

# **Investigations into the Effects of Blood Specimen Handling Procedures on Protein Integrity**

**Daniel Chelsky, Ph.D.**

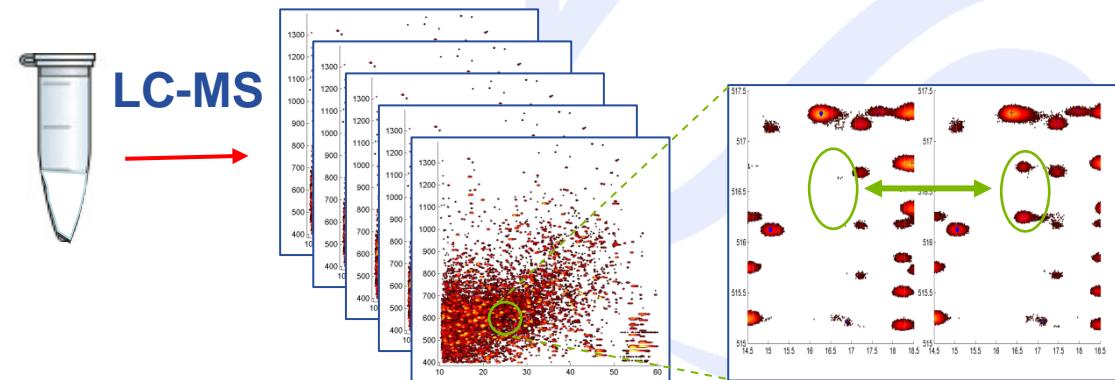
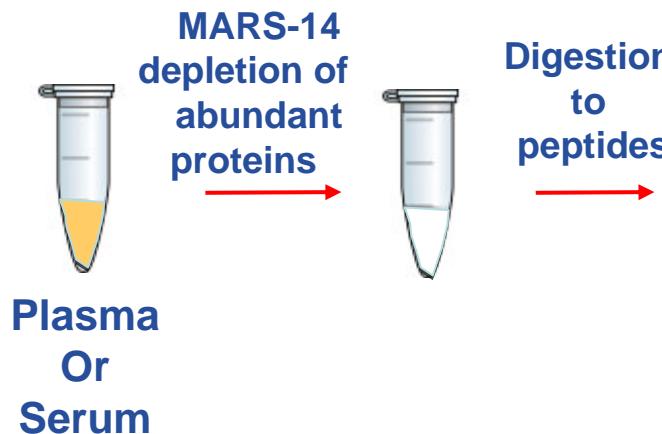
**BRN Symposium**

**February 23, 2012**

# PROGRAM GOALS

1. Characterize protein content and stability in blood samples after collection
  - a) Different collection tube types
  - b) Stability of blood prior to spin
  - c) Stability of plasma or serum after spin
2. Evaluate storage conditions
  - a) Freeze-thaw cycles
  - b) Time in freezer
3. Develop assay to assess archived plasma and serum
  - a) Multiplexed assay as an indicator of sample history

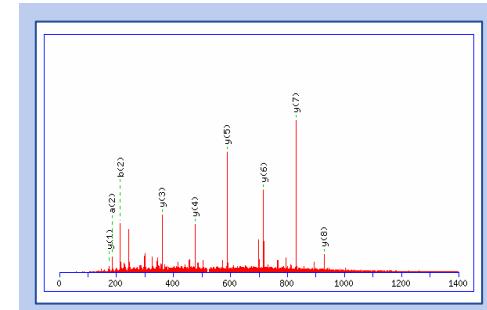
# ANALYTICAL APPROACH: MASS SPECTROMETRY-BASED PROTEOMICS



Informatics-based  
Quantitative Peptide Expression Profiling

Statistical Analysis

>gi|41327732|ref|NP\_958439.1|  
epidermal growth factor  
receptor isoform b [Homo  
sapiens]  
MRPSGTAGAALLALLAALCPASRALEKK**VCQGTSNKL**  
TQLGTEDHFLSLQRMFNNCEVVLGNLEITYVQRNYDL  
SFLKTIQEVAGYVIALNTVERIPLLENLQIIRGNMYYE  
NSYALAVLSNYDANKTGLKELPM**NLQEILHGAVRF**SN  
NPALCNVESIQWRDIVSSDFLSNMMSDFQNLHLGSCQKC  
DPSCPNGSCWGAGEENCQKLTK**IICAQQCSGR**CRGKSP  
SDCCHNQCAAGCTGPRESDCLVCRKFRDEAT.....



Protein Identification

Peptide Sequencing

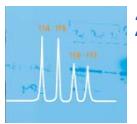
# MULTIPLEXED “MRM” MASS SPECTROMETRY ASSAYS



Select 1-350 proteins



1. Literature



2. Proteomics

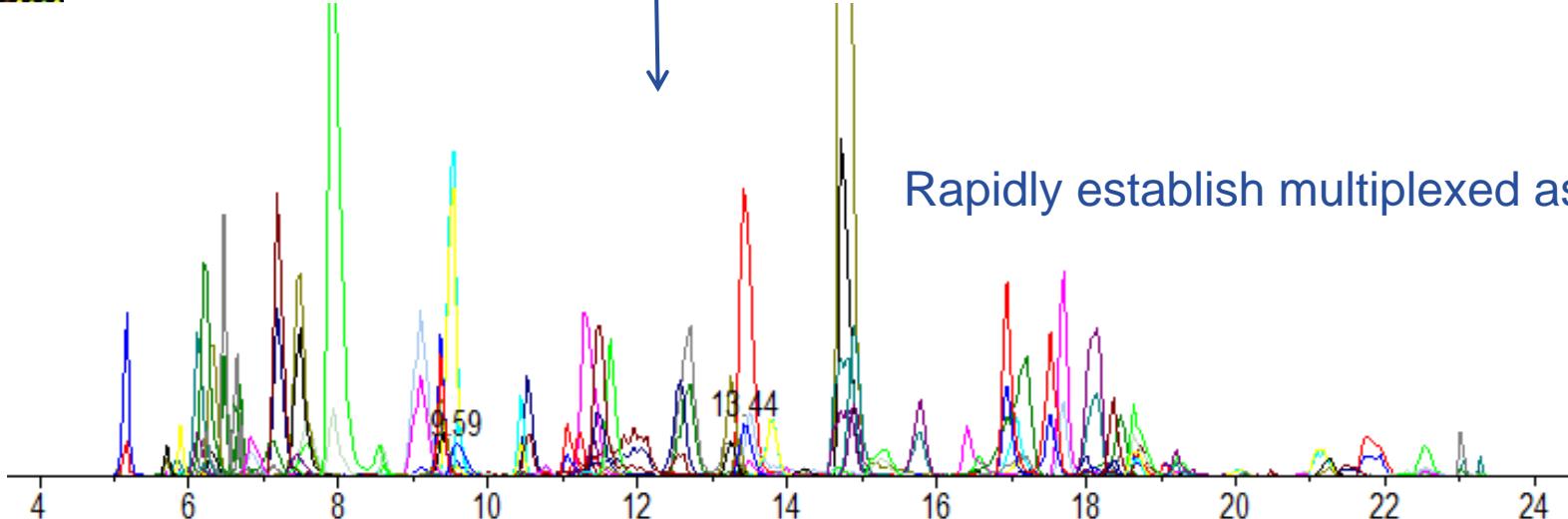


3. Transcript Profiling

## Protein Sequence

MSAIQAAWPSGTECIAKYNFHGTAEQD  
LPFCK**GDVLTIVAVTKDPNWYKAKNKV**  
GREGIIPANYVVKREGVKAGTKLSMP  
WFHGKITREQAER**LLYPPETGLFLVRE**  
STNYPGDYTLCLVSCDGKVEHYRIMYHA  
SKL**SDEEVYFENLK**MQLVEHYTSDAD  
GLCTRLLIKPKVMEGTVAAQDEFYRSGW  
ALNMKEKLQLQTIGK**GEFGDVMLGDYR**  
GNKVAVKCIKNDATA...

