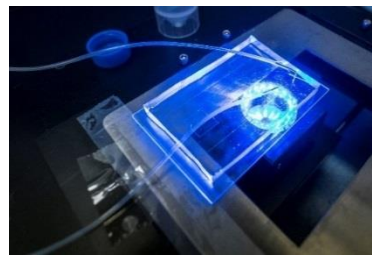# Providing Traceability for Protein Biomarkers

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Protein Metrology at NIST

Precision Medicine

Protein quantitation
- Spectrophotometric
- Mass spectrometry (AAA, MRM)

Protein characterization
- 3-D structure
- Post-translational modifications

Assay standardization/harmonization

Reference methods and reference materials

Biomanufacturing

Protein quantitation
- Spectrophotometric
- Mass spectrometry (future)

Protein characterization
- 3-D structure
- Post-translational modifications

New technologies

Reference materials and protocols

https://www.nist.gov/mml/biomolecular-measurement/bioanalytical-science-group

Current Protein Biomarkers of Interest

Cardiovascular disease

- Cardiac troponin I (cTnI)
- Brain natriuretic peptide (BNP)
- C-reactive protein (CRP)

Kidney disease

- Urine albumin

Bone health/nutrition

- Vitamin D binding protein
- Parathyroid hormone (PTH)

Forensics

- Insulin-like growth factor I (IGF-1)

NEW!

In development:
SRM 2925 Human Serum Albumin Solution
SRM 2926 Human Insulin-Like Growth Factor 1
SRM 2927 15N-Labeled Recombinant Human Insulin-like Growth Factor 1

Approaches to Quantitation

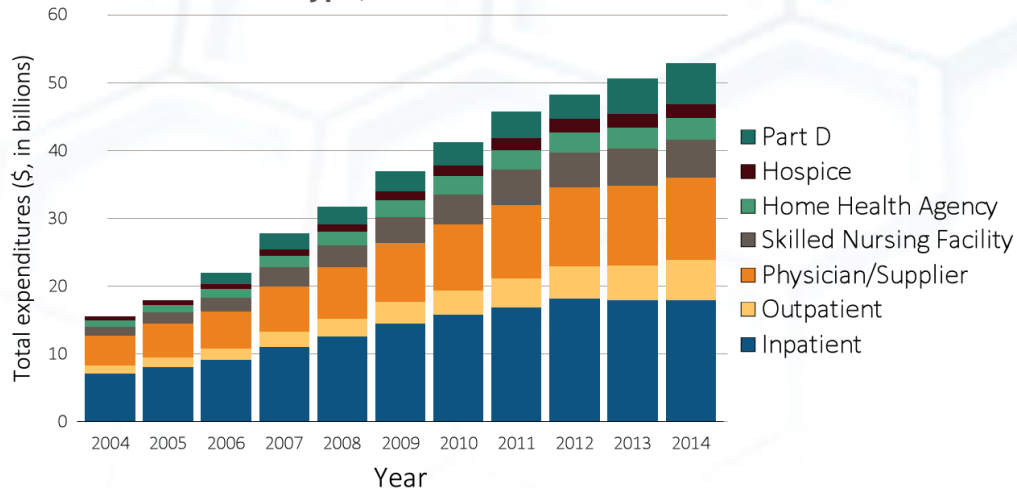| Labeled peptide	QconCAT*	Protein
Chemical synthesis with high purity	More closely resembles protein(s) of interest	Unlabeled or labeled forms can be used
Doesn't account for variability in sample prep	May not completely account for variability in sample prep	Closest to ideal internal standard
Synthesis of multiple peptides can be expensive	Useful for multiplex capabilities	May be difficult to obtain

* Artificial protein produced in cells, contains multiple linked peptides

Scott et al., Anal. Chem., 87 (2015) 4429-35

An Example: Urine Albumin

Trends in total Medicare Parts A, B, and D fee-for-service spending for CKD patients aged 65 and older, by claim type, 2004-2014

The problem:

- Chronic kidney disease is a growing problem in the U.S.

