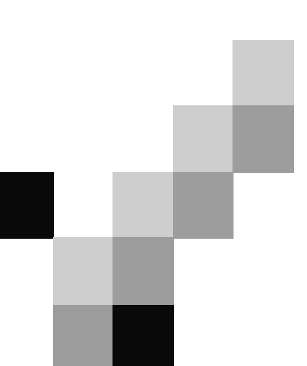

A Reference Measurement System for C-reactive Protein

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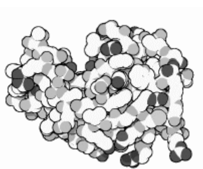
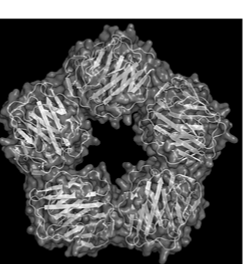


Definition of the Measurand: CRP



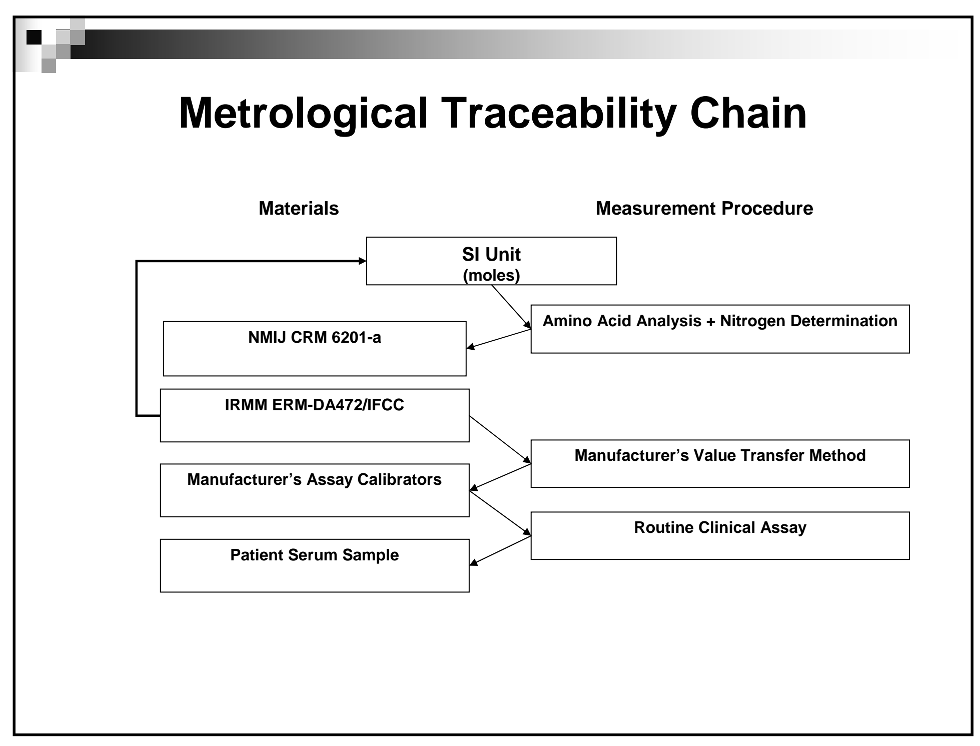
Human C-reactive Protein:

