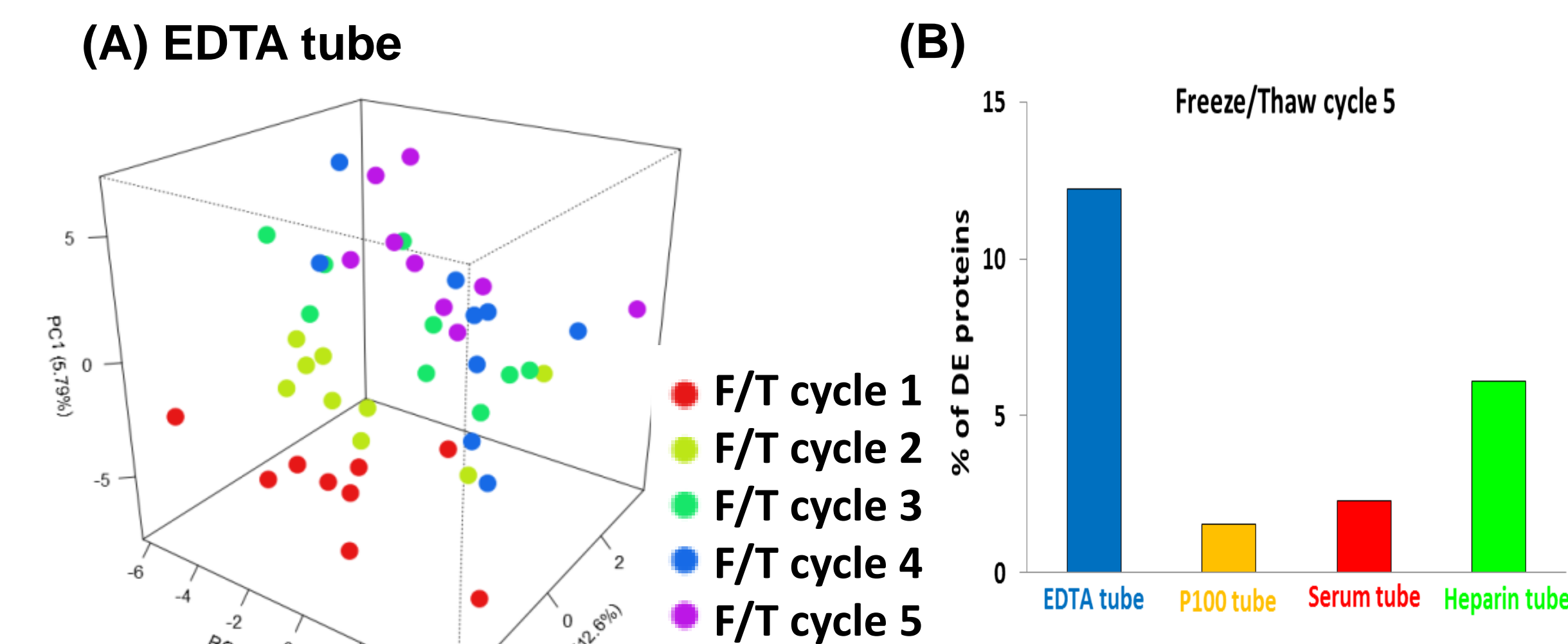


Figure 9. (A) Principal Component Analysis (PCA) of proteins in EDTA tubes against freeze/thaw cycle (discovery study). (B) Percentage of differentially expressed proteins seen in freeze/thaw cycle verification study

Summary

Marker type	Test condition	Tube type	UniProtID (Human)	AUC	Control	Test		
Blood on bench	20°C 48h	EDTA	PLSL	0.999				
			ANKRD1	0.999				
			S10A8	1.000				
			S10A9	1.000				
			LDHA	0.997				
			I4332	0.959				
		Serum	ENOA	0.991				
			S10A4	0.986				
			TLN1	0.811				
			PROF1	0.999				
			ECM1	0.918				
			FAO	0.844				
Heparin	20°C 48h	TRFB	0.834					
		LDHA	0.923					
		I4332	1.000					
		ENOA	0.991					
		S10A4	1.000					
		TLN1	1.000					
		Plasma/Serum on bench	20°C 48h	EDTA	PROF1	1.000		
					ECM1	0.824		
					FINC	0.805		
					LDHA	0.885		
					I4332	0.831		
					ENOA	0.889		
Serum	S10A4			0.909				
	TLN1			0.829				
	PROF1			0.972				
	CLUS			0.811				
	SIGN			0.855				
	CO3			0.998				
Time in Freezer	-20°C 6 month	EDTA	THIBB	0.818				
			CO3	0.897				
			ECM1	0.833				
			FINC	0.911				
			CFAD	0.851				
			CO3	0.818				
		Serum	ANACT	0.911				
			AFAM	0.849				
			ANT3	0.960				
			APOC2	0.893				
			APOC3	0.848				
			APOE	0.889				
Freeze/Thaw	5 cycle	EDTA	CFAD	0.956				
			FETUB	0.876				
			HEMO	0.907				
			ITIH4	0.942				
			KAIN	0.987				
			VITON	0.947				
		Serum	APOE	0.800				
			CHDP1	0.813				
			LEP	0.884				
			CABPA	0.813				
			SLP	0.903				
			AFM1	0.801				
Heparin	APOC2	0.940						
	APOC3	0.990						
	APOC4	0.801						
	APOC5	0.801						
	APOC6	0.813						
	APOC8	0.850						

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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