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Credentialing Plasma and Serum Biospecimen Banks for Proteomics Analysis

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Quality Metrics for Plasma/Serum

- How does collection, processing, and storage affect the molecular integrity of a specimen?
- Define the most important preanalytical variables to control for protocol design
- Establish database of protein abundance changes to use as metrics of sample integrity
- Panel of markers for testing samples from existing repositories

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Quantitative Measures of Protein Integrity

- Protein abundances
- Peptide abundances
- Modifications, e.g. oxidations
- Protein aggregation

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Preanalytical Variables in Blood Collection and Processing

- **Inter- and intra-individual variation**
  - Age, gender, history, genetics
- **Venipuncture**
  - Needle gauge, butterfly needle, tubing, adapter type
- **Phlebotomy**
  - Tourniquet technique
  - Patient position, arm position
  - Tube order- first *vs* last, discard tube
- **Collection device**
  - Gel or non-gel separator tube
  - Tube additives, e.g. anti-coagulants or clot activator
  - Manufacturer & device information
  - Tube temperature
- **Blood processing**
  - Time and temperature pre- and post-centrifugation
  - Centrifugation: speed, duration, temperature
  - Protocol for separation of blood from cells
  - Length of time before freezing
- **Storage**
  - Frozen before analysis: snap-frozen, slowly cooled
  - Storage temperature
  - Storage time prior to analysis
  - Number of freeze/thaw cycles

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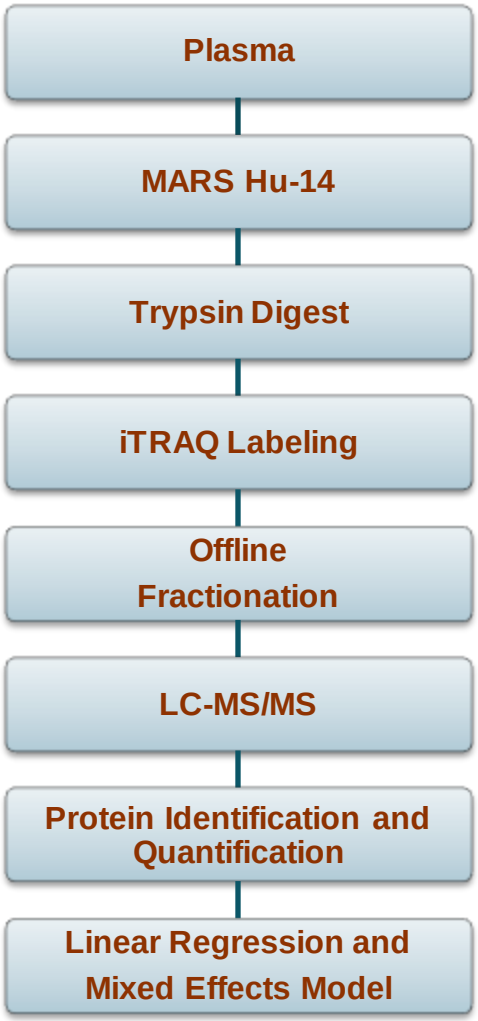
Comparative Proteomics

Assessing the impact of preanalytical variables at a global level



- Untargeted 'biomarker' discovery
- Immunodepletion of abundant plasma/serum proteins
- iTRAQ-8plex labeling for multiplexed relative quantification
- Standard protocols with QA processes

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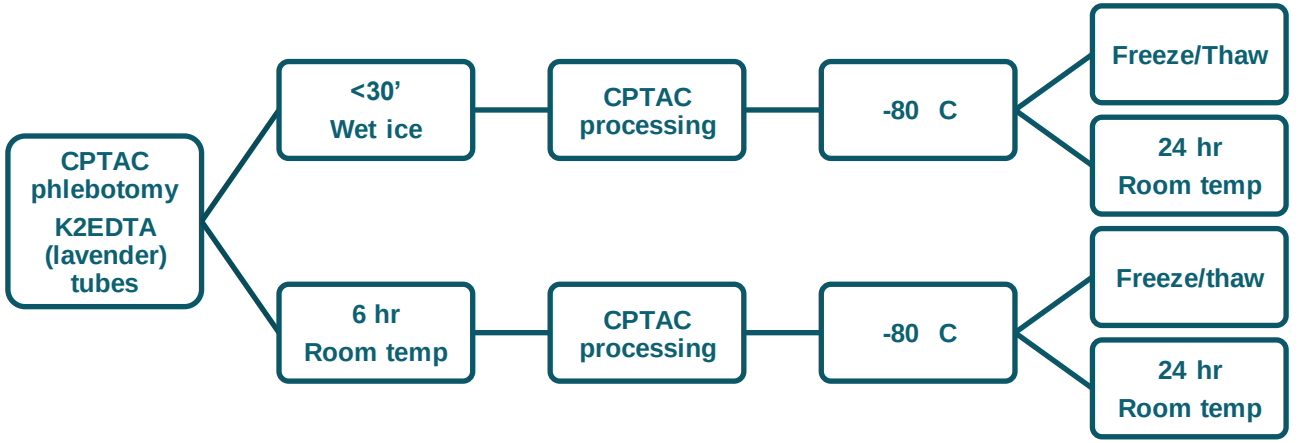
Study 1
Blood Collection and Processing
Effects of time and temperature

- 20 plasma samples
  - Healthy donors aged 20-40
- Two time points
  - 6 hours at room temperature prior to centrifugation
  - 24 hours at room temperature after thaw
- 2 freeze thaw cycles