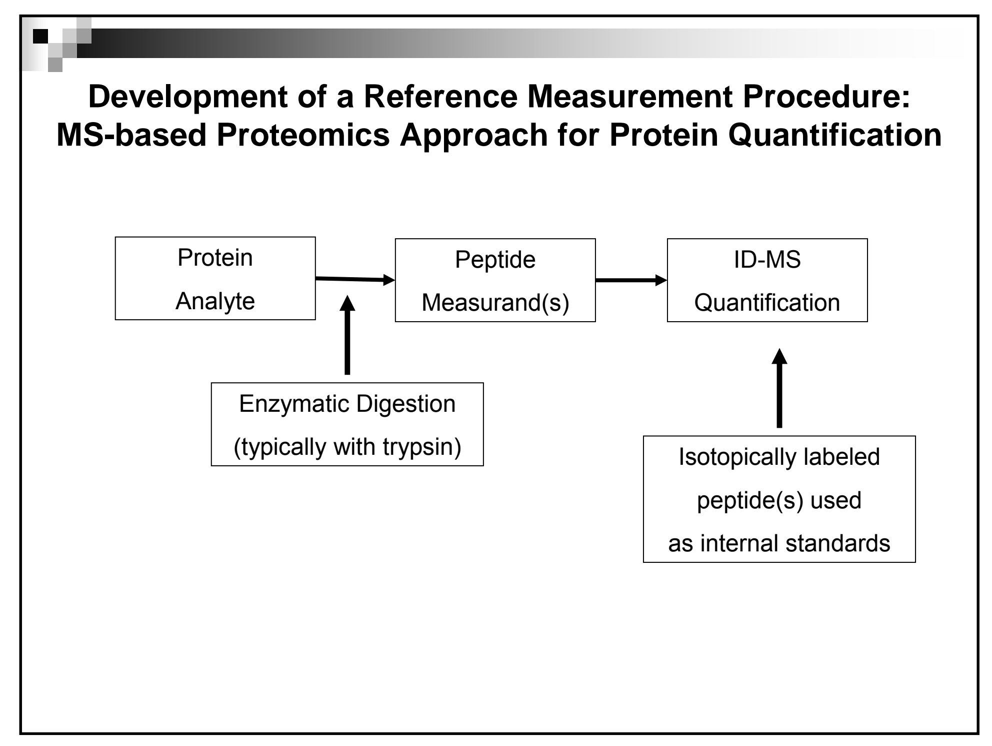
- Non-covalent pentamer with identical subunits
- Subunit consist of a single 206 amino acid chain
- Relative molecular mass of subunit ≈ 23047
- Minimal known post-translational modifications
  - N-terminal pyroglutamic acid
- Normal serum concentration ≈ 1 mg/L
- Serum CRP concentration slightly elevated above normal in atherosclerosis
- Serum CRP concentrations significantly elevated above normal in cases of infection and inflammation

Status of the Reference Measurement System

1) Measurand:	Well Defined
2) Primary Reference Material:	NMIJ CRM 6201-a
3) Secondary Reference Material:	IRMM ERM-DA472/IFCC
4) Reference Measurement Procedure:	???

Metrological Traceability Chain





Limitations of a MS-based Proteomics Approach to Protein Quantification

- No internal standard present during sample preparation (including disulfide reduction, cysteine alkylation, and enzymatic digestion)
- High possibility for interferences
- Specificity of the peptide measurand(s) for the analyte protein
- Measurand stability

A Higher Order Method:
MS-based Proteomics + Affinity Purification + Standard Addition

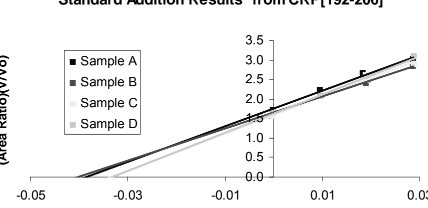
Measurement Approach:

1. Spike sample with a known amount of purified CRP
2. Perform monoclonal antibody-based purification of spiked and un-spiked sample
3. Digest isolated CRP in spiked and unspiked samples with trypsin
4. Add isotopically labeled synthetic peptides
5. Quantify using MRM-based isotope dilution LC-MS/MS

A Higher Order Method:
MS-based Proteomics + Affinity Purification + Standard Addition

Benefits:

1. Spiked CRP experiences the same affinity purification conditions and digestion condition as endogenous CRP and acts as internal standard
2. Affinity purification improves measurand stability and reduced likelihood for interferences



