CLINICAL PROTEOMIC TECHNOLOGIES FOR CANCER

Protein Quantitation by Targeted MS: the Bridge from Discovery to the Clinic

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A Functioning Pipeline for Cancer Biomarker Development Requires Both Discovery and Directed Assay Components

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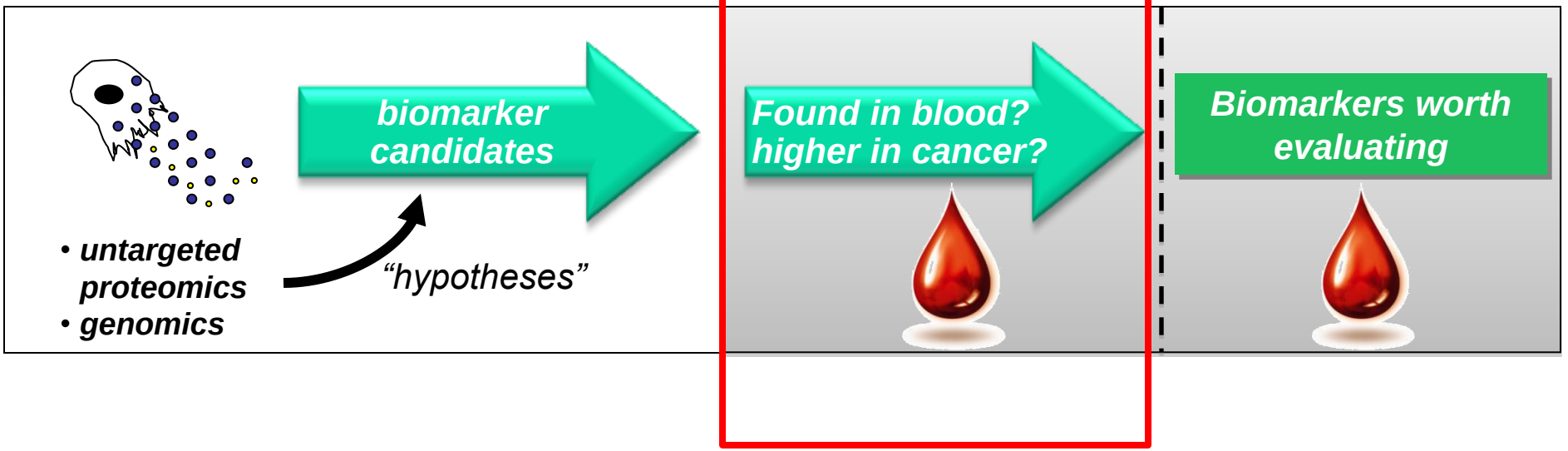
Bio-Specimens
- Plasma
- Tissue
- Proximal fluids

Discovery
- Tissue
- Proximal fluids

Verification
- Blood
- Population

Clinical Validation
- Blood
- Population



Adapted from Rifai, Gillette and Carr Nat. Biotech.2006

Technologies to bridge gap between Discovery and the Clinic are needed

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> 10,000 Analytes

Discovery Proteomics

10's Samples

LC/MS/MS

100's of Candidates

GAPS/BARRIERS

?

4 - 10 Analytes

Clinical Validation

1000's Samples

Immunoassay

Panel of Biomarkers

Antibodies alone are not sufficient to bridge gulf between Discovery and Validation

CLINICAL PROTEOMIC TECHNOLOGIES FOR CANCER

> 10,000 Analytes

Discovery Proteomics

10's Samples

LC/MS/MS

100's of Candidates

Validation by immunoassays is well established, but:

- Number of useful Abs: small
- Number of candidates: large
- Making new, immunoassay capable Ab's is expensive

New approaches are required

4 - 10 Analytes

Clinical Validation

1000's Samples

Immunoassay

Panel of Biomarkers

Need: ability to sort through large lists of biomarker candidates to identify most promising ones (without immunoassay)