Target 2-4  
peptides per  
protein



Rapidly establish multiplexed assay

# SAMPLE COLLECTION AND STORAGE VARIABLES



## 1. Type of collection tube

- Serum SST tube with gel and clot activator (red/grey top)
- Heparin tube (green top)
- EDTA tube (lavender top)
- P100
- EDTA tube (lavender top) with protease inhibitor cocktail added at the time of pipetting separated plasma

## 2. Variation in the 2 key bench times

- Before centrifugation
- After centrifugation but before pipetting and freezing
- 30 minutes, 2 days and 4 days
- 20°C or 37°C

## 3. Number of freeze-thaw cycles (up to 5)

## 4. Length of time in -20°C or -80°C storage

## 5. Cancer patients and age and gender-matched controls

- Prostate and breast cancer

# CONTROLLING A COMPLEX STUDY

## *Custom Tablet PC process control and monitoring*



**NCI Biospecimen Integrity Project - Sample Collector**

**Sample Tracking** Tb Study 1 - Sample Collected - Patient Has Departed      Current Time: **2:55:40 PM**

**Step 1: Arrange 5 draw tubes following this map, and scan draw barcodes on each tube:**

Pre-Label:	3	5	2	4	1
Tb #:	Tb 1	Tb 2	Tb 3	Tb 4	Tb 5
Draw Barcode:	571525 P9998HUA009_01P	571526 P9998HUA009_02P	571527 P9998HUA009_03P	571528 P9998HUA009_04P	571529 P9998HUA009_05P

**Step 2: Enter minutes since sample was drawn:**  **And click:**  **Tb started at:** **2:25:35 PM**

**Step 3: Enter Blood Level (cm):**

**Tb Timing - Blood Draw to Centrifugation**

Tb 1	Tb 2	Tb 3	Tb 4	Tb 5	
Required Blood Time:	30 Minutes	1 Hour	2 Hours	4 Hours	6 Hours
Count Down:	<b>00:04 OVER DUE</b>	<b>29:55</b>	<b>1:29:55</b>	<b>3:29:55</b>	<b>5:29:55</b>

When a timer is due (turns yellow), click button to proceed to centrifugation:

**Tp Timing Plasma Centrifugation to Pipetting**

Tp 1	Tp 2	Tp 3	Tp 4	Tp 5	
Required Plasma Time:	30 Minutes				
Count Down:	██████████	██████████	██████████	██████████	██████████

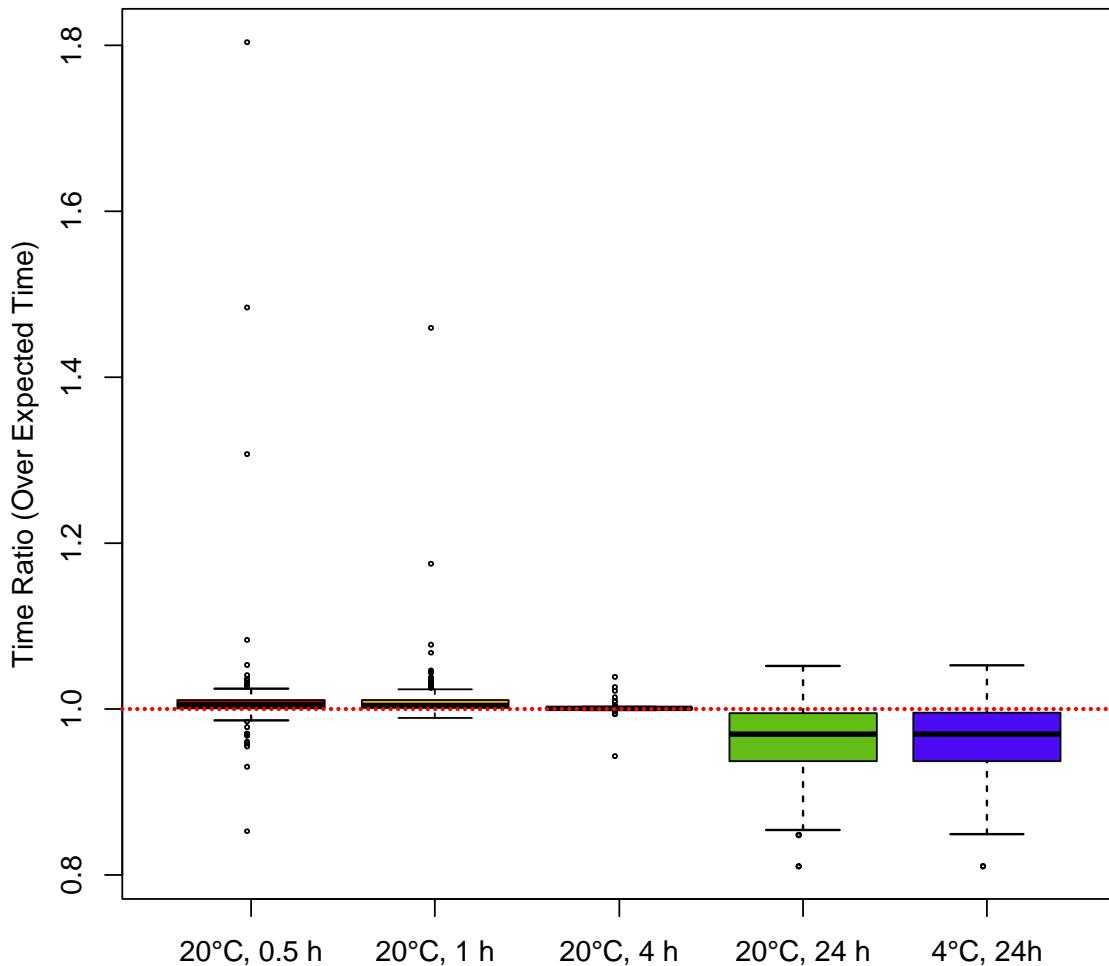
When a timer is due (turns red), click button to proceed to Pipeting:

Pipetting Done  Sample in Freezer  Pipetting Done  Sample in Freezer

Subject      Sample      Frozen Aliquot      Notes

# TABLET PC DATA ANALYSIS

## Variability of time on bench prior to centrifugation

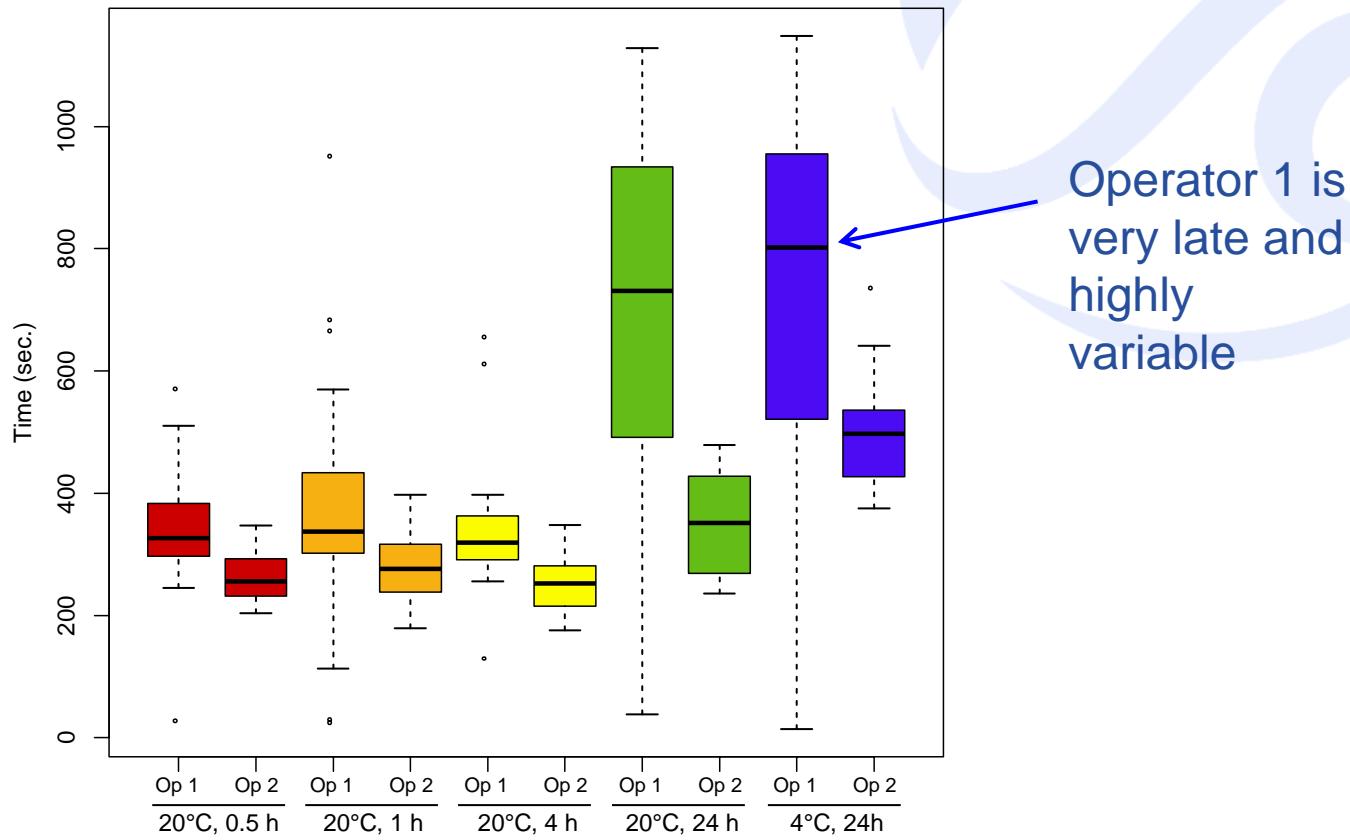


- Very close adherence to time points on first day
- Second day time points are off-set from protocol and more variable