- Large financial and quality of life impacts

- Urine albumin is an indicator of kidney dysfunction

- May give earlier indication of disease, useful for staging

- Existing reference ranges

Current challenges of affinity-based methodologies:

- Potential heterogeneity of albumin
- Assay specificity
- Biological variability of urine
- Assay bias varies with concentration


Clinical Chemistry 55:1
24–38 (2009)

Reviews

Current Issues in Measurement and Reporting of Urinary Albumin Excretion

W. Greg Miller,[1]* David E. Bruns,[2] Glen L. Hortin,[3] Sverre Sandberg,[4] Kristin M. Aakre,[4] Matthew J. McQueen,[5] Yoshihisa Itoh,[6] John C. Lieske,[7] David W. Seccombe,[8] Graham Jones,[9] David M. Bunk,[10] Gary C. Curhan,[11] and Andrew S. Narva,[12] on behalf of the National Kidney Disease Education Program–IFCC Working Group on Standardization of Albumin in Urine

Reference Measurement System
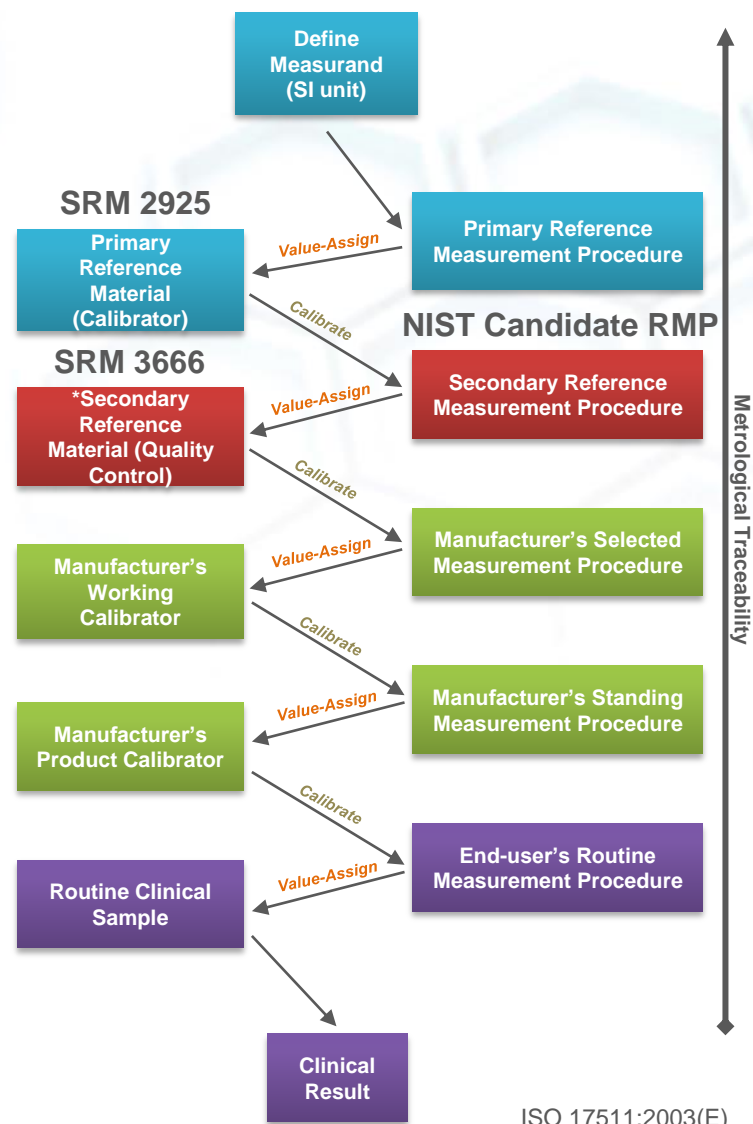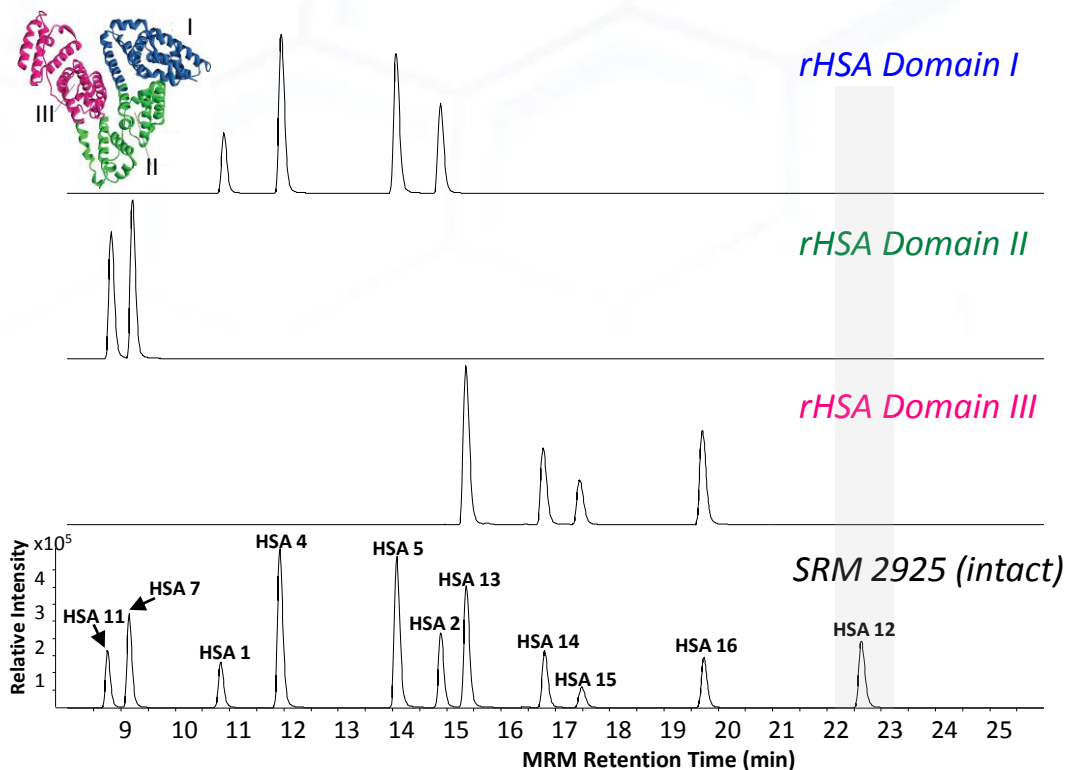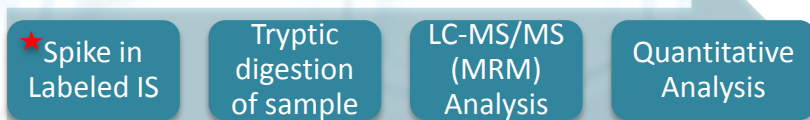
A.B. Green et al., J. Proteome Res., 13 (2014) 3930-3939