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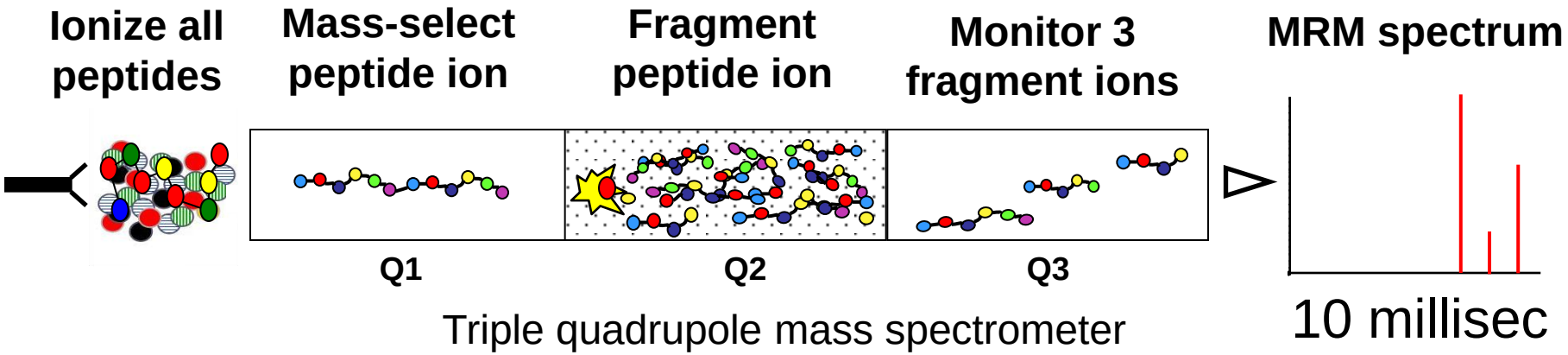
1. **Identify/develop the candidate list:**
   - **proteomics, microarray, literature mining**

2. **Credential/discard protein candidates ("Verification"):**
   - **MS-based assays to detect and quantify protein candidates in plasma without immunoassays**

3. **Assess performance of proteins in biological context**
   - **Sensitivity, specificity of candidates in patient plasma**

MRM-MS is fast, sensitive, highly specific

MRM-MS



How MRM-MS Works: peptides as surrogates of proteins for detection and quantitation

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Candidate Proteins

↓

Define "Signature peptides" for protein candidates

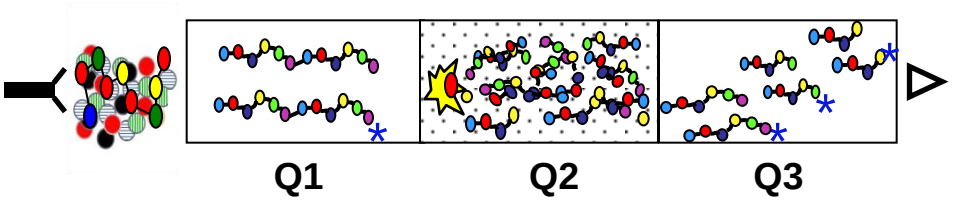
↓

Synthesize "heavy" isotope labeled internal standards

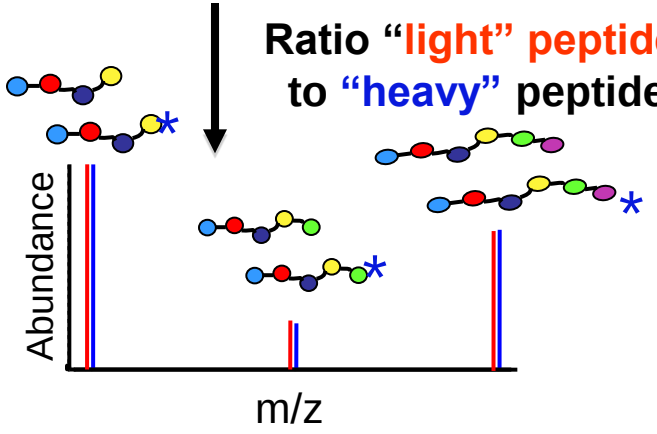
Spike "heavy" Internal stnd. peptides

Endogenous "light" peptides

- Observed ratios gives precise, relative quantitation across samples
- 10's to 100 peptides can be simultaneously quantified