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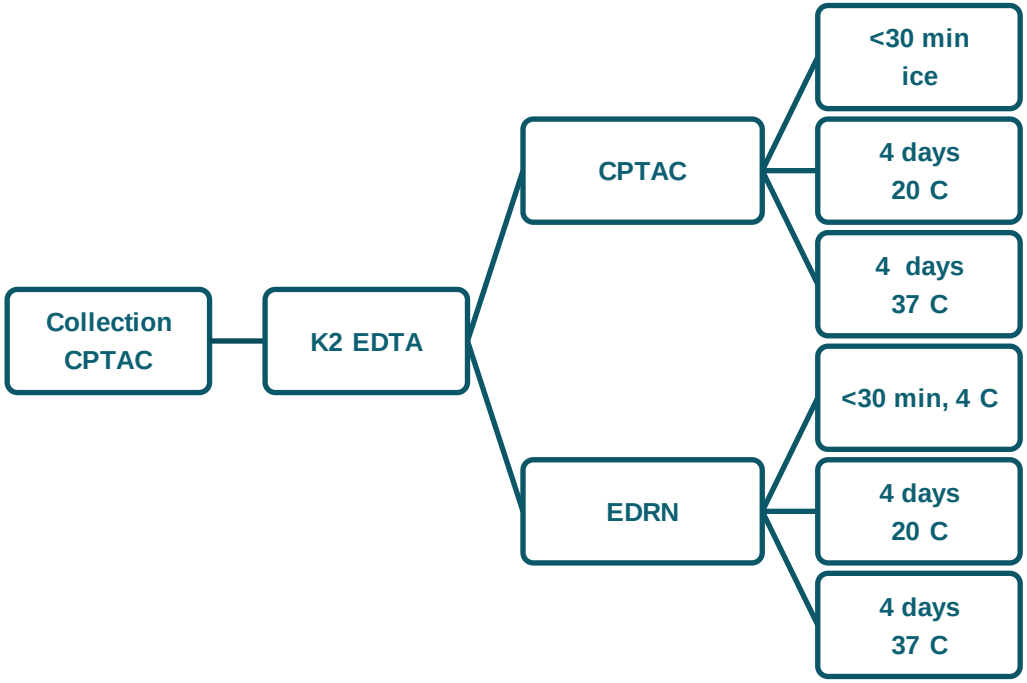
Study 1 Summary

Effects of time and temperature on plasma proteins

- No changes in total protein concentration
  - Pierce BCA Assay
- No proteins with significantly altered abundances using linear regression analysis
- Mixed effects model revealed minimal changes in plasma proteins at 6 hr pre-centrifugaton or 24 hr post-thaw
- No significant changes after 2 freeze thaws
- Under these conditions, proteins found in MARS depleted EDTA plasma appeared to be stable.
- Extending the time and temperature prior to processing may yield useful markers of sample integrity

Study 2
Blood Collection and Processing
Extended time and temperature prior to processing

- 4 days at 20 C and 37 C prior to centrifugation
- EDRN and CPTAC plasma processing



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Standardized Processing Protocols

Remove bias in the collection and processing of patient samples

- **CPTAC**

- Anticoagulant = EDTA
- Collection tubes pre-chilled on ice
- Holding temperature = ice
- Two centrifugation steps
  - 1500 x*g* @ 4 C → transfer plasma to fresh tube→ 2000 x*g* @ 4 C
- Freeze -80 C

- **EDRN**

- Anticoagulant = EDTA
- Collection tube temperature not specified
- Holding temperature = 4 C
- Single centrifugation
  - 1200 x*g* @ room temperature
- Freeze -80 C

http://proteomics.cancer.gov/
http://edrn.nci.nih.gov/resources/standard-operating-procedures/standard-operating-procedures/plasma-sop.pdf
J Proteome Res. 2009 January; 8(1): 113–117

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Study 2

EDRN and CPTAC plasma processing
4 days at 20 C and 37 C prior to centrifugation

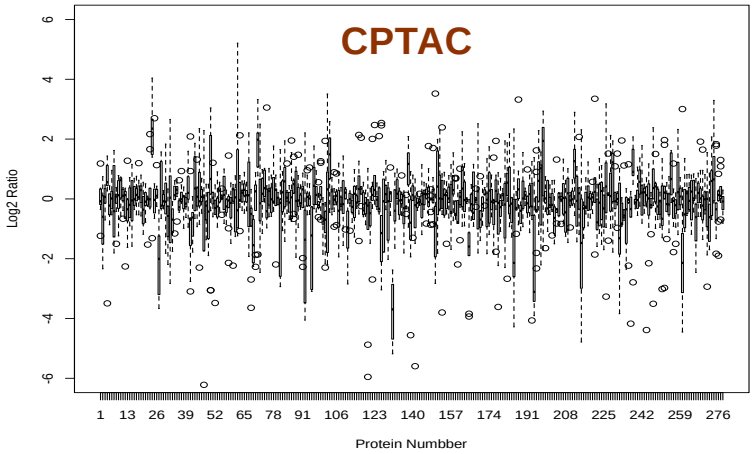
- **Data from multiple experiments normalized to a pool of samples**
  - CPTAC processing
- **First, compared each preanalytical and processing variable to an individuals own CPTAC SOP processed sample**
  - Mixed effect model using donor as a random effect
  - Corrected for multiple hypothesis testing using BH FDR
- **Protein filtering**
  - ≥5 samples
  - unused protscore ≥1