Exploratory: Cardiac Troponin I

- Cardiac troponin I and T (cTnI,cTnT) are the preferred biomarkers of acute coronary syndromes

- Large number of commercial assays, target different epitopes of cTnI

- Circulating cTnI is heterogeneous, undergoes modifications and degradation

- NIST introduced SRM 2921 Human Cardiac Troponin Complex in 2004

- SRM is not commutable with a number of immunoassays, matrix-based material might be preferable

- SRM 2921 may be suitable as a calibrator for a higher-order method
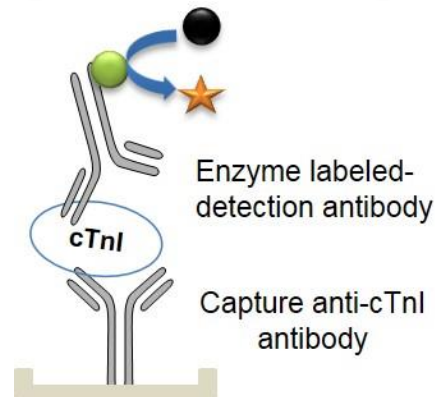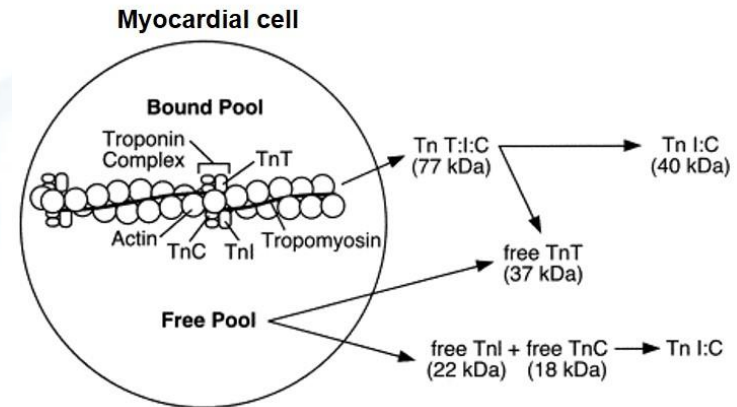
SRM 2921

Freda, B.J.; et al. Journal of the American College of Cardiology, 40, 2065-2071 (2002)