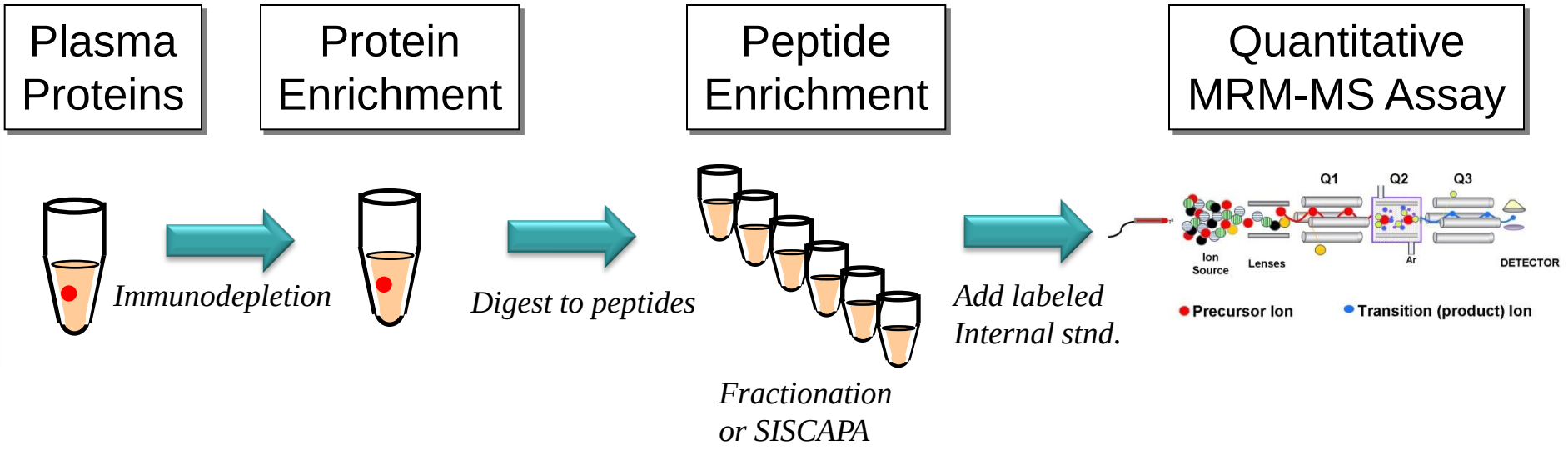
Ratio "light" peptide to "heavy" peptide



Benefits of MRM-MS for protein assays

- New application of existing technology
- Short assay development timeline
- Can be highly multiplexed
- High molecular specificity
- Ease of detecting and avoiding interferences (unlike immunoassays)
- Quantify low abundance proteins (1 ng/mL) in plasma
- Reproducibility approaching clinical assays
- Does not require immunoassay-grade antibodies
- Large deployed instrument base

MRM-MS coupled to peptide and protein enrichment enables assay of proteins in plasma at ng/mL levels



10-100 uL plasma

- **Low (1-10) ng/mL LOQ of candidate biomarkers in plasma achieved by protein and peptide enrichment**

Keshishian et al. (2007) Mol Cell Proteomics
Whiteaker et al. (2007) Anal Biochem

CPTAC is determining if MRM technology in transferable and reproducible across labs