Study conducted to assess compliance with pre-set time lines for sample processing by analysis of PC Tablet data

# TABLET PC DATA ANALYSIS

## Comparison of two operators

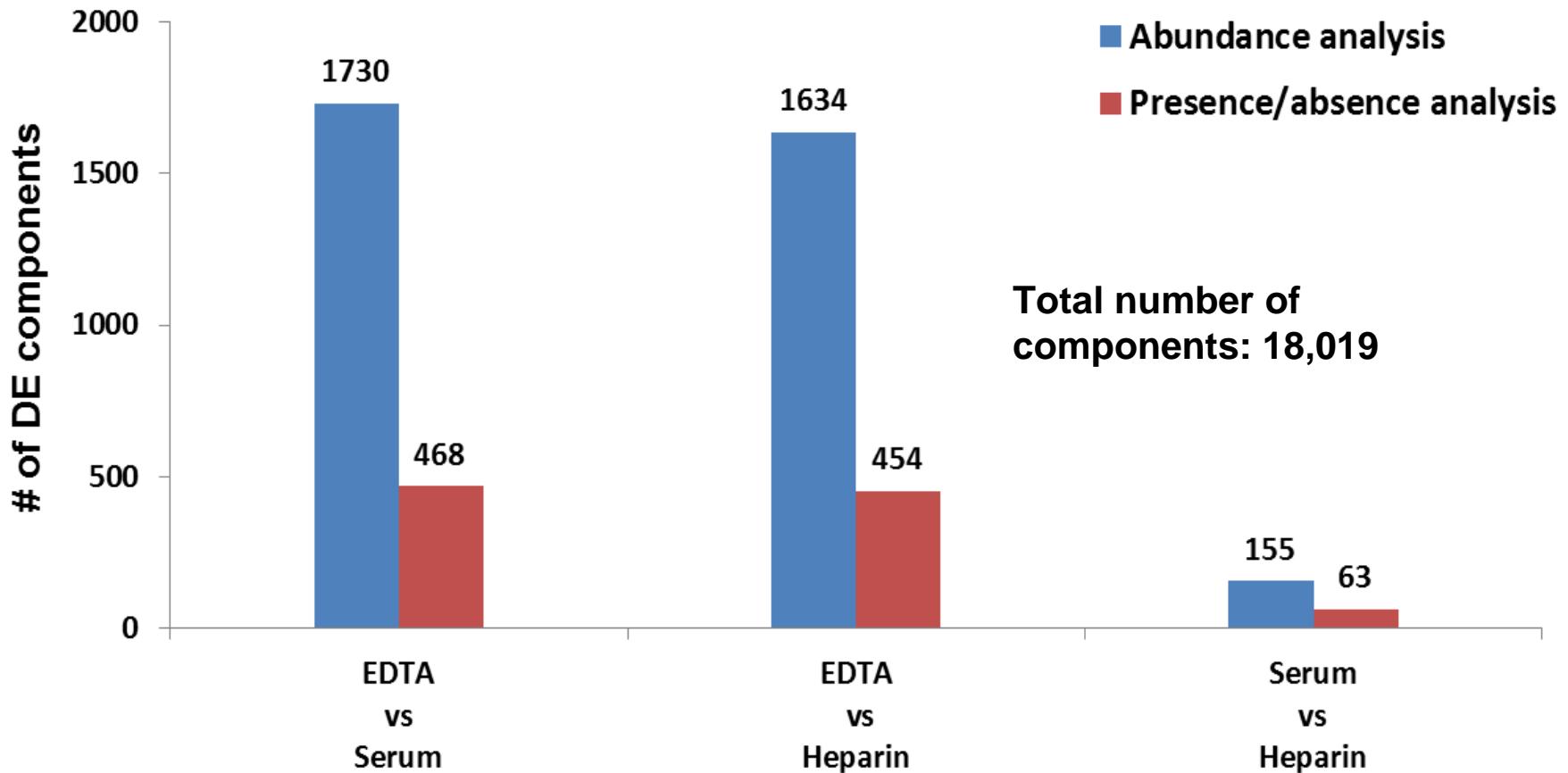


Operator 1 is  
very late and  
highly  
variable

Large difference in performance of 2 different operators

# COLLECTION TUBE COMPARISON

*Protein content differs between tube types*

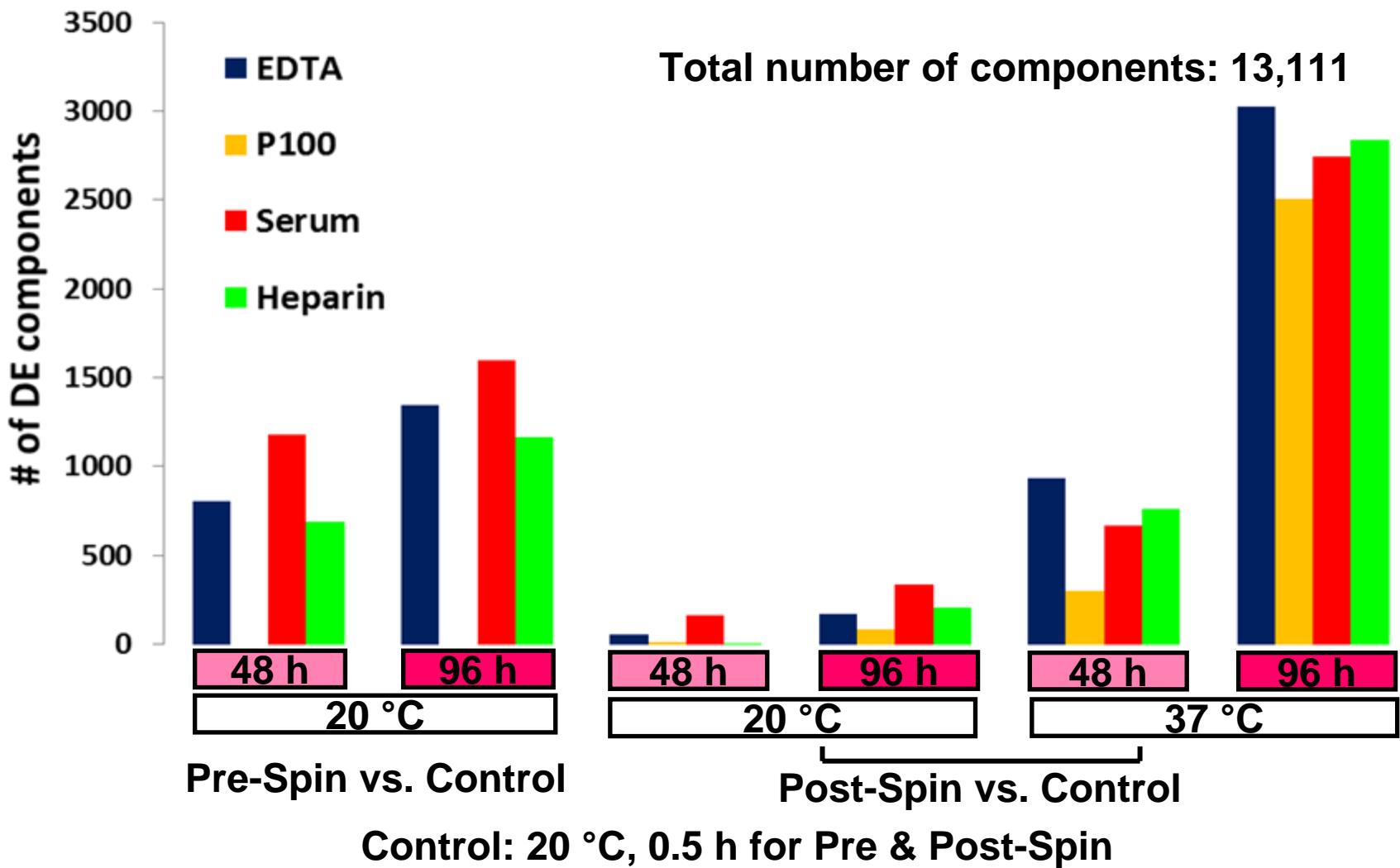


Major difference can be traced to lower protein concentration in EDTA tubes.  
May be due to effect of EDTA on cells prior to spin.

