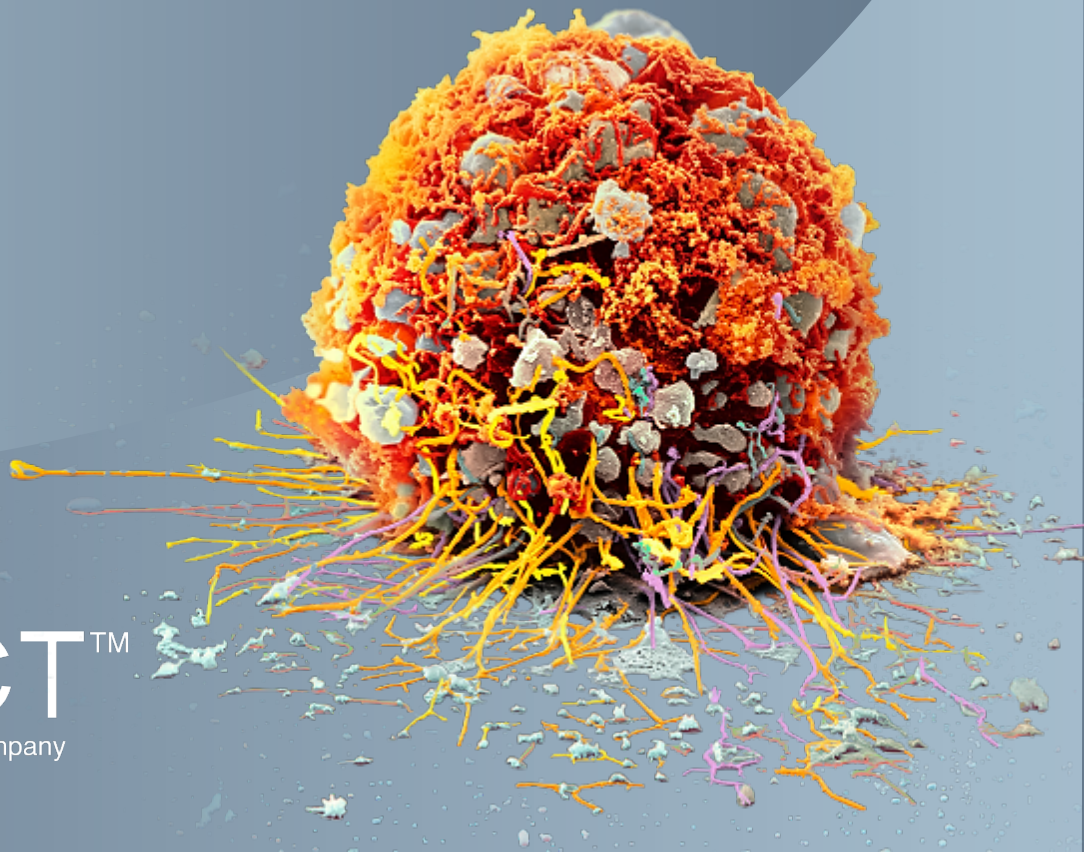
CRYOXTRACT INSTRUMENTS

Preserving Biospecimen Integrity



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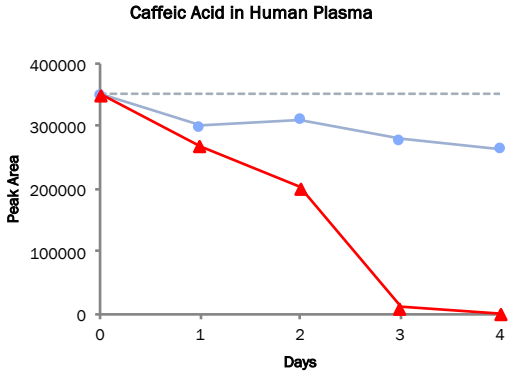
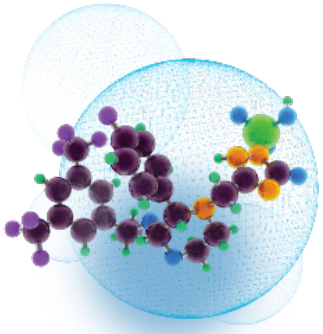
BIOANALYSIS OF UNSTABLE DRUGS AND BIOMARKERS



