Credentialing Plasma and Serum Biospecimen Banks for Proteomics Analyses

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Factors affecting protein integrity for biomarker research

- Proteolysis
- PTMs
  - Phosphorylation
  - Glycosylation, N-linked and O-linked
  - Oxidation
    - cysteine disulfides, methionine sulfoxide, carbonyls, nitrotyrosine, cross links
  - Nitration
  - Acetylation
  - Methylation
  - Acylation
  - Sulfation
- Aggregation and precipitation

Serum & Plasma Pre-analytical Variables

- Patient information
  - 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 prior to centrifugation
  - Centrifugation: speed, duration, temperature
  - Protocol for separation of blood from cells
  - Aliquotting before analysis, time and temperature
  - 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

Modified from Rai, et al. 2005

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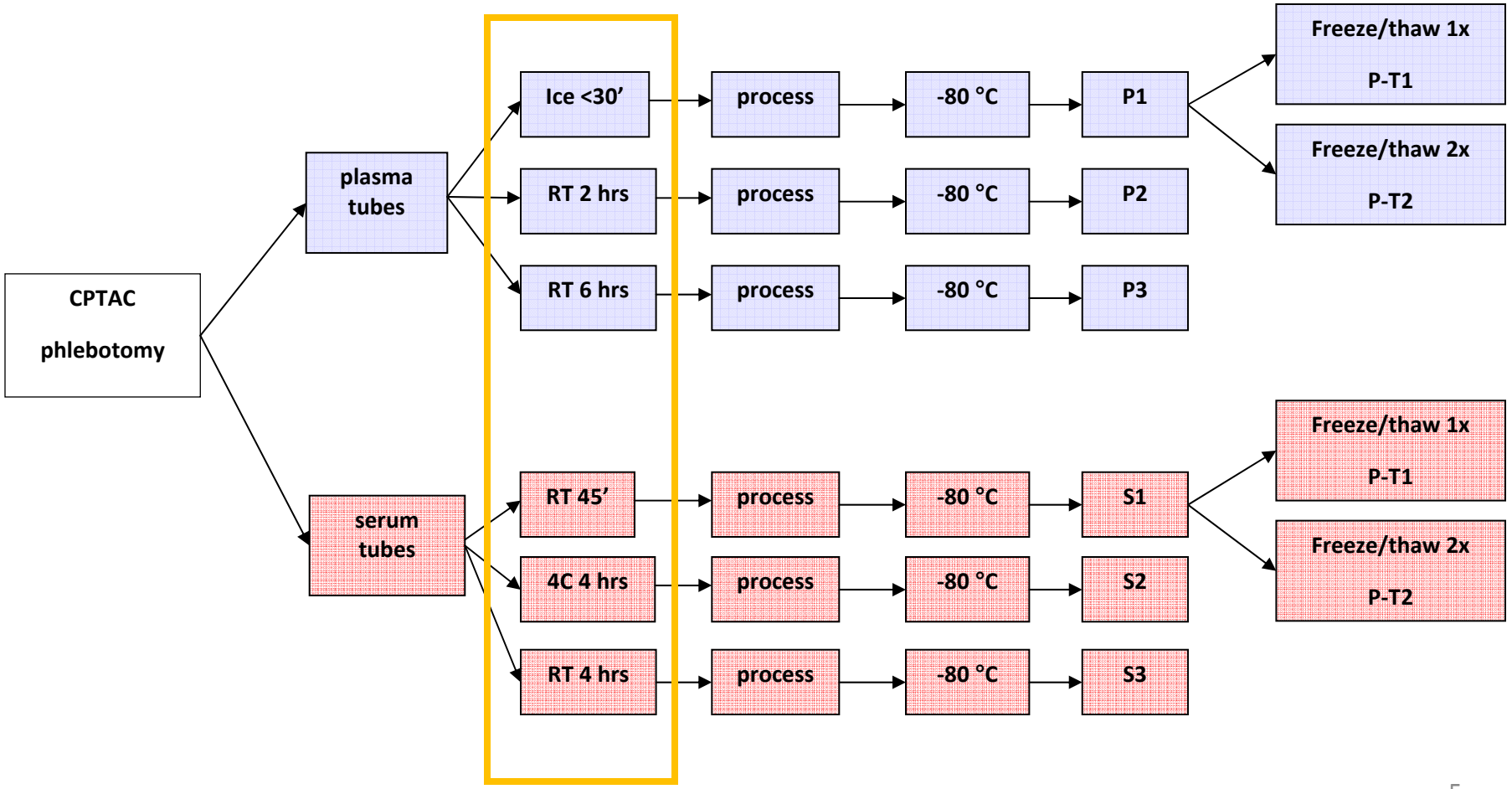
Objectives