Quantification of cTnI

Technical challenges

- cTnI is a low abundance protein in serum
  - Serum cTnI levels around 1-10 ng/mL after myocardial infarction
  - Near 1 pg/mL in healthy individuals
- Large dynamic range of protein concentrations in serum
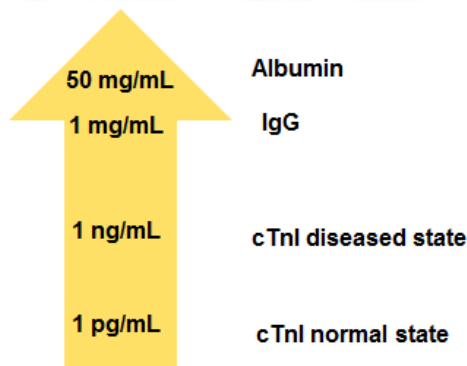
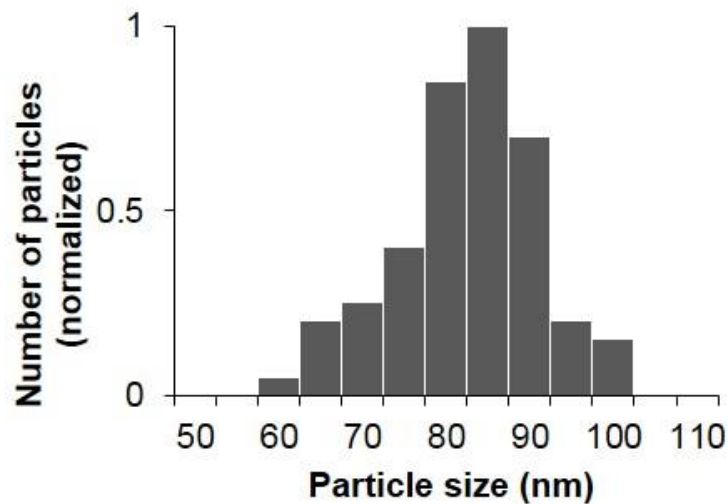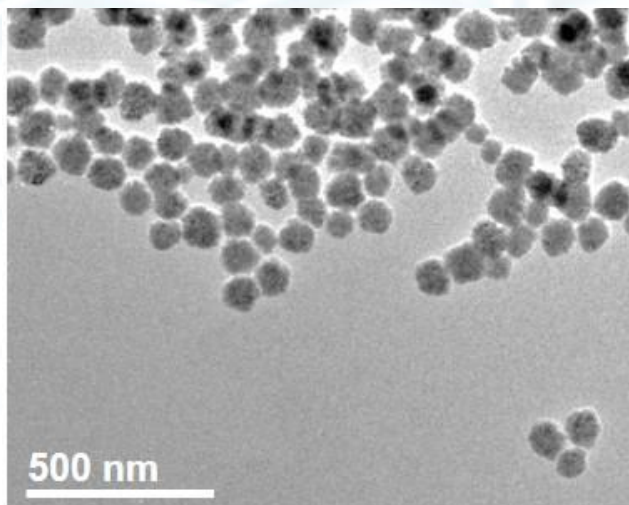*cTnI in human serum is undetectable by direct LC-MS/MS-based approaches*

Approach

- Use magnetic nanoparticle-based immunoaffinity enrichment prior to LC-MS/MS analysis
- Reduce sample complexity
- Increase analyte: background ratio

Schneck, N.A.; et al. Nanomedicine, 10, 443-446 (2015)
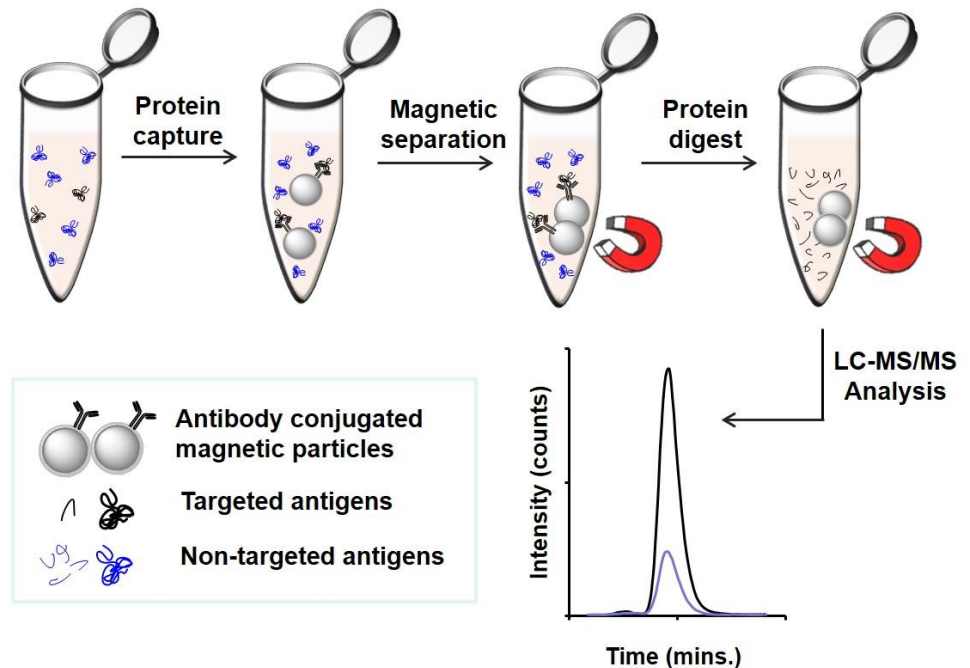
Synthesis of Modified Nanoparticles