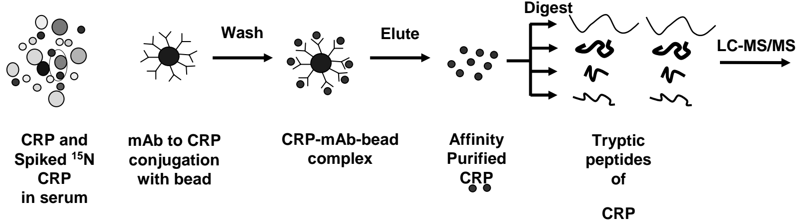
Limitations of “Proteomics + IP + Standard Addition”

- Treatment of spiked sample may not be identical to that of unspiked sample
- Use of multiple data points to determine analyte concentration increases measurement uncertainty

A Higher Order Method (part 2): Complete Protein ID-MS

Measurement Approach:

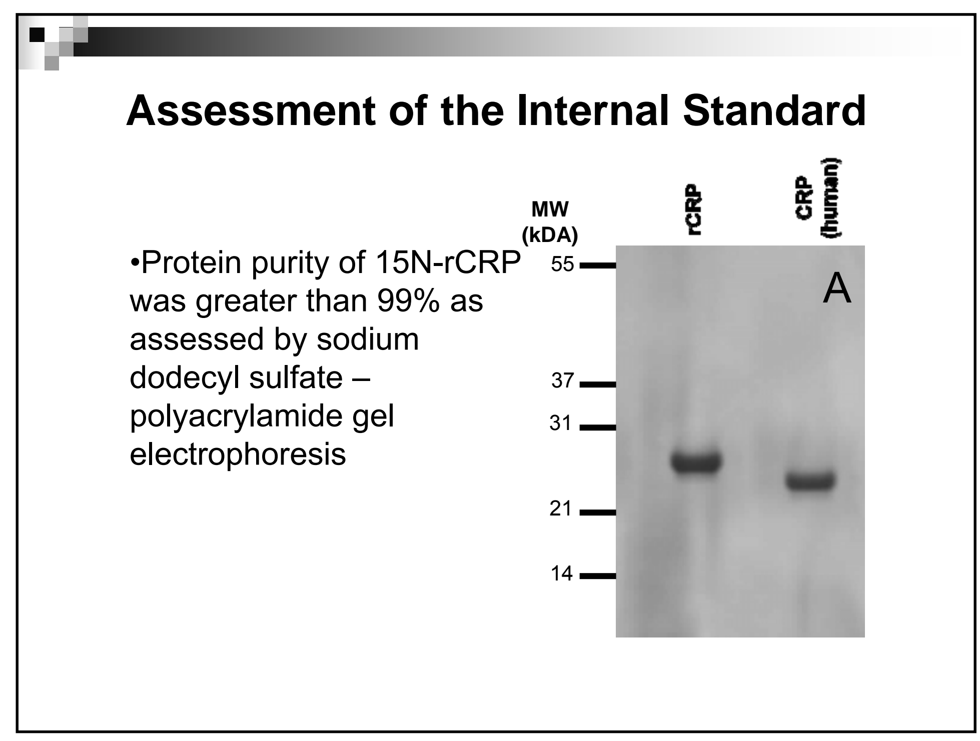
1. Spike sample with isotopically-labeled rCRP
2. Perform monoclonal antibody-based purification spiked sample
3. Digest isolated CRP in spiked sample with trypsin
4. Quantify using MRM-based isotope dilution LC-MS/MS on multiple CRP tryptic peptides using calibrators prepared from labeled and unlabeled CRP (i.e., double isotope dilution mass spectrometry)