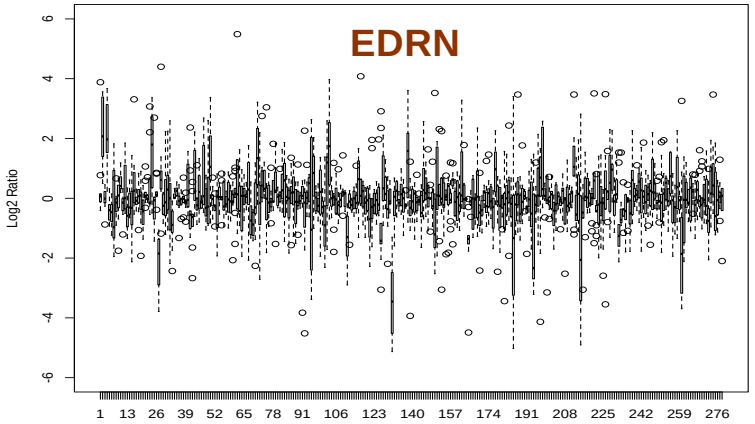
EDRN plasma compared to CPTAC plasma

No significant differences in protein abundances

Processing:
1. EDRN
2. CPTAC

Treatment:
1. SOP
2. 20 C 4 days
3. 37 C 4 days





sorted by fdr_p value

1. Box plots show relative protein abundance ratios to the pooled CPTAC standard.
   - The p-value was generated by a mixed effects (ME) model treating processing and treatment as fixed effects and donor as random.
2. ME model with processing as fixed and time and donor both random:
   - No significant proteins found
3. ME model: fixed effect was processing, random was donor, taking time points separately:
   - No significance for any of the 3 time points.
4. No proteins found at extreme ratios, i.e. in only one sample type.

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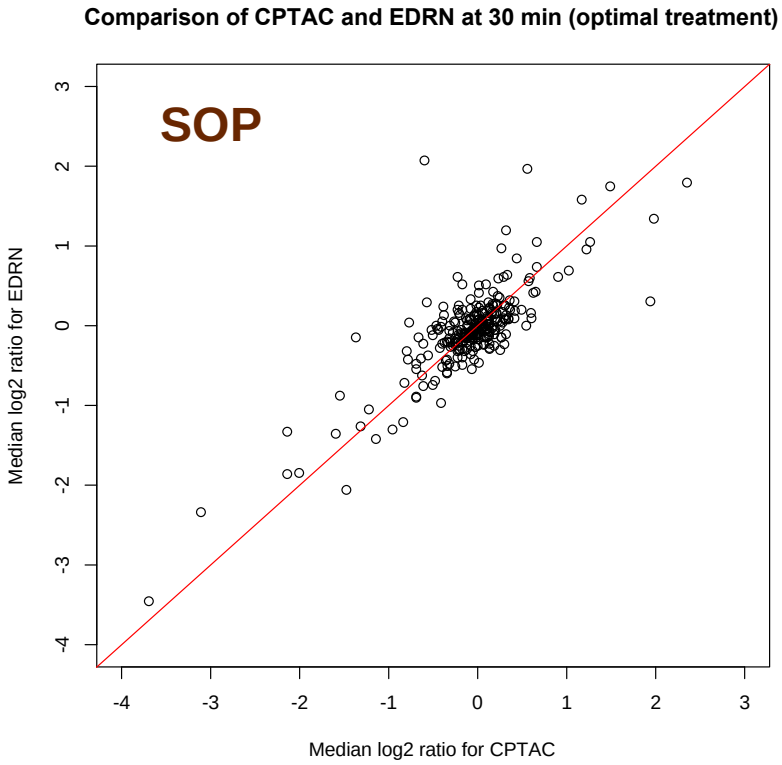
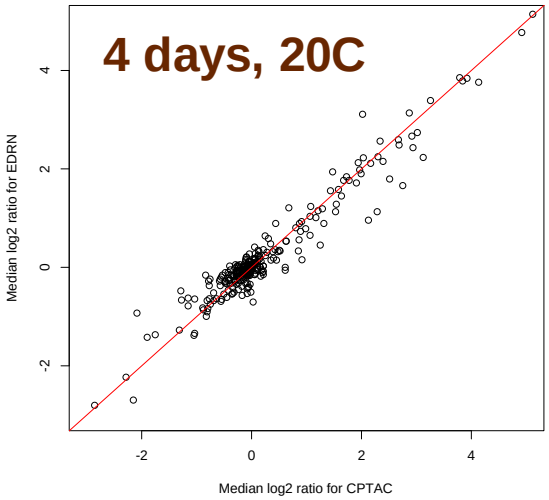
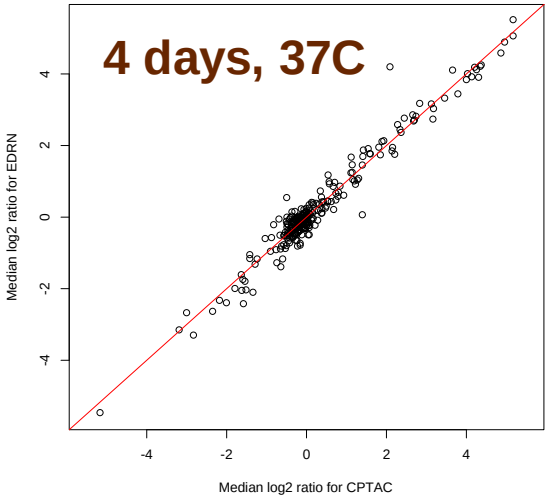
Plasma Processing Effects