# EFFECT OF TIME AND TEMPERATURE ON BENCH STUDY DESIGN

- Pre-spin incubation for 0.5, 48 or 96 h
- Post-spin incubation for 48 or 96 h (after 0.5 h pre-spin incubation)
- 0.5 h incubations at 20°C
- 48 and 96 h incubations at 20 or 37 °C
- 4 tube types (EDTA, P100, Heparin, Serum)

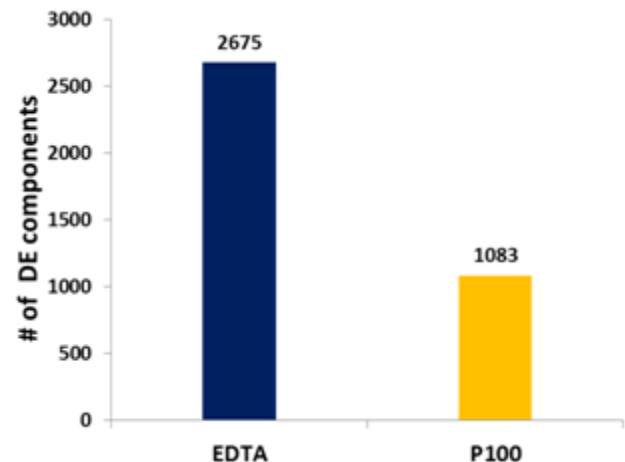
# TIME AND TEMPERATURE EFFECTS



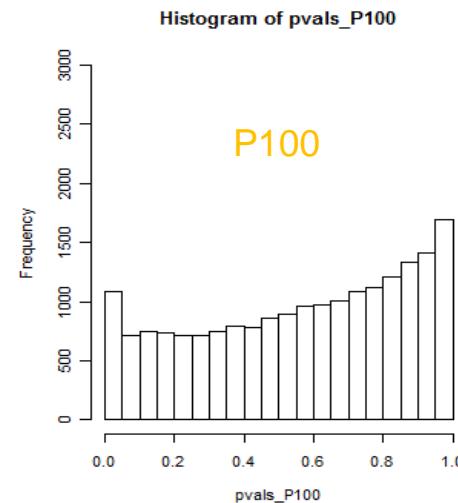
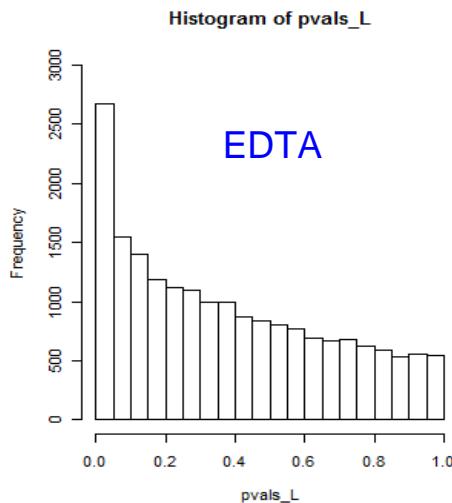
# IMPACT OF P100 TUBES (CONTAIN PROTEASE INHIBITORS)