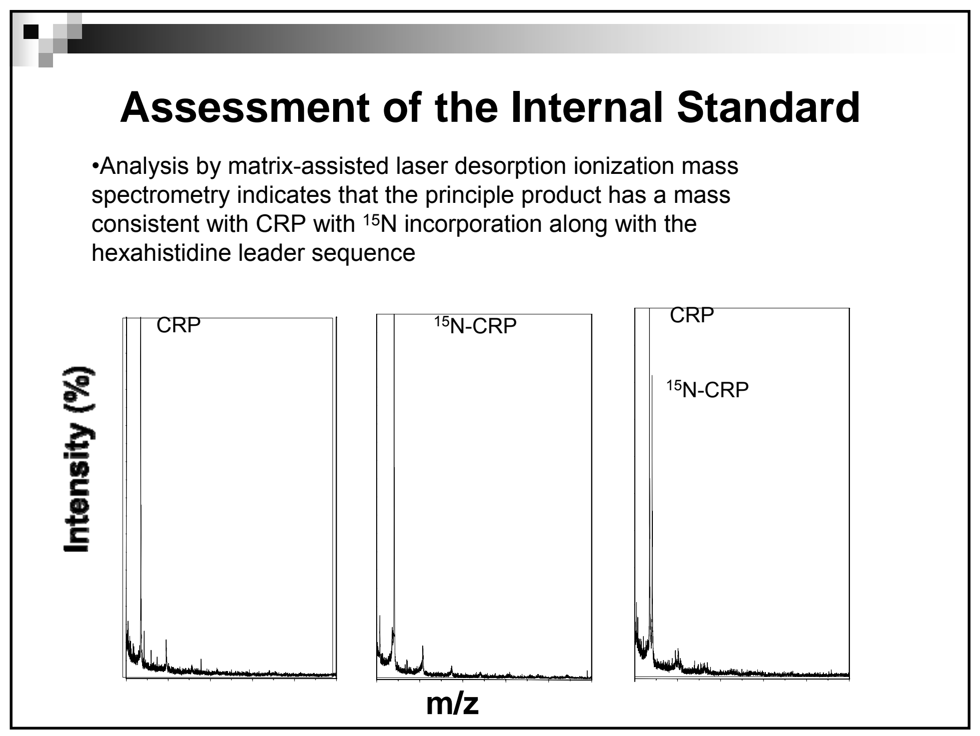


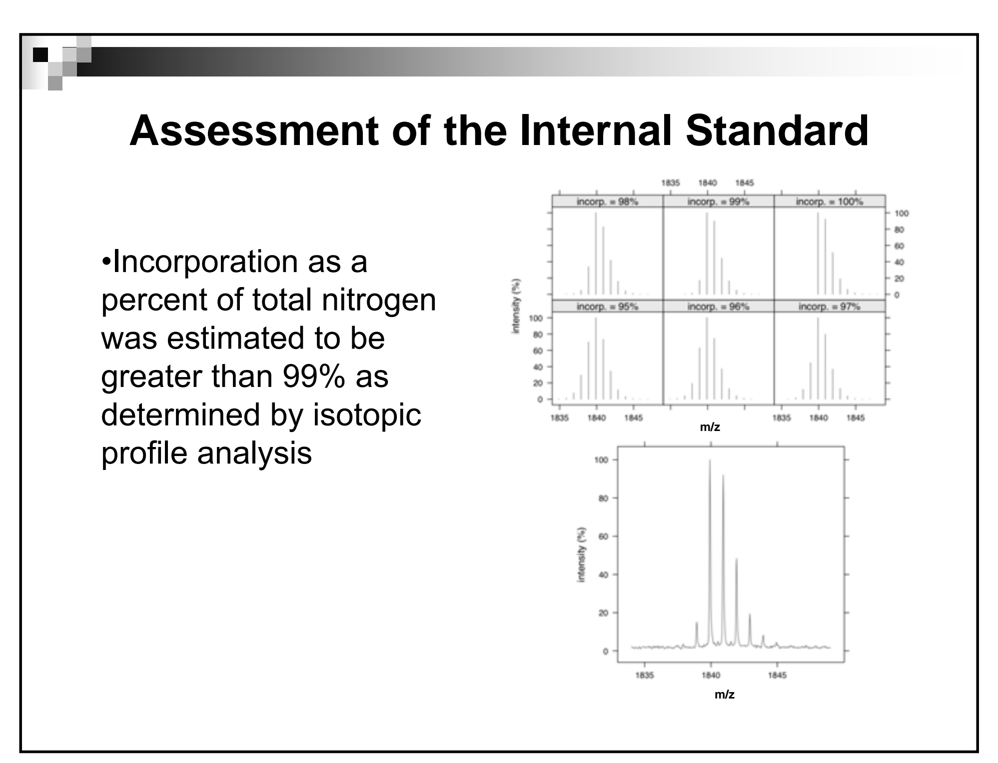
Preparation of the Internal Standard

Recombinant ^{15}N-labeled human C-reactive protein:

- Expressed in *E. coli* with media containing ^{15}N-ammonium chloride as the sole nitrogen source
- Expressed with a hexahistidine affinity tag on leader sequence
- Purified by sequential immobilized metal and phosphatidylcholine affinity chromatography purifications
- Hexahistidine affinity tag enzymatically cleaved leaving 4-amino acid leader sequence on the N-terminus of CRP

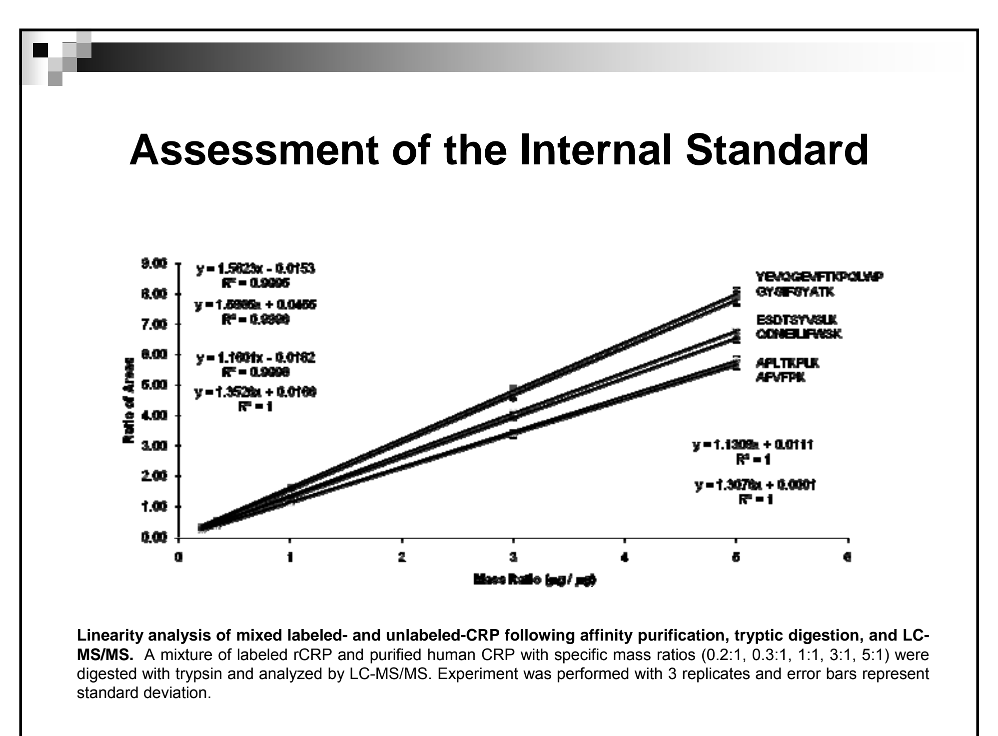



Assessment of the Internal Standard
•Analysis by matrix-assisted laser desorption ionization mass spectrometry indicates that the principle product has a mass consistent with CRP with 15N incorporation along with the hexahistidine leader sequence
CRP
15N-CRP
CRP
15N-CRP
Intensity (%)
m/z


Assessment of the Internal Standard
•Incorporation as a percent of total nitrogen was estimated to be greater than 99% as determined by isotopic profile analysis
incorp. = 98%
incorp. = 99%
incorp. = 100%
incorp. = 95%
incorp. = 96%
incorp. = 97%
intensity (%)
m/z