- Generate a defined set of biospecimens collected and processed into serum and plasma under well defined, well controlled conditions.
  - Plasma and Serum
    - Blood collection using CPTAC protocol
    - Process using CPTAC or EDRN protocol
  - Training set
    - "Gold standard" samples
    - Processing and storage variables
      - Time, temperature, freeze-thaw cycles
- Establish normal ranges for candidate markers of protein damage
  - Proteolysis, oxidation, aggregation
  - Defined as those values observed for gold standard reference specimens whose processing was perfectly executed according the CPTAC and EDRN protocols
- Panel of reference markers that can be easily assayed in plasma or serum that serve as acute sentinels of plasma and serum degradation resulting from proteolysis, oxidation, and aggregation

Blood Processing Workflow



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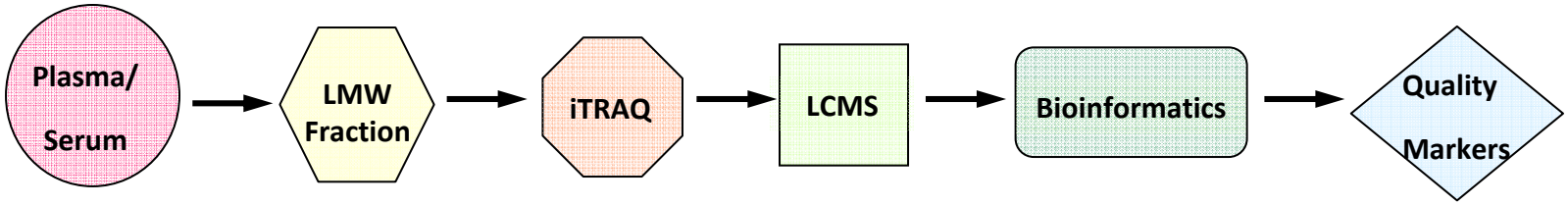
Qualitative Measures of Protein Integrity

- MS-based proteomics strategy
  - identify products of *ex vivo* proteolytic degradation
- ELISA assays
  - quantify the levels of protein oxidation and nitration
- Size Exclusion Chromatography
  - evaluate the extent of sample aggregation