EDRN and CPTAC plasma processing

Median protein ratio for all proteins across all donors





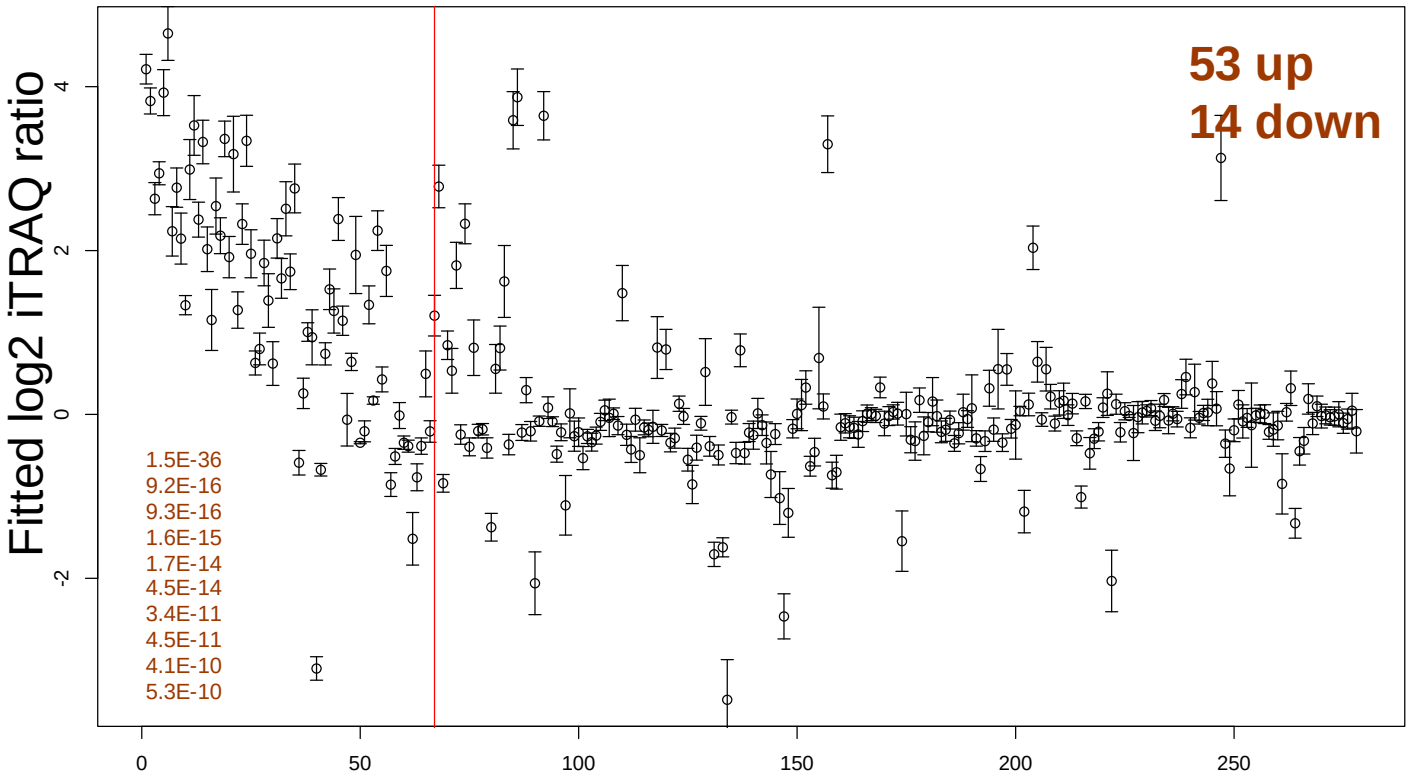


Protein Abundance Changes at 4 days, 20 C

Processing:
1. EDRN
2. CPTAC

Treatment:
1. SOP
2. 20 C 4 days
3. 37 C 4 days

Proteins ranked by fdr_p value