Example of nanoparticle functionalization and antibody coupling scheme:

Modify nanoparticle with silane agents

Silane coupling (Aminopropytriethoxysilane)
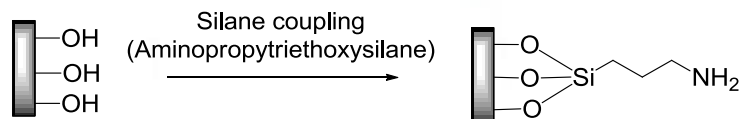
Add activation or crosslinking agent

Crosslinking agent (Glutaraldehyde)
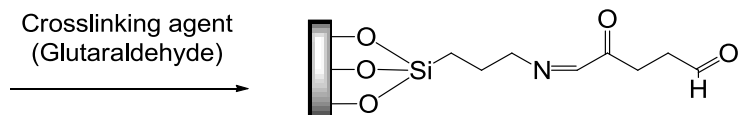
Immobilize antibody onto modified nanoparticle

Reducing agent (Sodium cyanoborohydride)

Schneck, N.A. et al. Anal. Bioanal. Chem., 408, 8325-8332 (2016)

MATERIAL MEASUREMENT LABORATORY

Development of LC-MS/MS Method
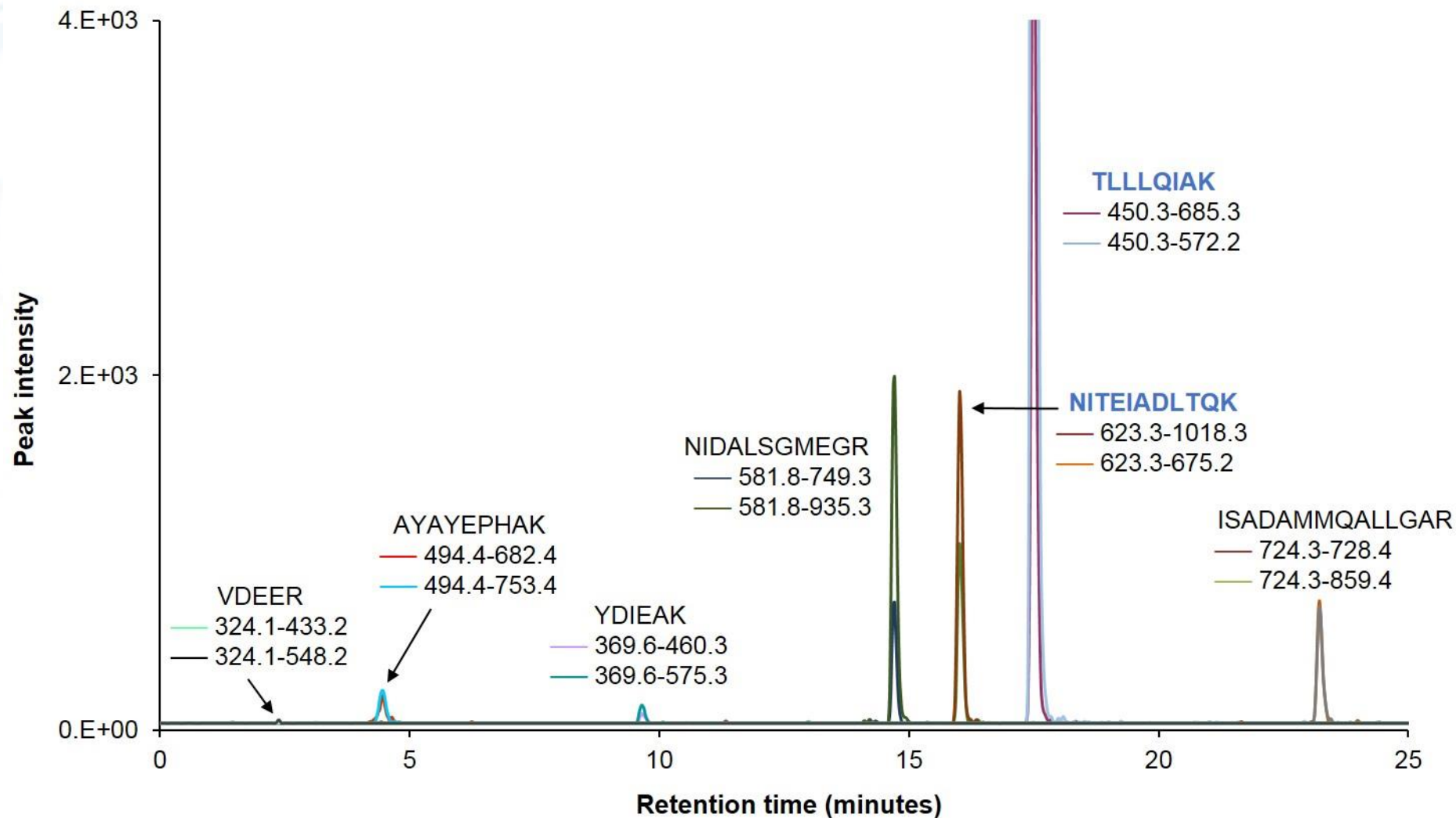
Very few peptides with adequate signal, without potential modifications