P100 tube shows ~60% less changes than EDTA tube

Total components: 20,000



Number of components changing in intensity over time for each tube type

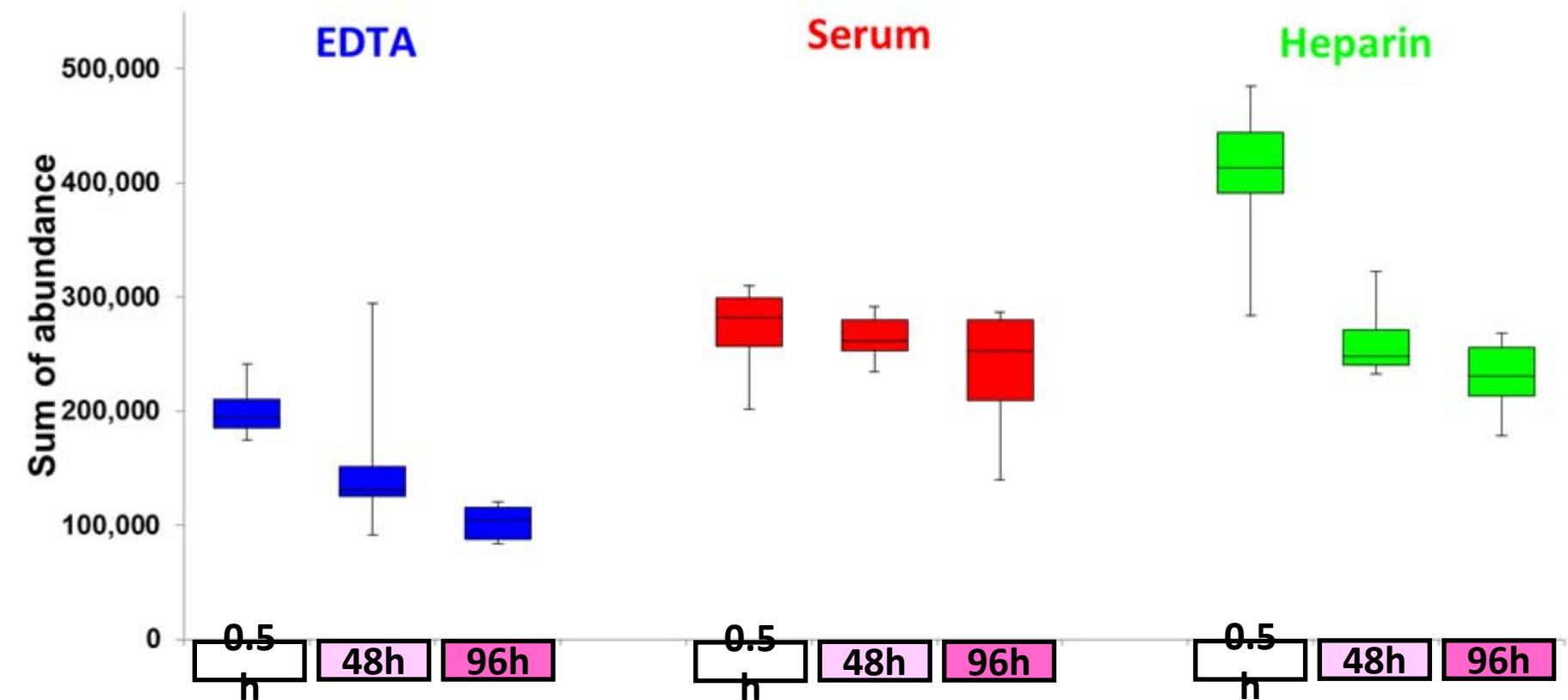


P-value distributions for differential expression over time

# DECREASING OVER INCUBATION TIME

## WHOLE BLOOD AT 20 °C

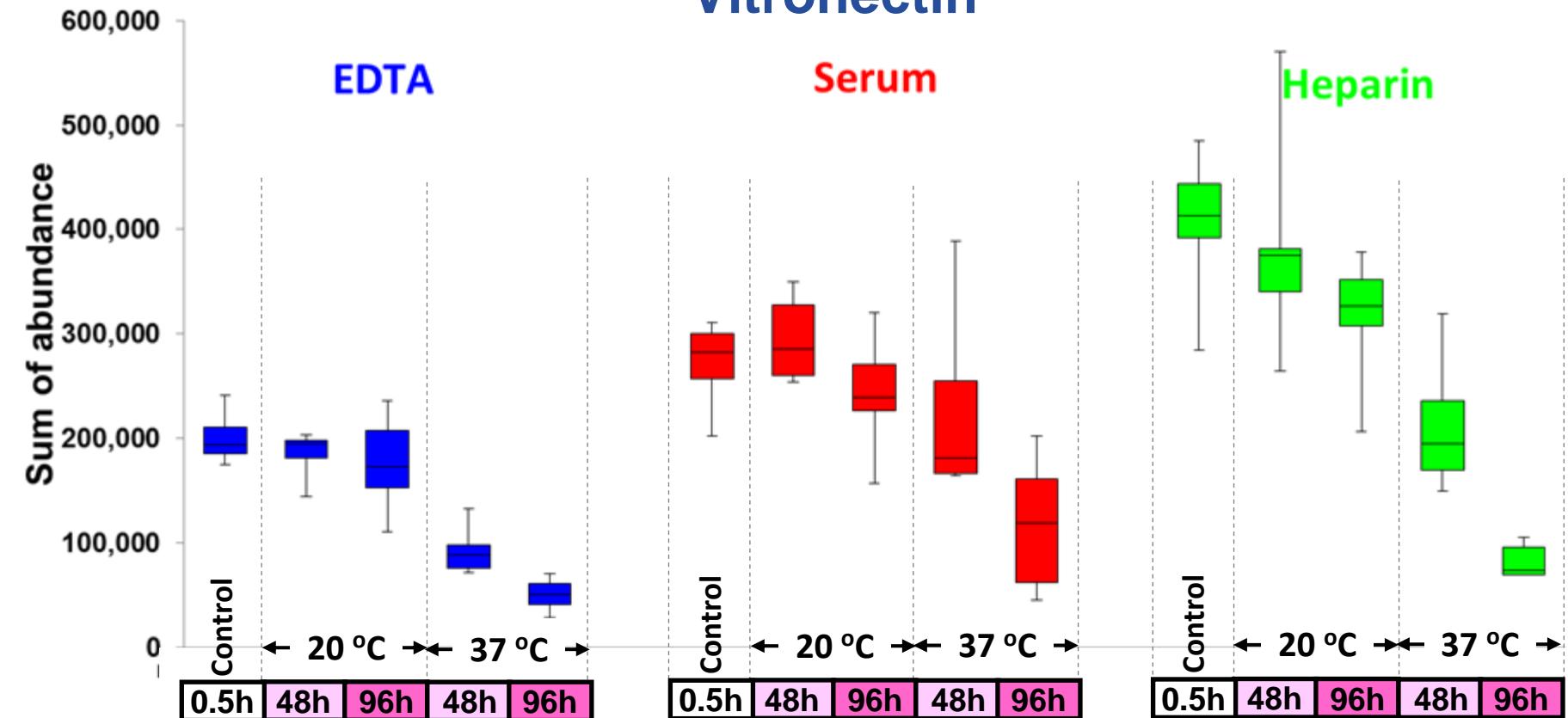
### Vitronectin



Identified unique peptides : 11

# DECREASING OVER INCUBATION TIME POST SPIN AT 20 AND 37°C

## Vitronectin



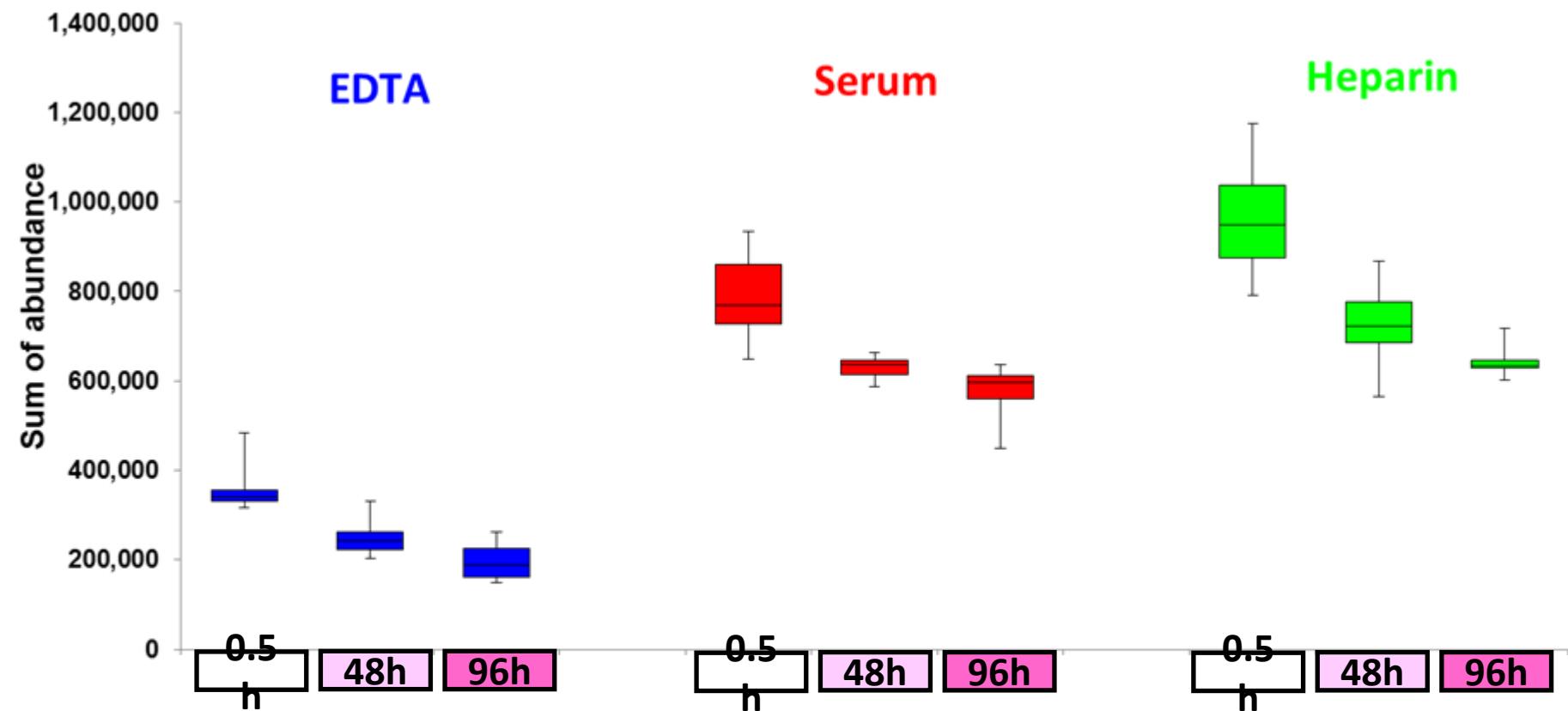
Control: 20 °C, 0.5 h for Post-Spin

Identified unique peptides : 11

# DECREASING OVER INCUBATION TIME

## WHOLE BLOOD AT 20 °C

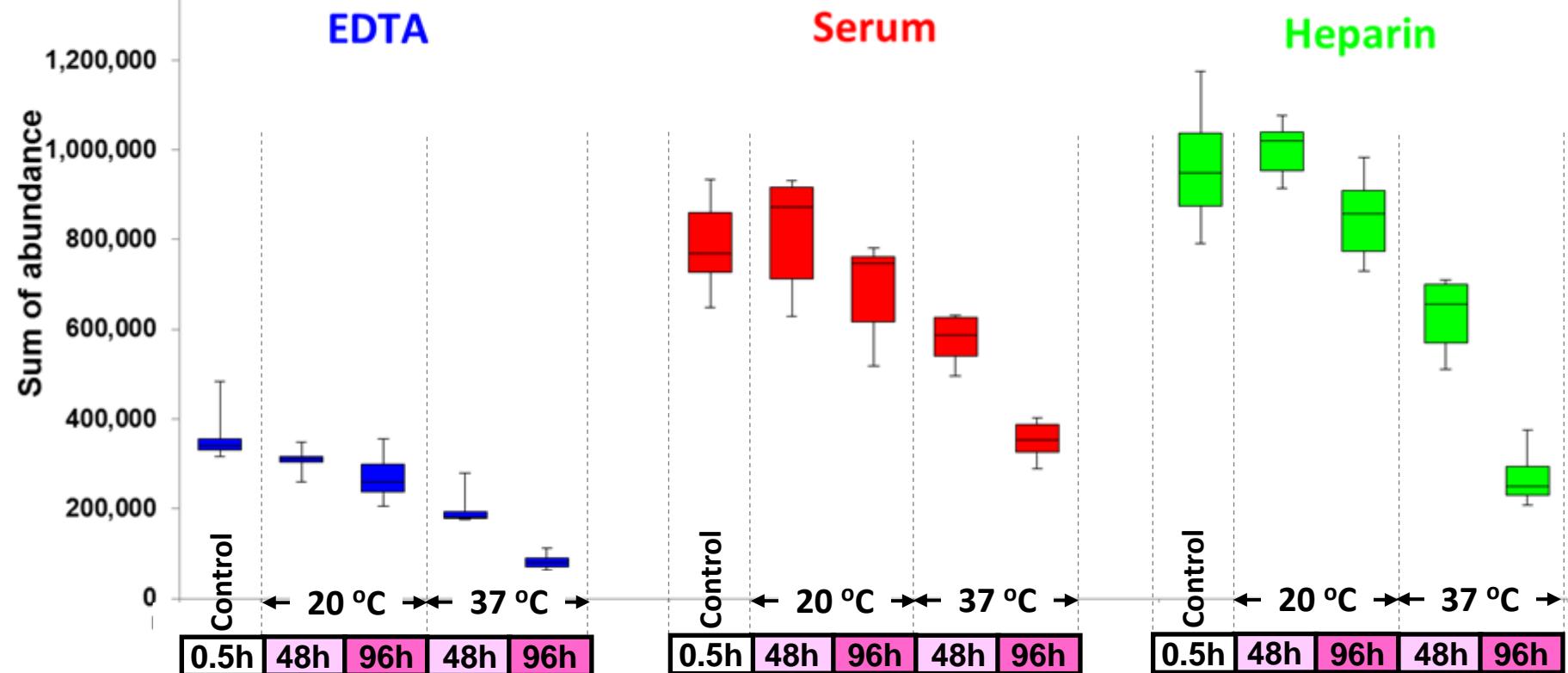
### Ceruloplasmin



Identified unique peptides : 18

# DECREASING OVER INCUBATION TIME POST SPIN AT 20 AND 37°C

## Ceruloplasmin

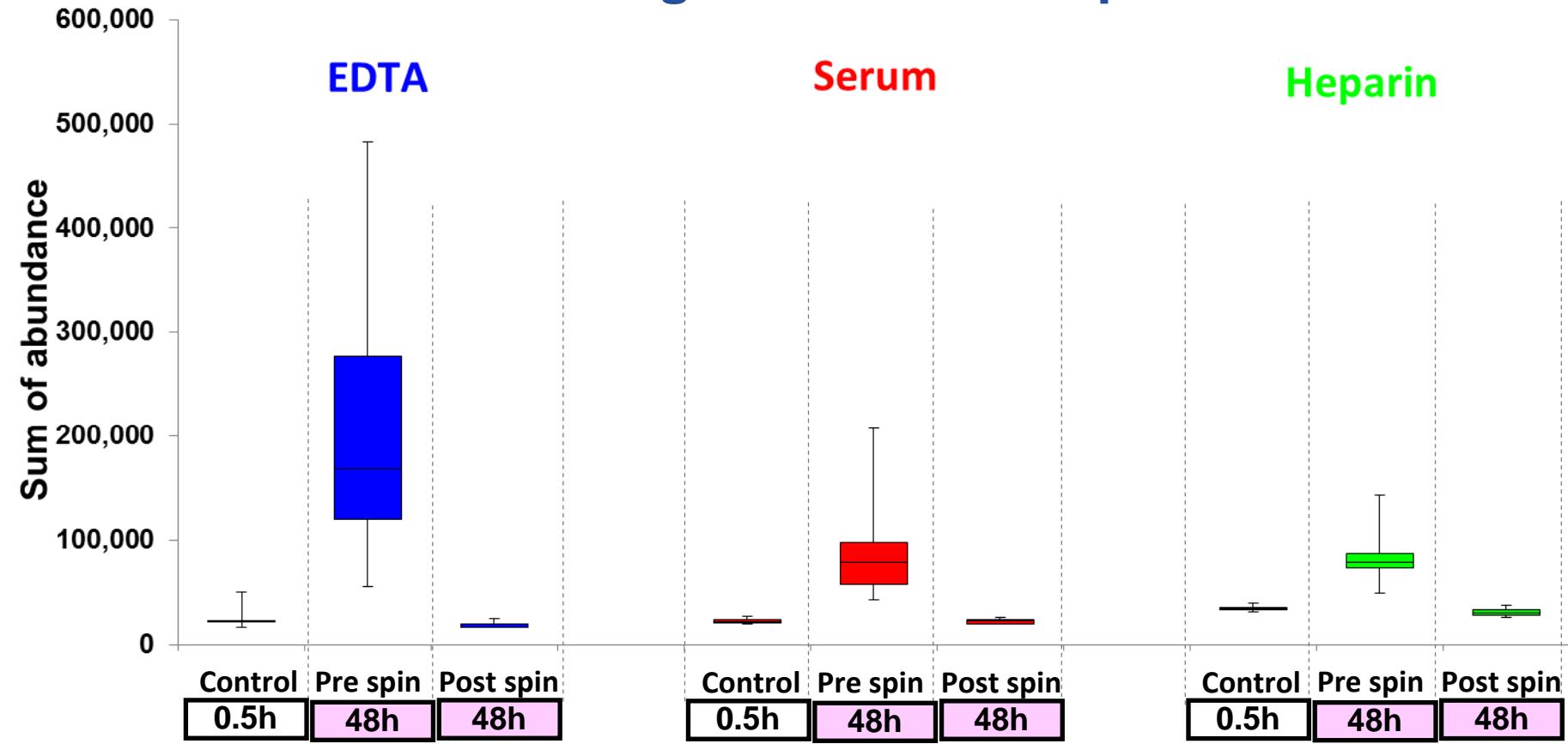


Control: 20 °C, 0.5 h for Pre & Post-Spin

Identified unique peptides : 18

# WHOLE BLOOD CELL LYSIS MARKER

## Hemoglobin subunit alpha

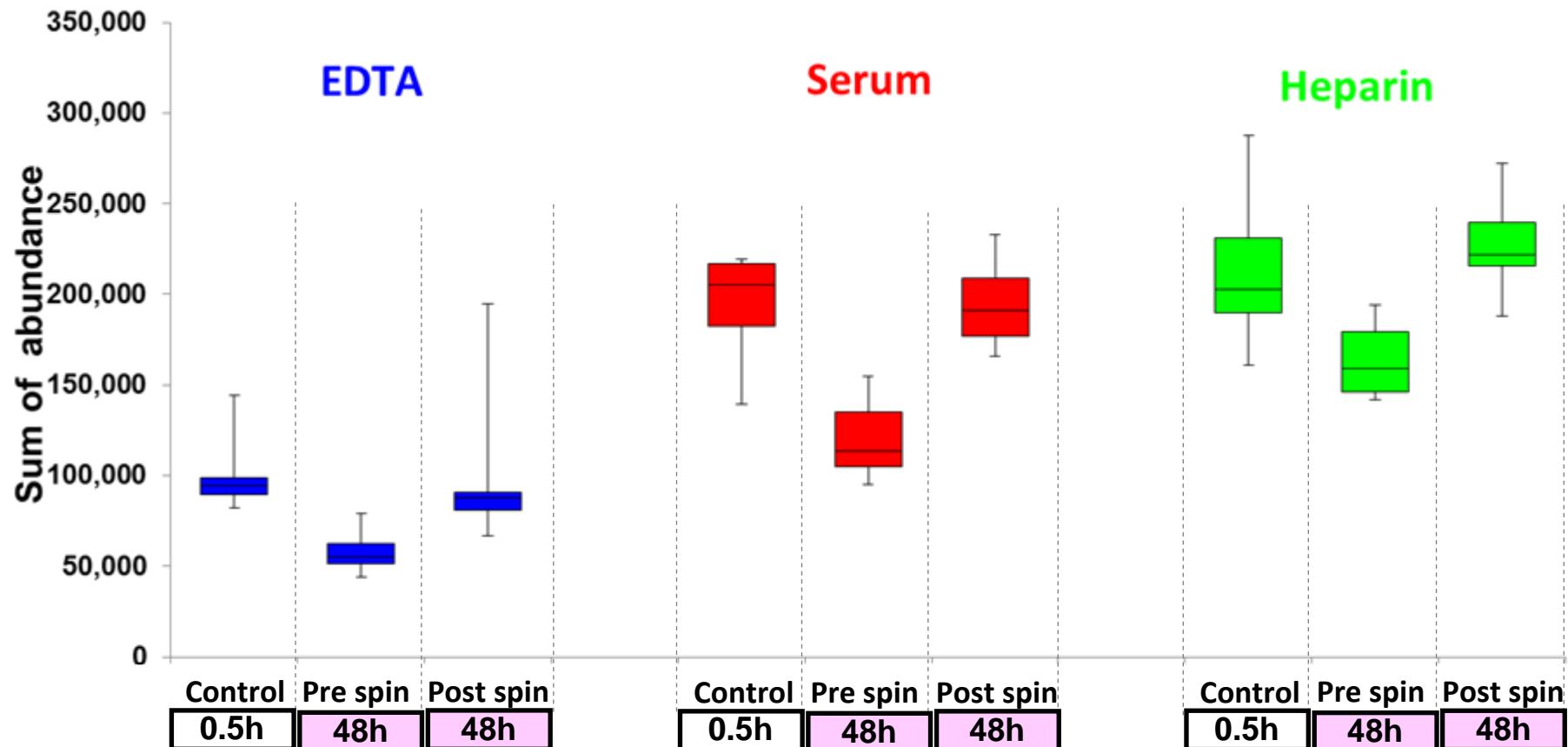