red line, p=0.05



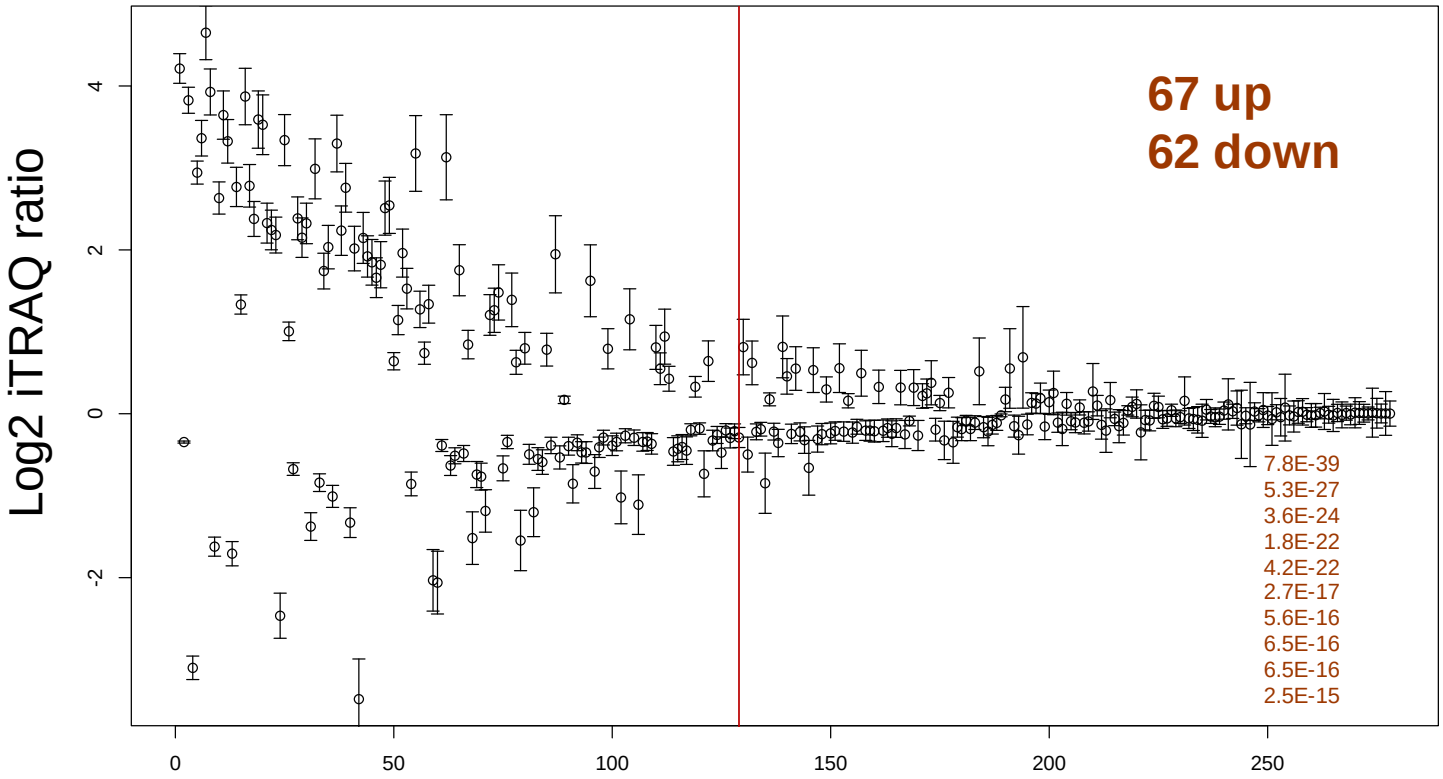
ME with processing and treatment as fixed effect and donor as random

Protein Abundance Changes at 4 days, 37 C

Processing:
1. EDRN
2. CPTAC

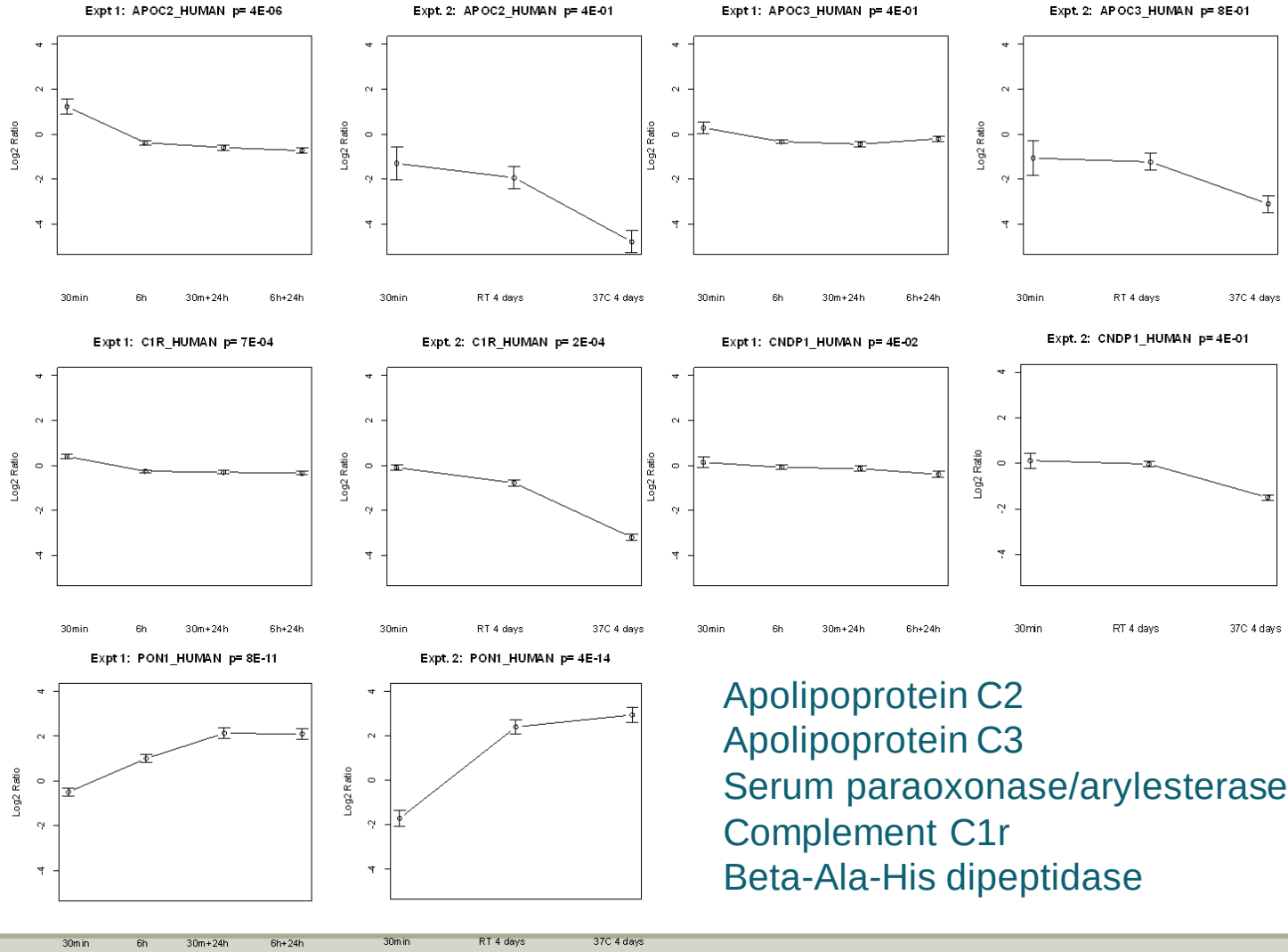
Treatment:
1. SOP
2. 20 C 4 days
3. 37 C 4 days