Quantification Approach

Amino acid sequence of human cTnI

ADGSSDAAREPRPAPAPIRRRSS
NYRAYATEPHAKKKSKISASRKLQ
LKTLLLQIAKQELEREAEERRGEK
GRALSTRCQPLELAGLGFAELQDL
CRQLHARVDKVDEERYDIEAKVTK
NITEIADLTQKIFDLRGKFKRPTLR
RVRISADAMMQALLGARAKESLDL
RAHLKQVKKEDTEKENREVGDWR
KNIDALSGMEGRKKKFES

cTnI MRM transitions

K.TLLLQIAK.Q	
450.3++	685.4+ (y6)
	572.4+ (y5)

K.NITEIADLTQK.I	
623.3++	1018.3+ (y9)
	675.2+ (y6)

R.AYATEPHAK.K	
494.4++	753.4+ (y7)
	682.4+ (y6)

Enzymatic digestion (e.g. trypsin)

LC-MS/MS with MRM analysis
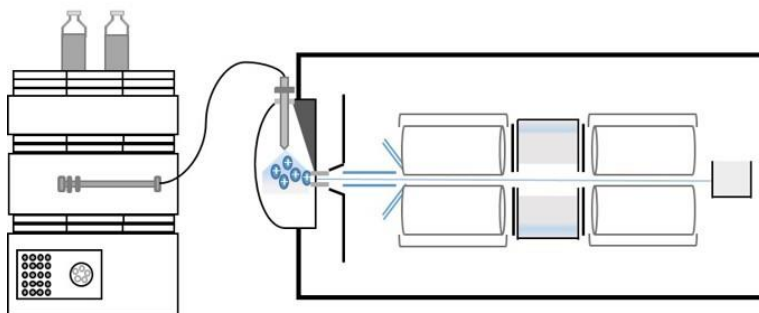
Extracted ion chromatogram