Control: 20 °C, 0.5 h for Pre & Post-Spin

Identified unique peptides : 9

# ADDITIONAL PRE-SPIN MARKER

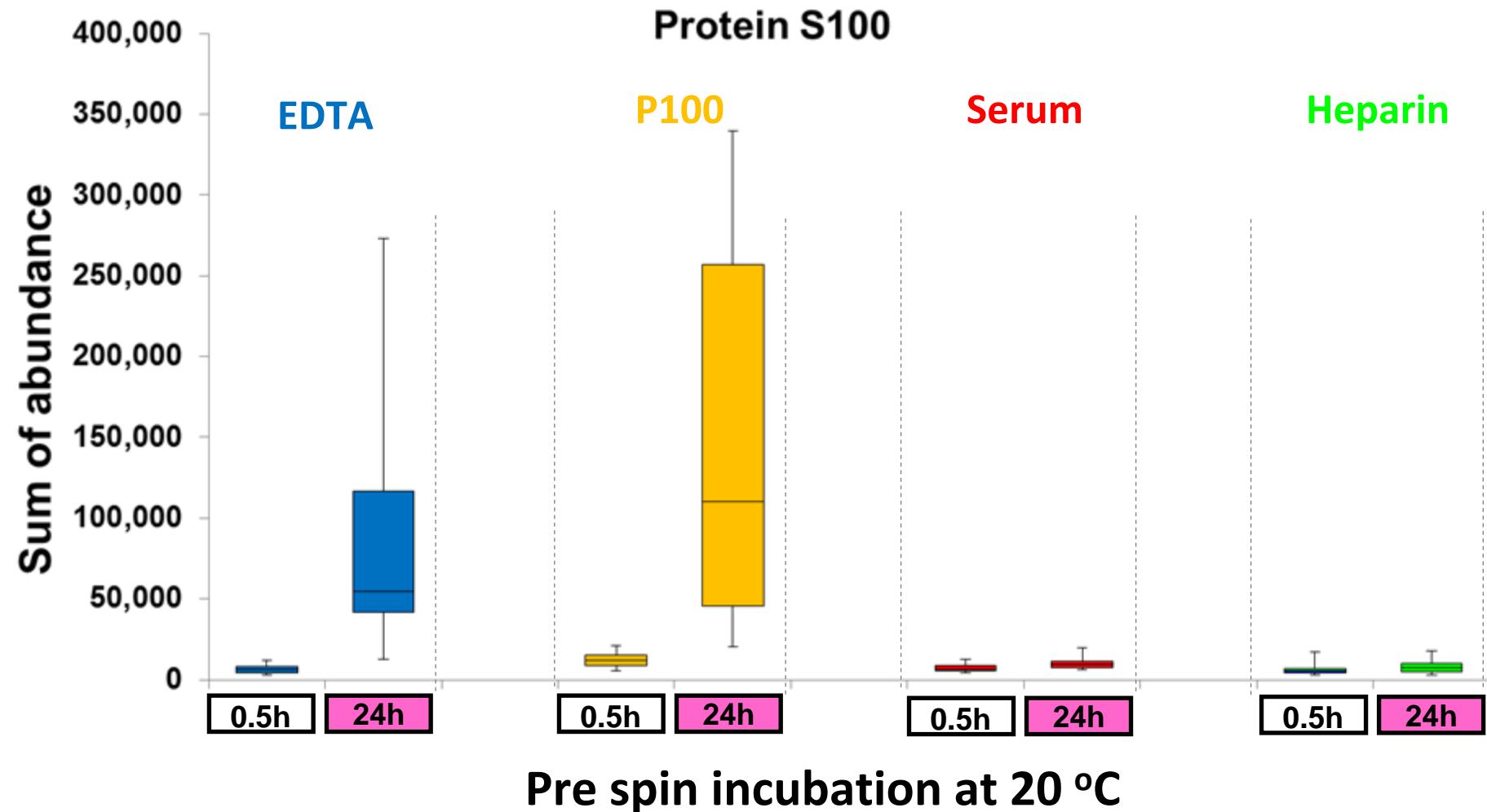
## Protein AMBP



Control: 20 °C, 0.5 h for Pre & Post-Spin

Identified unique peptides : 6

# EDTA TUBES AFFECT CALCIUM ION DEPENDENT LEUKOCYTE BINDING PROTEIN



Identified unique peptides : 4

# CONCLUSIONS

- Proteomic changes occur more rapidly in whole blood than in plasma or serum
- At least 7-10% of proteome changes in abundance (mostly down) during incubation of whole blood for 48 h.
- Only 1-2% of proteome changes in abundance during incubation of plasma or serum 48 h
- P100 tubes show protection of protein levels compared to other tubes
- Multiple markers of blood and plasma/serum storage identified

# FREEZE-THAW ANALYSIS

## Study Design

- **1-5 cycles tested**
  - **1 h at RT, 1 day in -80°C for each cycle**
  - **10 subjects, 4 tube types**

# FREEZE-THAW ANALYSIS DOWN-REGULATED PROTEINS

Protein intensity comparison:  