Proteins ranked by fdr_p value
red line, p=0.05



ME with processing and treatment as fixed effect and donor as random

5 Proteins found in both Study 1 and Study 2



Apolipoprotein C2
Apolipoprotein C3
Serum paraoxonase/arylesterase 1
Complement C1r
Beta-Ala-His dipeptidase

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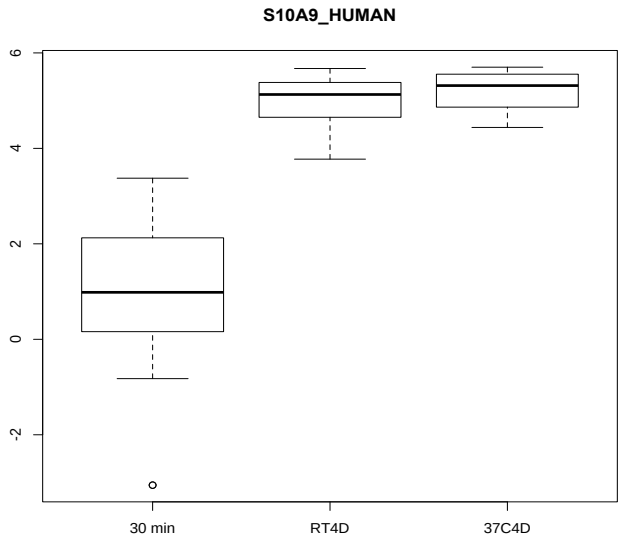
Developing analytical assays for protein integrity

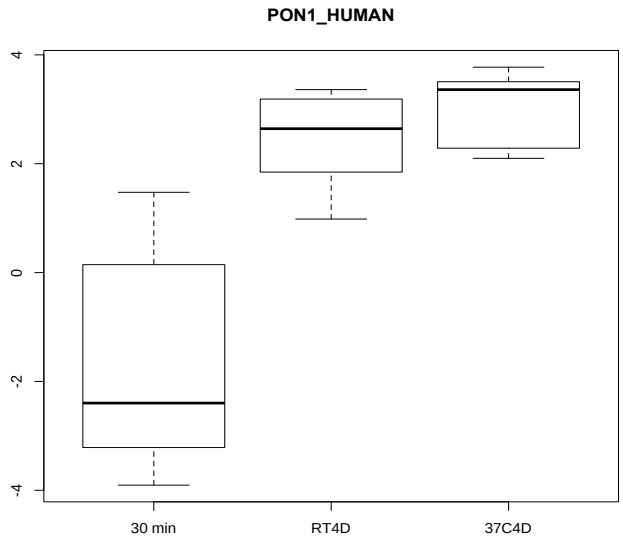
- **Previous analyses were done using a mixed-effects model treating donor as a random effect**
  - This treatment removes the inter-donor variation for each peptide or protein.
  - Possible because all time and temperature treatments were applied to all donors, allowing pairing of SOP and other treatments.
- **Reanalyze without donor grouping**
  - Apply a simple regression model, i.e. not treating donor as a random effect.
  - Less sensitive: observe changes larger than the inter-donor variability.
- **Challenge for sample bank testing**
  - There will not be a comparator/standard from each individual
  - Abundance ranges

Preanalytical changes due to treatment that are greater than the inter-donor variability