Assessment of the Internal Standard

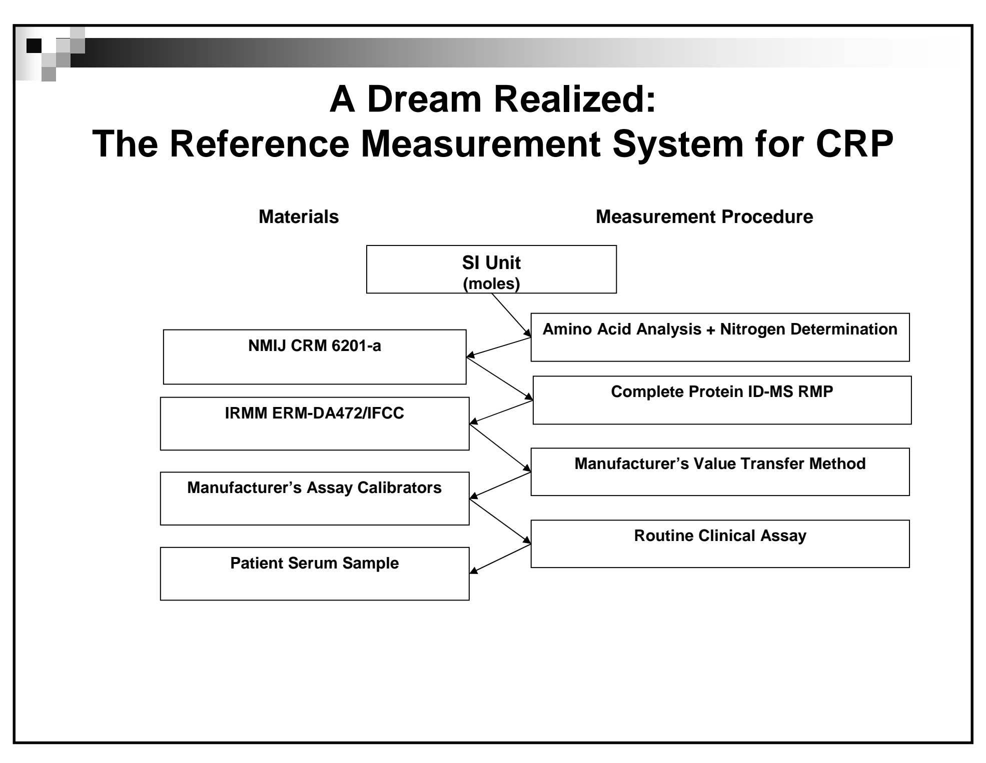
- Equivalence of ^{15}N-labeled rCRP to purified human CRP under digestion conditioned assessed by comparing expected to observed tryptic peptide ratios in blends of labeled and unlabeled CRP
- Equivalence of 15N-labeled CRP to purified human CRP under affinity purification conditioned assessed by comparing expected to observed tryptic peptide ratios in affinity purified blends of labeled and unlabeled CRP
- Equivalence of 15N-labeled CRP to endogenous human CRP under digestion and affinity purification conditions assessed by comparing expected to observed tryptic peptide ratios in affinity purified ^{15}N-rCRP spiked serum samples



Linearity analysis of mixed labeled- and unlabeled-CRP following affinity purification, tryptic digestion, and LC-MS/MS. A mixture of labeled rCRP and purified human CRP with specific mass ratios (0.2:1, 0.3:1, 1:1, 3:1, 5:1) were digested with trypsin and analyzed by LC-MS/MS. Experiment was performed with 3 replicates and error bars represent standard deviation.

The Complete RMP for CRP

1. Spike serum sample with ^{15}N-labeled rCRP
2. Perform monoclonal antibody-based affinity purification (on magnetic beads) of CRP
3. Digest isolated CRP in spiked sample with trypsin
4. Quantify using MRM-based isotope dilution LC-MS/MS on 6 CRP tryptic peptides using calibrators prepared from labeled and unlabeled CRP (i.e., double isotope dilution mass spectrometry)



Future Applications of Complete Protein ID-MS

- Albumin in Urine (with ^{15}N-labeled rHSA)
- Cardiac Troponin I (with ^{15}N-labeled recombinant troponin complex)
- Prostate Specific Antigen (with ^{15}N-labeled recombinant PSA + ^{15}N-labeled recombinant PSA-ACT complex)

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Thank You