Each FT cycle vs. FT cycle 1

List of 4 down-regulated proteins  
Numbers indicate differential intensity

Uniprot_ID	Protein	2 vs 1	3 vs 1	4 vs 1	5 vs 1	Protein Description
FIBG_HUMAN	FGG	0.95	0.89	0.84	0.83	Fibrinogen gamma chain precursor
FINC_HUMAN	FN1	0.84	0.73	0.67	0.61	Fibronectin precursor
TSP1_HUMAN	THBS1	0.72	0.54	0.47	0.41	Thrombospondin-1 precursor
VWF_HUMAN	VWF	0.73	0.57	0.51	0.53	von Willebrand factor precursor

FN1: fibronectin

Known to precipitate with  
fibrinogen (FGA, FGB, FGC)  
and likely TSP1 and VWF on  
cooling

# FREEZE-THAW ANALYSIS

## UP-REGULATED PROTEINS

Protein intensity comparison:  
Each FT cycle vs. FT cycle 1

List of 4 up-regulated proteins  
Numbers indicate differential  
intensity

Uniprot_ID	Protein	2 vs 1	3 vs 1	4 vs 1	5 vs 1	Protein Description
ACTB_HUMAN	ACTB	1.24	1.43	1.54	1.66	Actin, cytoplasmic 1
ALBU_HUMAN	ALB	1.03	1.42	1.35	1.47	Serum albumin precursor
APOA1_HUMAN	APOA1	1.18	1.28	1.27	1.27	Apolipoprotein A-I precursor
TPM4_HUMAN	TPM4	2.16	3.00	3.69	4.25	Tropomyosin alpha-4 chain