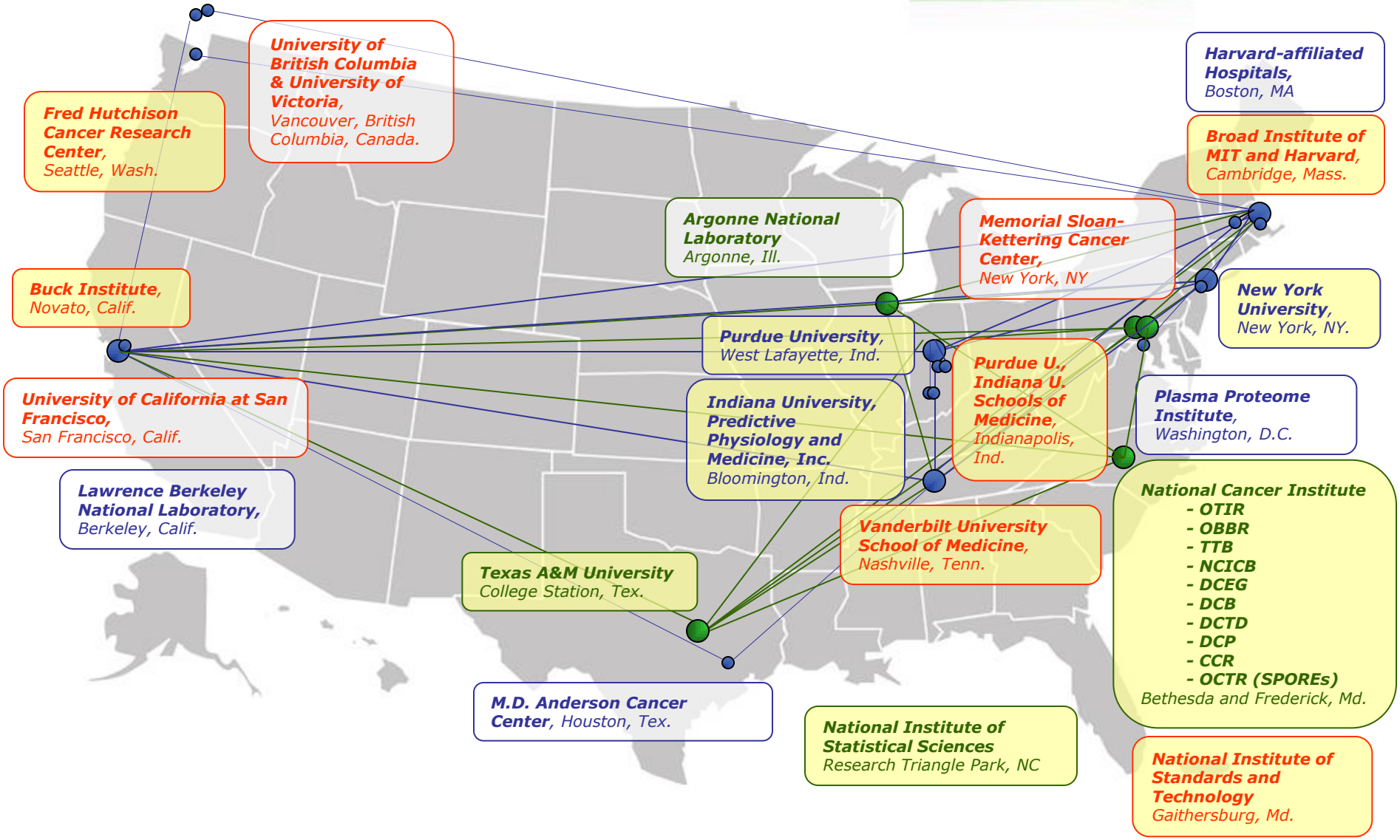
- **Prior studies indicated that individual labs could achieve excellent reproducibility and Limits of Quantitation (LOQ) in plasma, but…**

- **Reproducibility and transferability of these assays across the labs not demonstrated preventing widespread adoption**

CPTAC conducted the first multi-laboratory study to assess performance of multiplexed, MRM-based assays