41 proteins at 20 C, 4 day
85 proteins at 37 C, 4 day
39 found in both

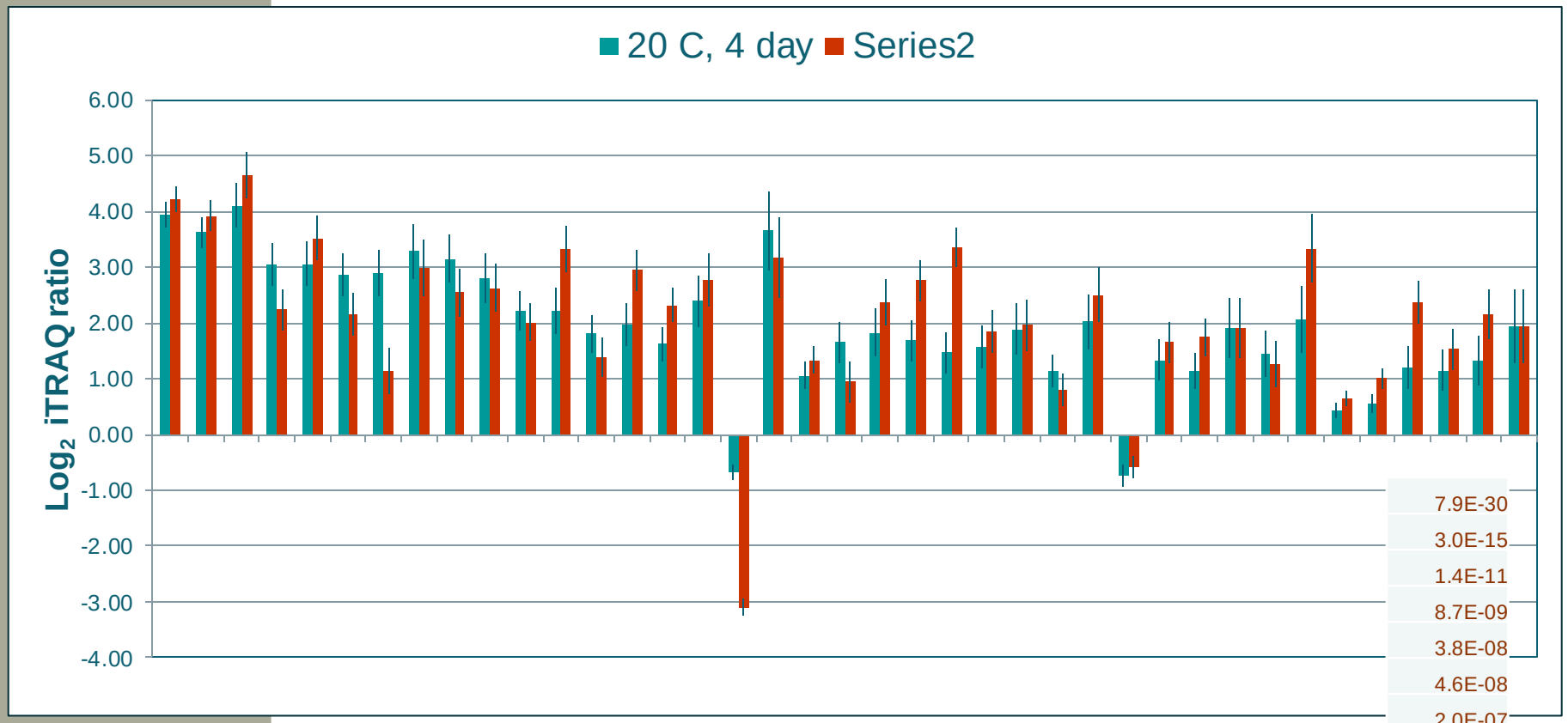
Treatment:
1. SOP
2. 20 C 4 days
3. 37 C 4 days





Significant protein abundance changes

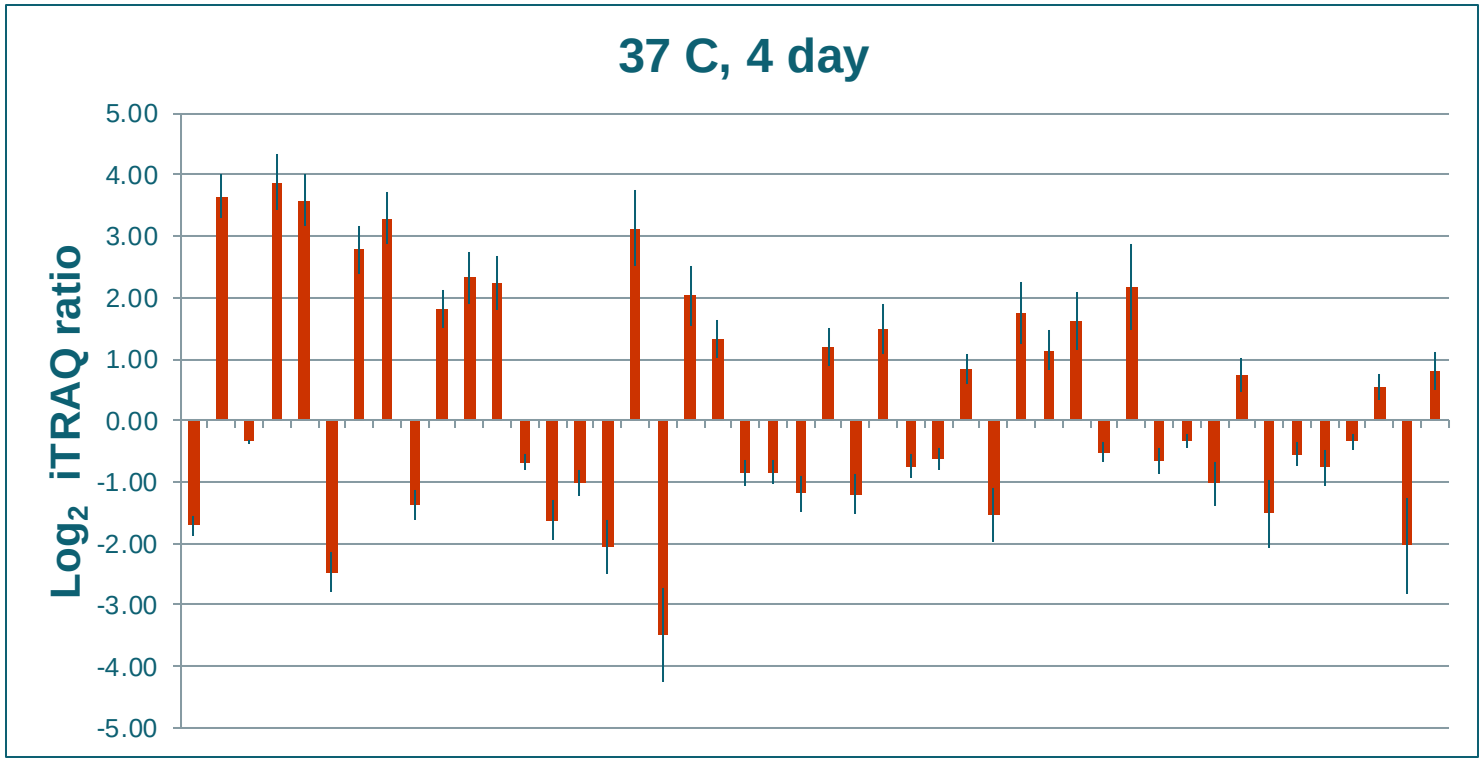
Proteins found in both 20 C and 37 C samples