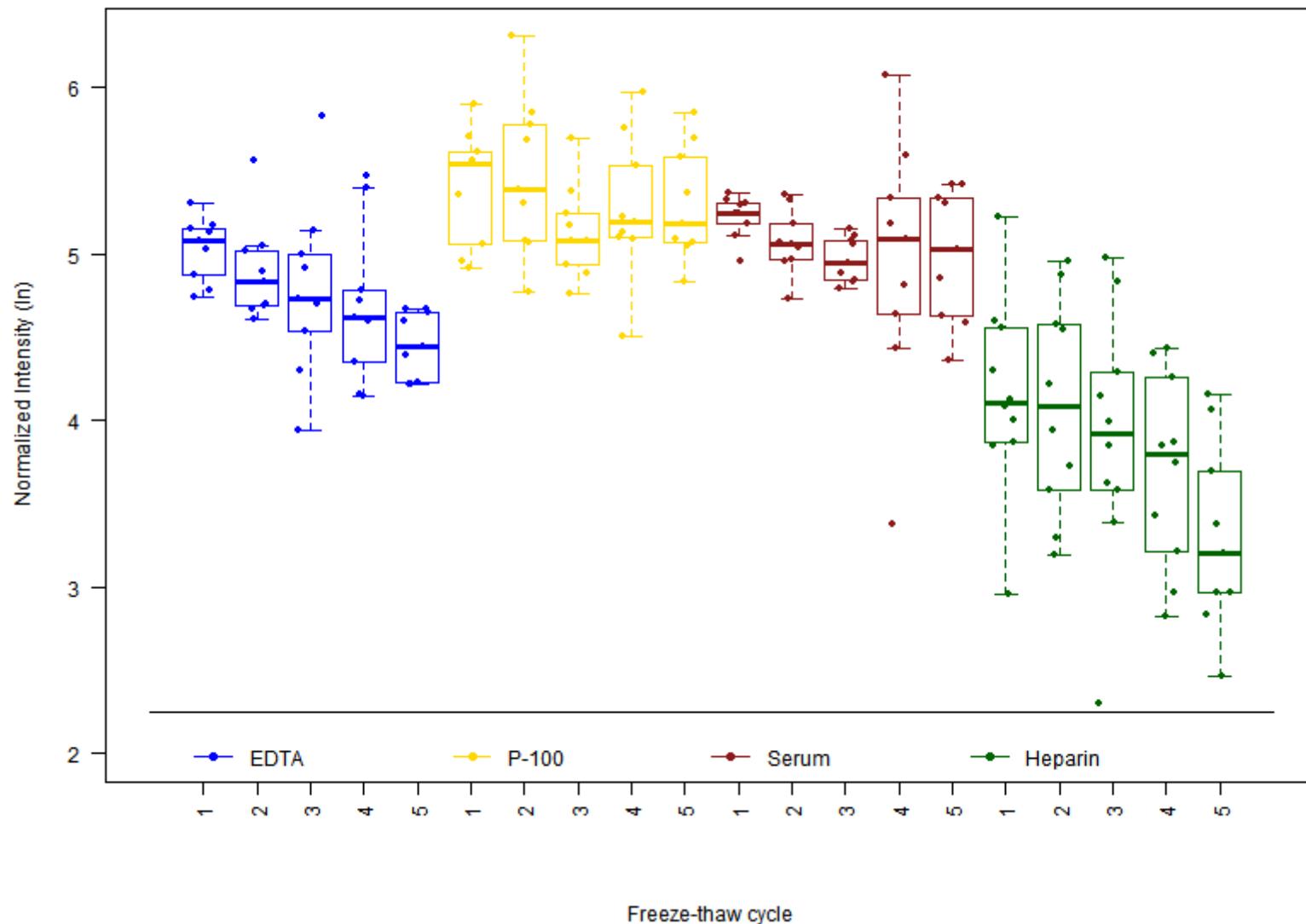
Actin B and TPM4 are likely due to lysis of contaminating cells

APOA1 and Albumin likely due to denaturation and reduced depletion by MARS14

# FREEZE-THAW ANALYSIS TUBE COMPARISON

## FIBRONECTIN

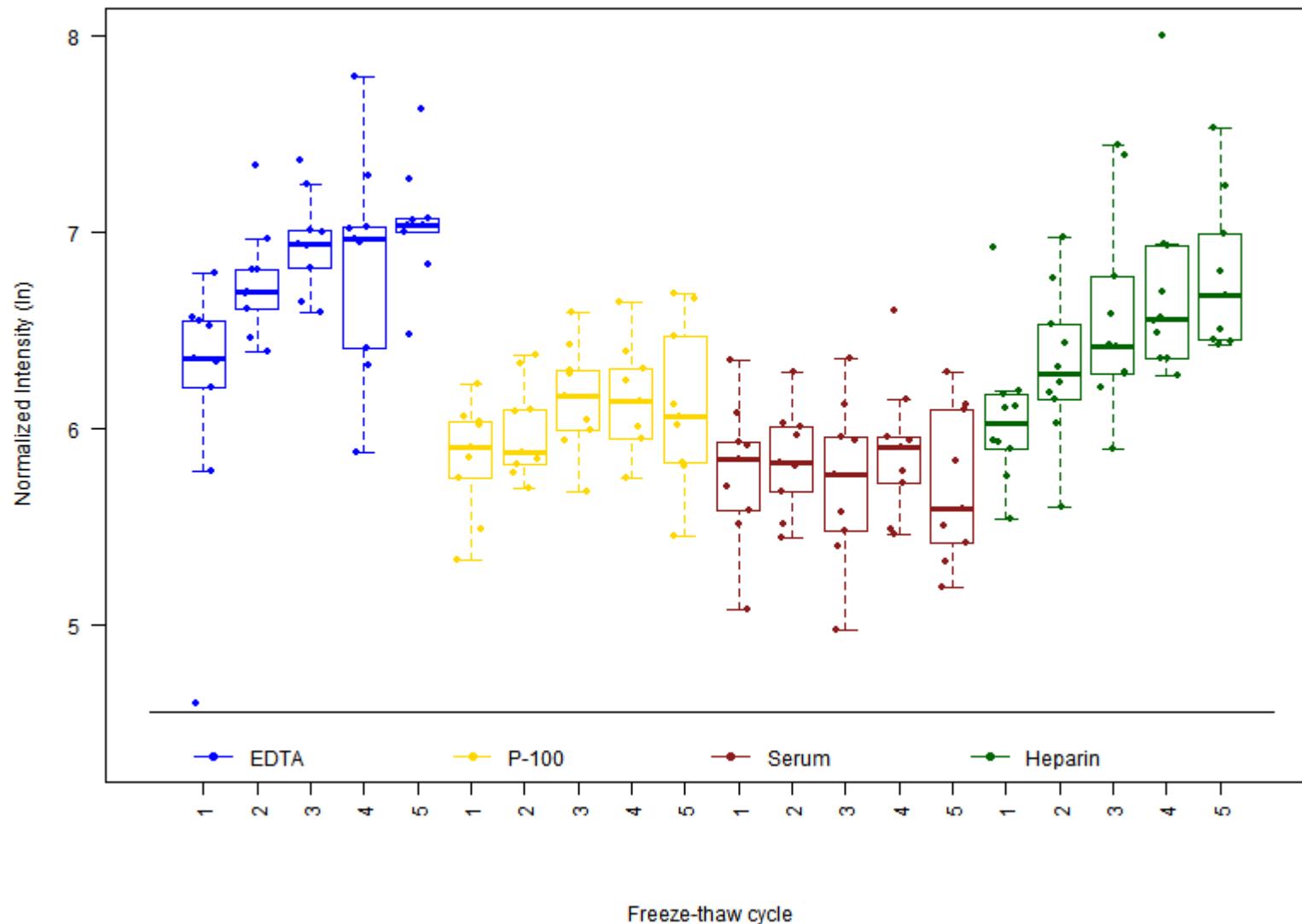
FN1-48514154



# FREEZE-THAW ANALYSIS TUBE COMPARISON

## ACTIN

ACTB-48508888



## FREEZE-THAW CONCLUSIONS

- 194 proteins identified (2,484 unique peptides)
- 8 proteins show significant change (p-value, q-value < 0.05; DI > 1.5)
- Number of changes observed increases with # freeze-thaw cycles
- Changes are evident even after 1 F/T
- Most changes are only significant (>1.5 fold change) after 4 F/T cycles

# ACKNOWLEDGMENTS

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**CAPRION  
PROTEOMICS**