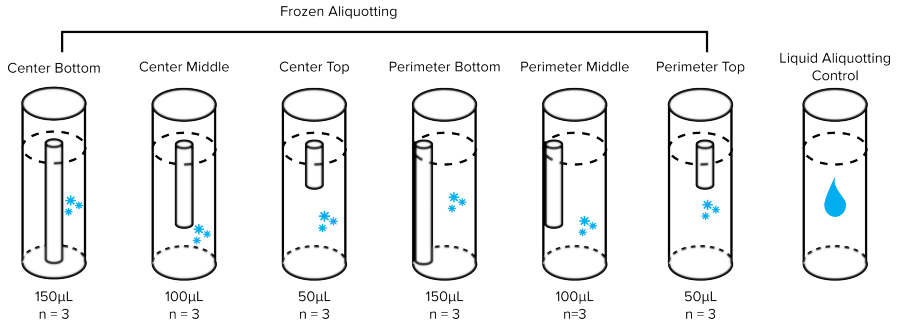
LC-MS/MS quantitation of Caffeic acid spiked into human EDTA-plasma comparing multiple freeze-thaws to frozen aliquotting.



Problem: Unstable drug structures are extremely difficult to quantitate in PK trial samples yielding high assay CVs, leading to ISR concerns, delays & failures, risking $MMs. Chemical stabilization is costly & difficult to implement at trial arms. Some molecules degrade entirely with a single freeze-thaw.

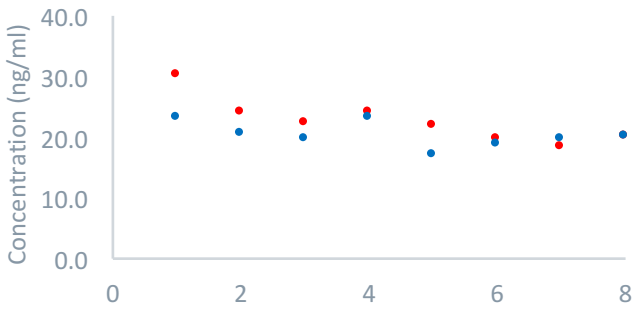
Solution: Frozen aliquotting avoids F-T cycles & stabilizes labile structures, avoiding room temperature liquid phase, dissolving frozen portions of plasma, serum or blood directly into a stabilized environment (solvent crash buffer). This improves reproducibility of analytical results, lowering ISR concerns for challenging molecules.

Challenge: Heterogeneous distribution of a target analyte in the frozen matrix may result in too much analytical variability for frozen aliquotting to be applied effectively in a bioanalysis application. Therefore, studies were conducted to assess the factors contributing to, and the severity of, sample heterogeneity. Both small and large molecules were evaluated in serum and EDTA plasma by comparing cores generated from various lateral and vertical positions from frozen samples in 2ml cryovials prepared at different freezing rates.



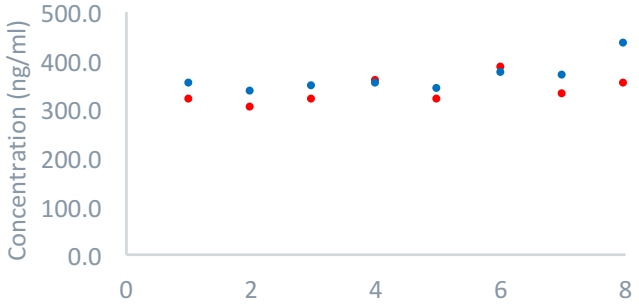
COMPARISON OF FROZEN ALIQUOTS TO LIQUID ALIQUOTS BY ELISA

Soluble P-Selectin
M.W. ~140 kDa
EDTA Plasma
*Full cores



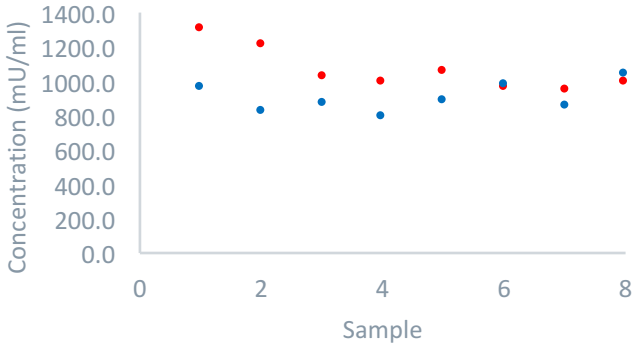
	Liquid Aliquots	Frozen Aliquots
Avg. Conc.	**22.9**	**20.6**
Std. Dev.	**3.48**	**1.98**
CV	**15.2%**	**9.6%**