TLLLQIAK
— 450.3-685.3
— 450.3-572.2

NITEIADLTQK
— 623.3-1018.3
— 623.3-675.2

AYATEPHAK
— 494.4-682.4
— 494.4-753.4

Peak Intensity (y-axis): 0.E+00, 5.E+03, 1.E+04, 2.E+04
Retention Time (minutes) (x-axis): 5, 7, 9, 11, 13, 15, 17, 19
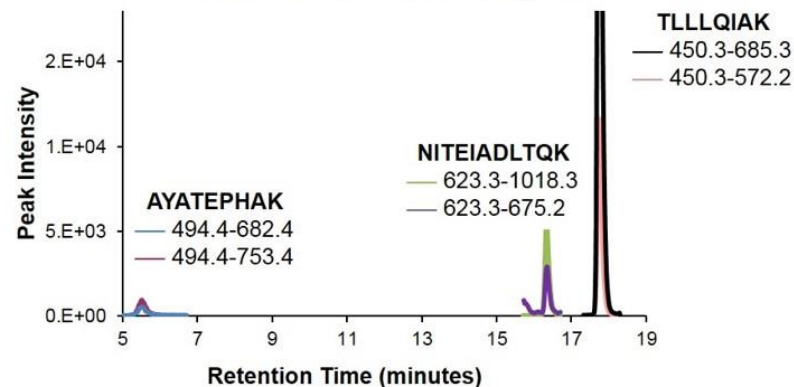
Sample Preparation
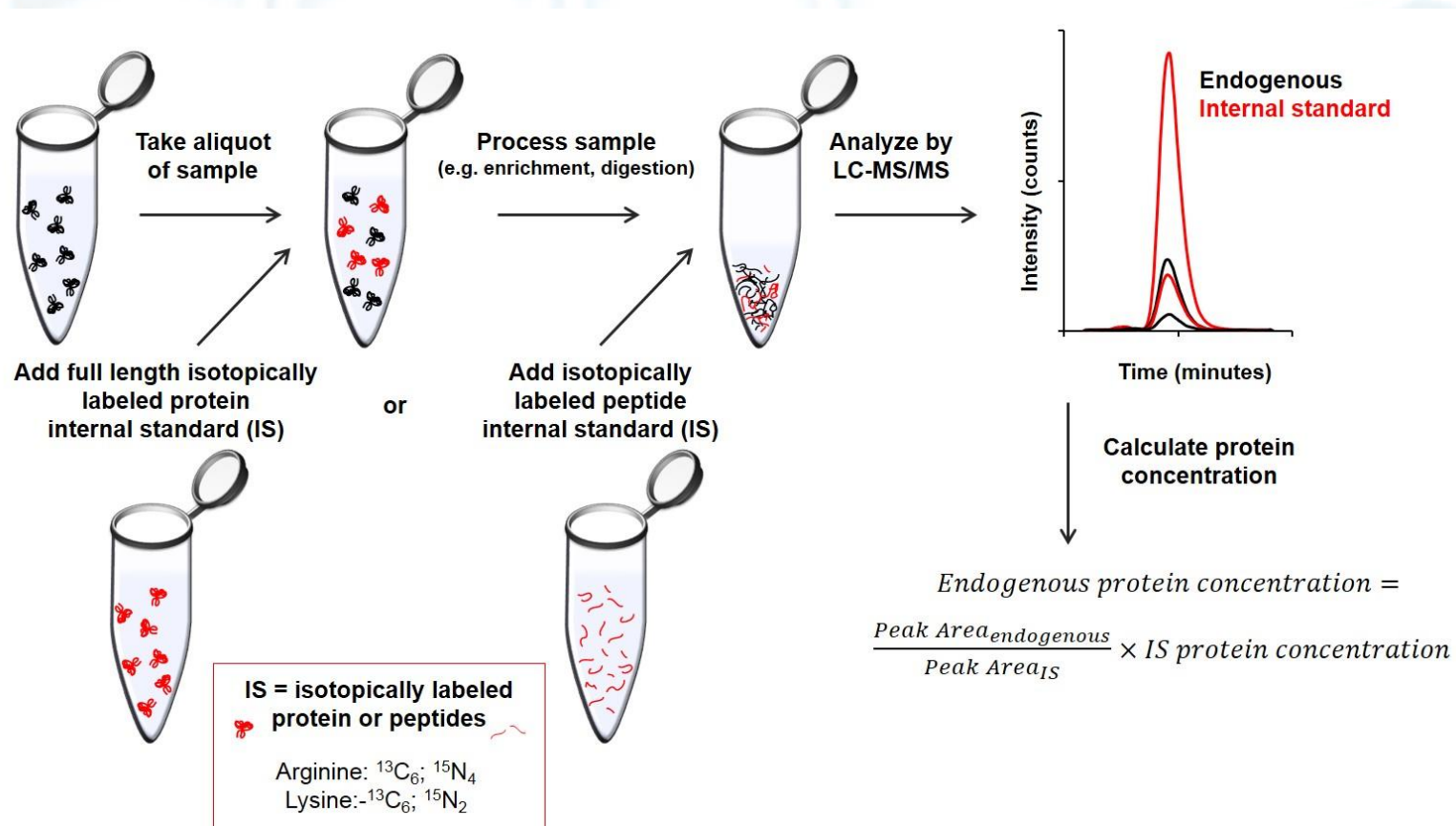
Full-length labeled protein used for quantification
Labeled peptides used for recovery studies

Calibrants prepared by spiking cTnI-free plasma with
SRM 2921 Human Cardiac Troponin Complex