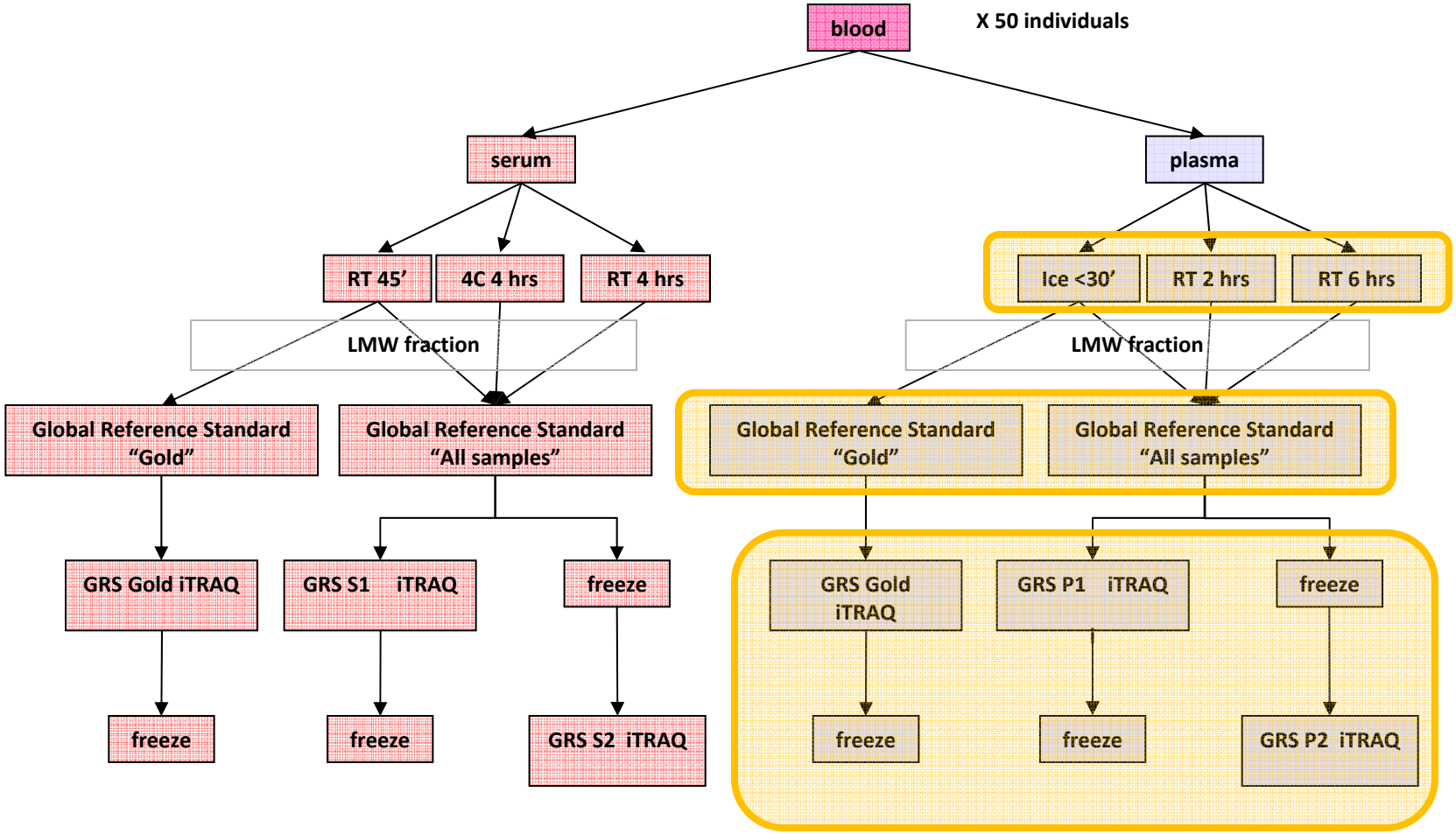
Comparison to Gold Standard Samples

Quantitative Measures of Proteolysis

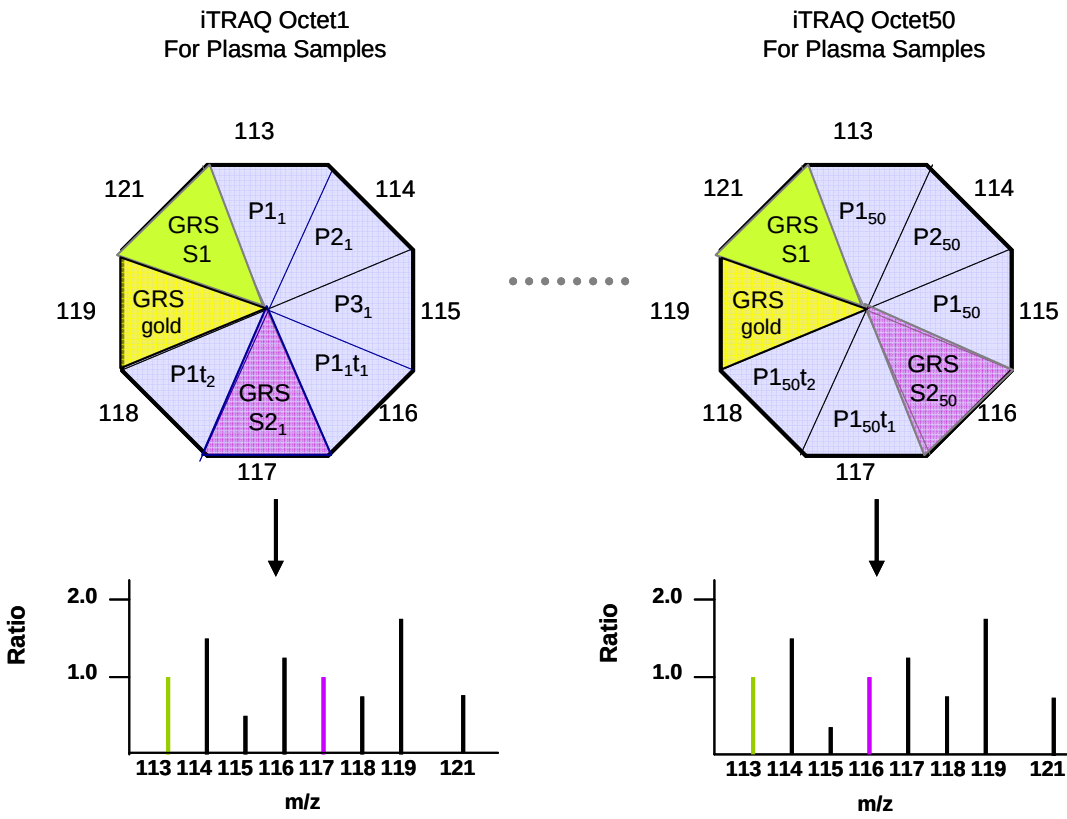


- iTRAQ labeling workflow
- Multiplexed assay
- Peptide identification and relative quantitation
- Unbiased, non-targeted strategy

Global Reference Standards for iTRAQ Quantitation



iTRAQ Experiments
Multiplex 8 samples in 1 LCMS run



- 5 Samples
- 3 Reference Standards
  - Gold Standards
  - All samples
    - labeled in bulk
    - labeled individually

Comparison to Gold Standard Samples

Oxidation and Nitration

- ELISAs
  - Formation of carbonyl groups on protein amino acid side chains is one of the early markers for protein oxidation
    - OxyELISA (Millipore), OxiSelect (Cell Biolabs)
    - Microgram amounts of protein
    - Lower limit of sensitivity of 0.2 nmol carbonyl/mg protein.
  - Reactive nitrogen species can be formed from nitric oxide, hydrogen peroxide and other pro-oxidants
    - RNS can target selective tyrosine residues in proteins to form 3-nitrotyrosine adducts
    - Nitrotyrosine ELISA (Northwest Life Science Specialties)
    - Lower limit of detection is 2 nM

Comparison to Gold Standard Samples

Aggregation

- Size Exclusion Chromatography with UV detection
  - measure the fraction of protein aggregates present in the total protein content of plasma and serum
  - The SEC column functions as a molecular sieve that separates species by size
    - column with very high M_r exclusion limit ($4x10^7$) to accommodate very large protein aggregates.
    - Measure "aggregate percentage value" for each sample:

      the ratio between the void volume peak area and total area under all peaks

Comparison to Gold Standard Samples

Statistical Analysis

- Confirmatory aim of quantifying effects of pre-analytical variables
  - Oxidation assay (nM)
  - Nitration assay (nM)
  - iTRAQ data (ratio)
  - SEC data (ratio)
- For each of these four outcomes there are two main goals:
  - Quantify the effect of procedural variables
  - Describe a normative distribution for a given sample handling procedure for quality assurance use in existing banks
- Exploratory aim to discover a "signature" combination of peptide quantities that indicates degradation (proteolysis)

Credentialing Plasma and Serum Biospecimen Banks

- Plasma samples
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- Serum samples
  - Laura Esserman
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