Apolipoprotein B
M.W. ~550 kDa
EDTA Plasma
*Full cores



	Liquid Aliquots	Frozen Aliquots
Avg. Conc.	**336.2**	**362.6**
Std. Dev.	**25.59**	**28.96**
CV	**7.6%**	**8.0%**

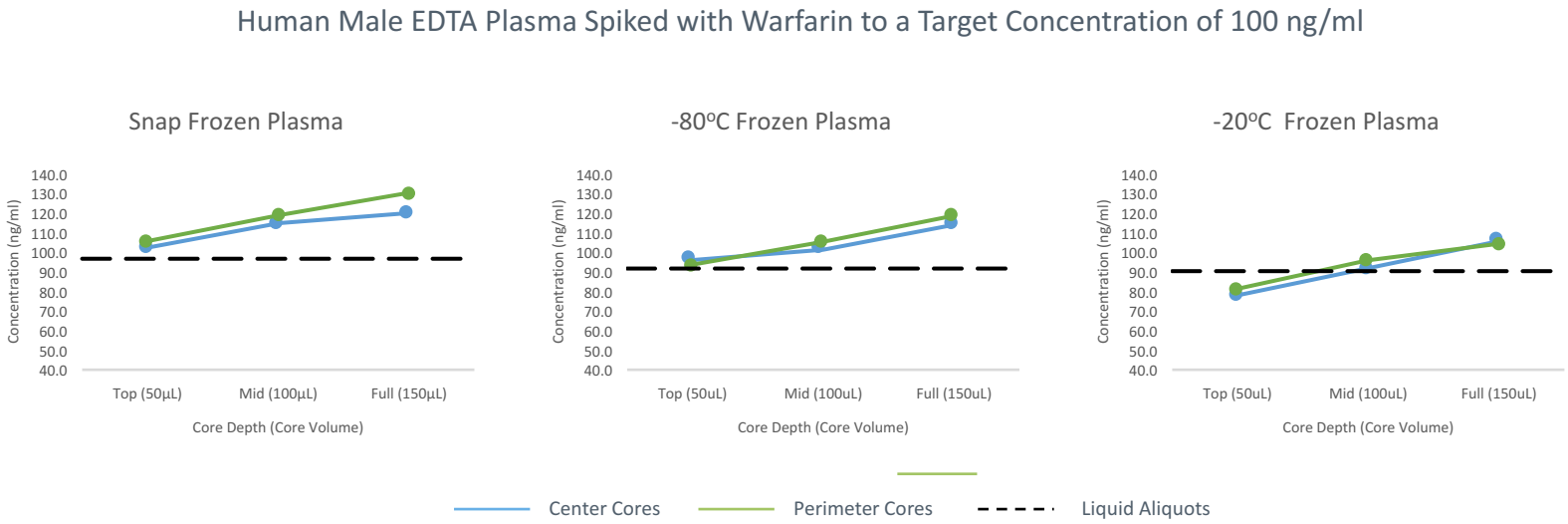
von Willebrand Factor
M.W. ~500-20,000 kDa
Citrate Plasma
*Full cores



	Liquid Aliquots	Frozen Aliquots
Avg. Conc.	**1061.2**	**897.9**
Std. Dev.	**121.40**	**80.86**
CV	**11.4%**	**9.0%**