cTnI Quantification in Patient Plasma Samples

Achievements

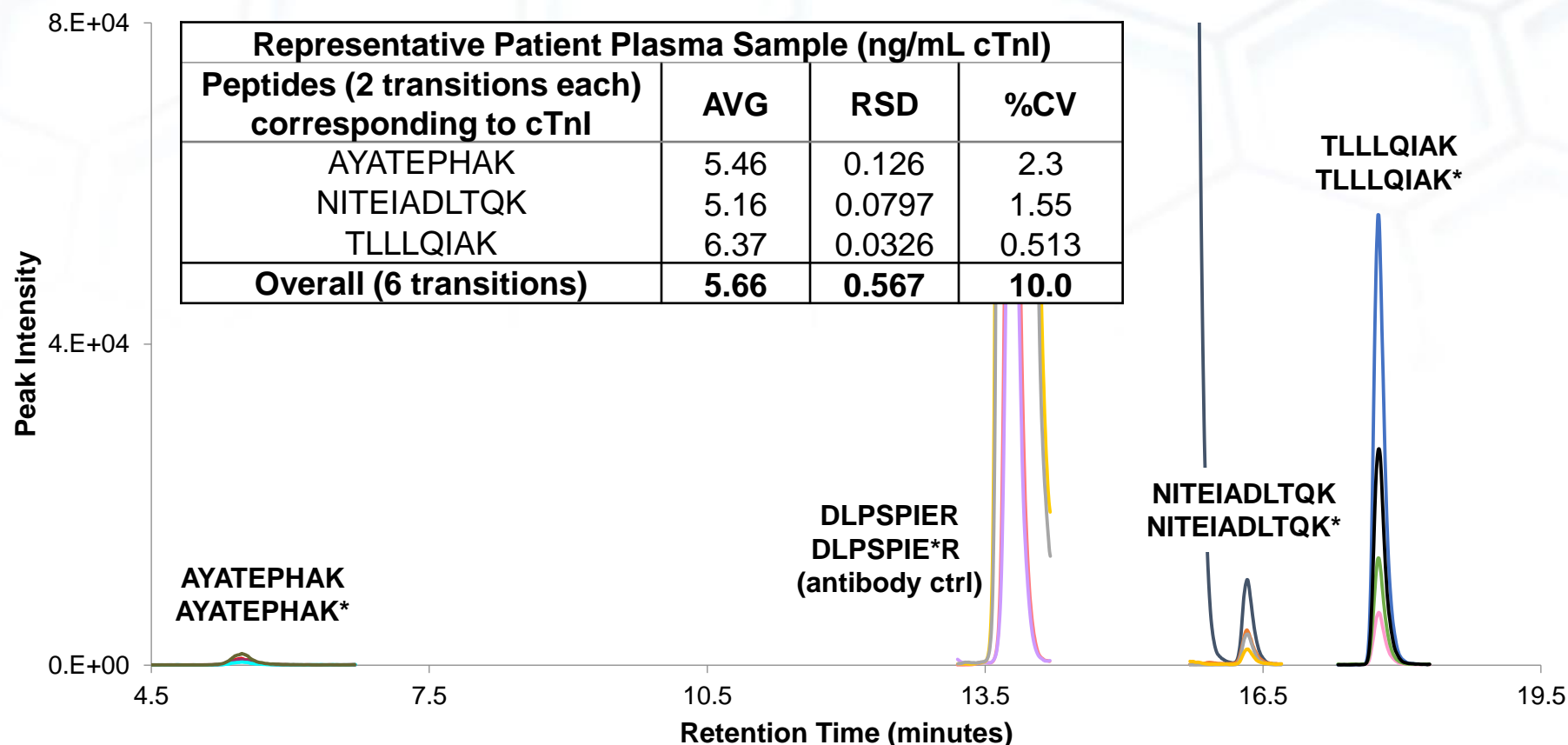- Using magnetic nanoparticle enrichment and isotope dilution-LC-MS/MS (with an isotopically labeled protein internal standard), cTnI was measured in five myocardial infarction patients with concentrations between 4-11 ng/mL and CV's ≤ 15% using 3 peptides for quantification

Representative Patient Plasma Sample (ng/mL cTnI)			
Peptides (2 transitions each) corresponding to cTnI	AVG	RSD	%CV
AYATEPHAK	5.46	0.126	2.3
NITEIADLTQK	5.16	0.0797	1.55
TLLLQIAK	6.37	0.0326	0.513
Overall (6 transitions)	**5.66**	**0.567**	**10.0**

Ongoing Challenges for Protein Biomarkers
(from a mass spectrometry perspective)

- Appropriate primary standards/calibration materials

- Producing CRMs for both immunoassays and clinical mass spec

- Sources of isotopically labeled protein internal standards

- Instrument limitations (sensitivity)

Anal. Chem. **2009**, *81*, 8610–8616

**Reference Measurement Procedure Development
for C-Reactive Protein in Human Serum**

Eric L. Kilpatrick*,† and David M. Bunk‡

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Acknowledgments

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Sang Bok Lee, University of Maryland

Free resources:

NIST Mass and Fragment Calculator Software
Calculates mass of input peptide or program and ions for various charge states
https://www.nist.gov/services-resources/software/nist-mass-and-fragment-calculator-software
Isotope and mass calculators

Isotope Enrichment Calculator
Determines the percentage of ^{15}N enrichment of stable isotope-labeled peptides or proteins
https://www.nist.gov/services-resources/software/isotope-enrichment-calculator