Linear regression model, effects at 4d20 and 4d37
Proteins sorted by fdr_p value of 4d20 proteins

Significant protein abundance changes

Proteins found only in 37 C samples



4.9E-32
6.4E-17
1.1E-13
4.0E-06
3.7E-10
8.9E-06
2.8E-02
2.6E-06
2.1E-05
3.4E-06

Linear regression model, effects at 4d37
Sorted by fdr_p value

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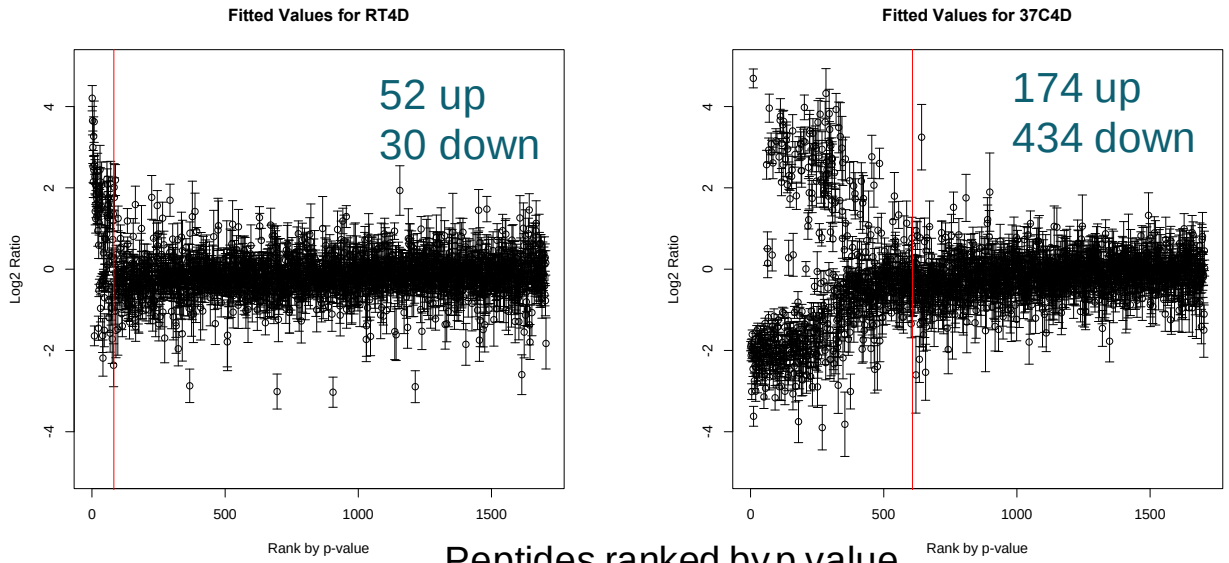
Study 2 Peptide Analysis

- **Peptide Filtering Criteria**
  - ≥ 95% confidence in ID
  - PTMs and semi/non-tryptics included
  - Ratios >0.01 and <100
  - Must have such ratios for at least 5 donors for all 6 treatments and processing groups
- **Mixed effects model and linear regression analyses with multiple testing correction**

Changes in Peptide Abundances 4 Days Prior to Plasma Processing



Peptides ranked by p value
red line, p=0.05

- 76% of peptides were mapped to proteins identified as changing in 20 C, 4 day samples, all in same direction.
- 51% were found in proteins we identified as changing in 37C 4D, 99% in the same direction.
- Peptides not matched to changing proteins: What are they? N- or C-terminal peptides? Modifications?

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Long term storage effects

- **UCSF AIDS Bank**
  - 2 aliquots of serum from 25 individuals
  - -80 C for ~27 years
  - One of each pair had been thawed and refrozen
- **Magee Women's Research Institute**
  - 2 samples from 25 individuals, plasma and serum
  - -80 C for 15-18 years
  - Thawed and refrozen up to 4 times
  - Recently collected samples using same protocol

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Summary

- No significant changes in protein and peptide abundances were seen between MARS-depleted plasma samples processed using the EDRN and CPTAC protocols.
- Only a few proteins and peptides were found with altered abundance at 'shorter' time points
  - 6 hrs prior to plasma processing
  - 24 hr after plasma thaw
- Many proteins were found with significant changes in abundance in samples held for 4 days prior to plasma processing.
  - 41 proteins at 20 C and 85 proteins at 37 C
  - Some are known to be non-specifically immunodepleted
    - Targeted approach using undepleted plasma
- Markers of long term storage in progress. Appears to be some overlap with processing markers.

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