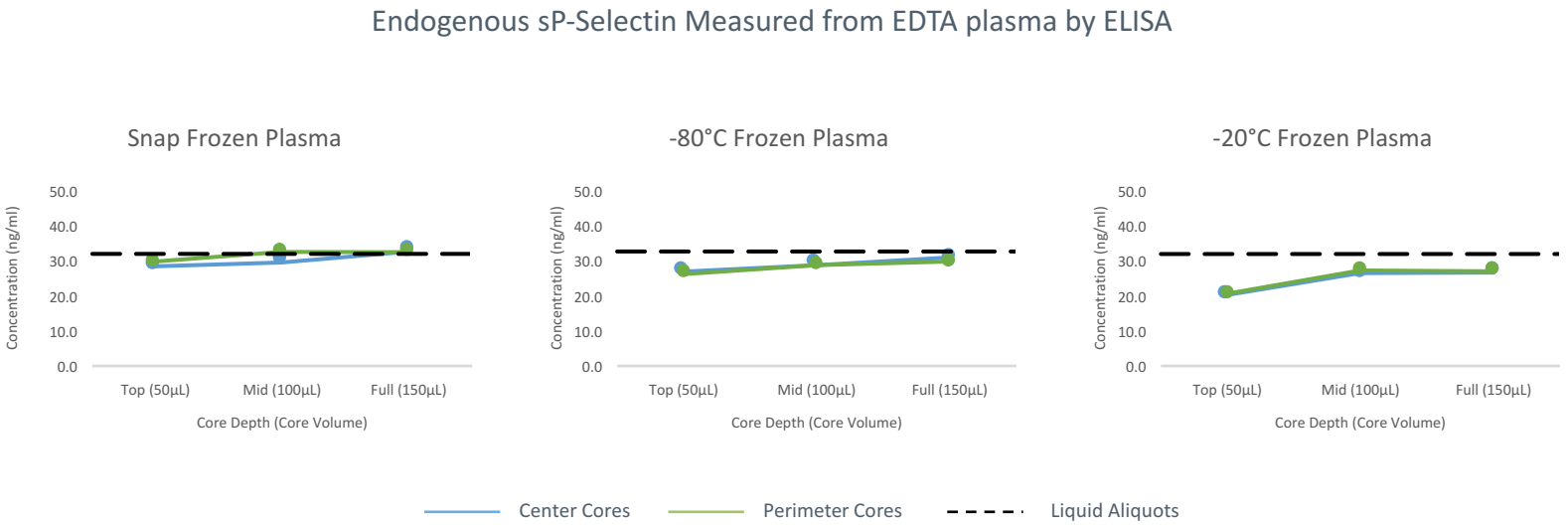
* Full cores refers to cores generated by coring down to the bottom of the sample tube.

DISTRIBUTION DATA – SMALL MOLECULES



- No Evidence of radial gradients forming
- Increased analyte sedimentation was observed to be inversely associated with freeze rate
- Analyte concentration from liquid aliquots tended to be less than frozen cores and may suggest an additional sedimentation/precipitation effect in the thawed control samples. Additional experiments are underway
- Experiments utilizing warfarin spiked serum, atenolol spiked serum, and atenolol spiked plasma revealed similar trends

DISTRIBUTION DATA – LARGE MOLECULES



- No Evidence of radial gradients forming when comparing cores generated from the perimeter and center of the tubes at three coring depths (i.e. top, mid, and full).
- Increased analyte sedimentation was observed to be inversely associated with freeze rate as evident from increases in concentrations with increases in coring depth.
- Concentration data from thawed liquid controls largely agree with full and mid length cores across all freeze rates and suggest that the amount of sedimentation is minimal.
- Experiments for other large molecule and small molecule targets in both EDTA plasma and serum show similar trends.

RECOMMENDATIONS FOR DEVELOPING & VALIDATING QUANTITATIVE ASSAYS USING FROZEN ALIQUOTTING

Qualify a working parent sample volume for the assay that will support the required assay performance

Figure 1: Sample Volume vs sP-Selectin Concentration

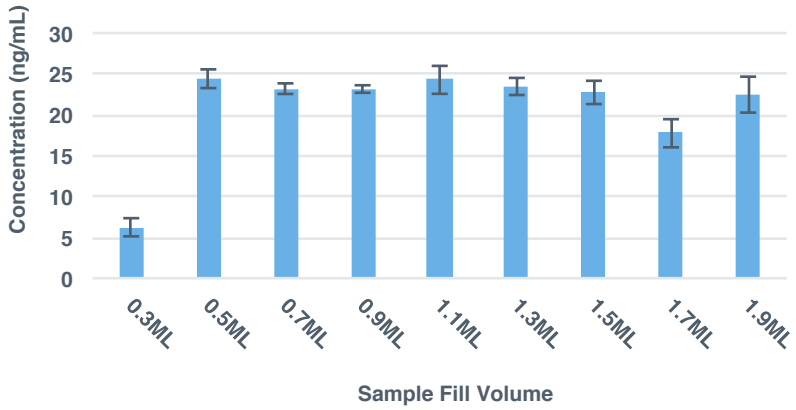


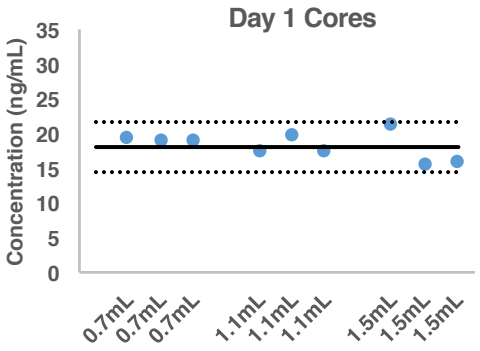
Table 1: Impact of Volume Range on Concentration Measurements for sP-selectin from Frozen Aliquotted Citrate Plasma

Fill Volume	Avg. Conc. (ng/ml)	Standard Deviation	Coefficient of Variation
0.3 - 1.9mL	20.9	5.7	27.2%
0.5 - 1.7mL	22.7	2.5	10.8%
0.7 - 1.5mL	23.4	1.3	5.4%
0.9 - 1.3mL	23.6	1.3	5.4%

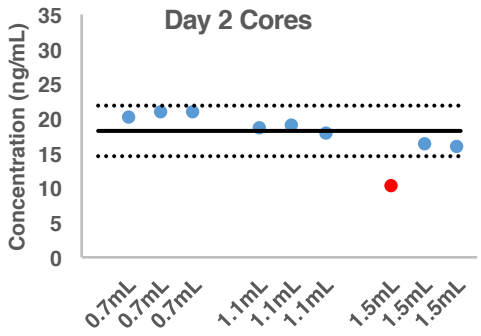
- Assay requires 50uL of sample for analysis
- -80C frozen citrate plasma (pooled) evaluated (*endogenous sP-Selectin concentration only)
- 0.3ml to 1.9ml starting parent sample volume evaluated (n=6 per volume)
- 0.7ml to 1.5ml with a target volume of 1.1ml selected
- Non-validated commercial assay

RECOMMENDATIONS FOR DEVELOPING & VALIDATING QUANTITATIVE ASSAYS USING FROZEN ALIQUOTTING

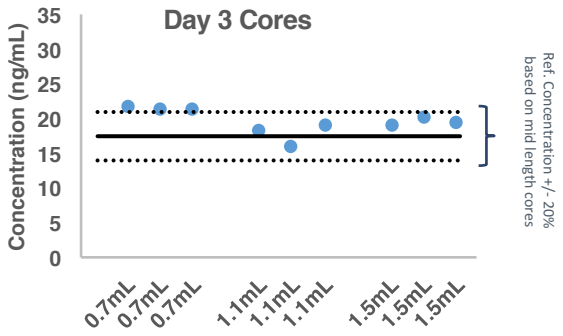
Confirm assay performance and reproducibility for selected target volume and range



	Cores (n=9)
Avg. Conc.	18.1
CV	9.7%
% Diff	3.5 - 4.5%



	Cores (n=8)
Avg. Conc.	18.5
CV	9.6%
% Diff	10.8 – 13.7%



	Cores (n=9)
Avg. Conc.	19.3
CV	9.1%
% Diff	9.2 – 17.9%

- 50uL analysis volume from 0.7ml, 1.1ml, and 1.5ml samples (n=3 per volume)
- -80C frozen citrate plasma (pooled) evaluated (*endogenous sP-Selectin concentration only)
- Standard curve generated from cored calibration standards prepared at target volume (1.1ml) and frozen at -80C
- Cores generated over three days, analyzed once.

• Note: Outlier removed due to insufficient core volume

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DISCUSSION

- Analyte settling is believed to occur due to gravimetric sedimentation and an analyte's decreasing solubility as the sample cools during the initial freezing.
- Thawing samples prior to analysis may result in additional analyte precipitation and sedimentation. Presumably, this effect may be further exacerbated by thawing on ice or low temperature environments. This may impact:
    - Sample mixing efficiency and pipetting error
    - Co-precipitation / variable extraction recoveries
    - Bioanalytical assay measurement reproducibility
- To date, observations of vertical stratification have been subtle but should still be evaluated for each new assay with consideration for how the pre-analytical study samples can/will be collected and frozen.
- Full cores will be most representative of an aliquot generated from a homogeneous liquid sample and can be used when assay/work is amenable to thawing and sub-aliquotting a frozen core.
- When a specific volume of sample is required for analysis, assay performance can be tuned by qualifying and selecting an optimal parent sample volume range in conjunction with the desired freezing rate.
- The impact of sample homogeneity for semi-quantitative profiling (e.g. global metabolomics) is currently being evaluated.