- step-wise assessment of the sources of variability in MRM assays
- establish performance achievable within and between labs
- studies done in non-enriched plasma where potential for interference is highest

Verification WG participants



Study designed to systemically assess sources of variability

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Samples:

- 11 synthetic signature peptides (heavy and light) from 7 proteins
- Peptides or proteins spiked at 9 concentrations into plasma

Studies: Three distinct studies conducted by 8 labs in parallel

- In each study, labs generated peptide response curves in plasma
- Each study introduced additional sources of variability in sample preparation relevant to assays development

Increasing study complexity

Study I: Light and heavy peptides spiked into digested plasma	Study II: Digested proteins spiked into digested plasma	Study III: Proteins spiked into plasma

Centralized sample preparation

Labs do all sample prep. (Simulated biomarker study)

Intra-lab and Inter-lab reproducibility across the 3 studies at limit of quantitation

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Intra-lab Reproducibility for SSDLVALSGGHTFGK at LOQ

Study I: 4.0 - 8.9%

Study II: 4.6 - 7.3%

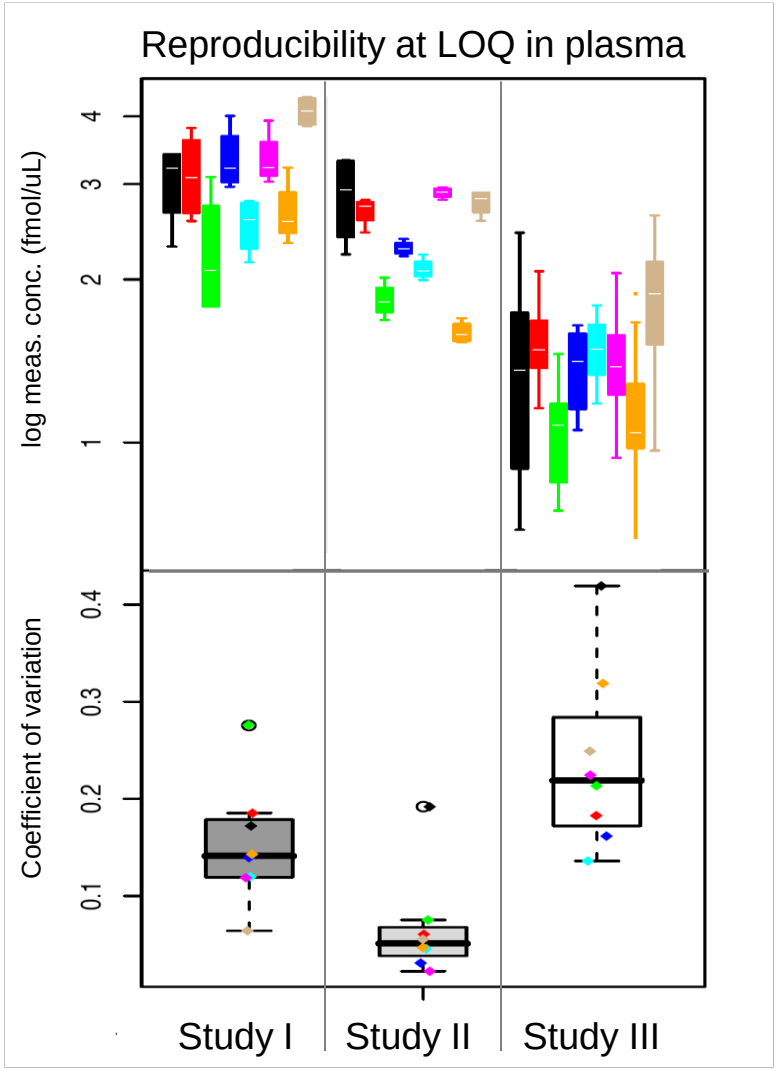
Study III: 8.4 - 21.4%

Inter-lab Reproducibility for SSDLVALSGGHTFGK at LOQ

Study I: 14.1%

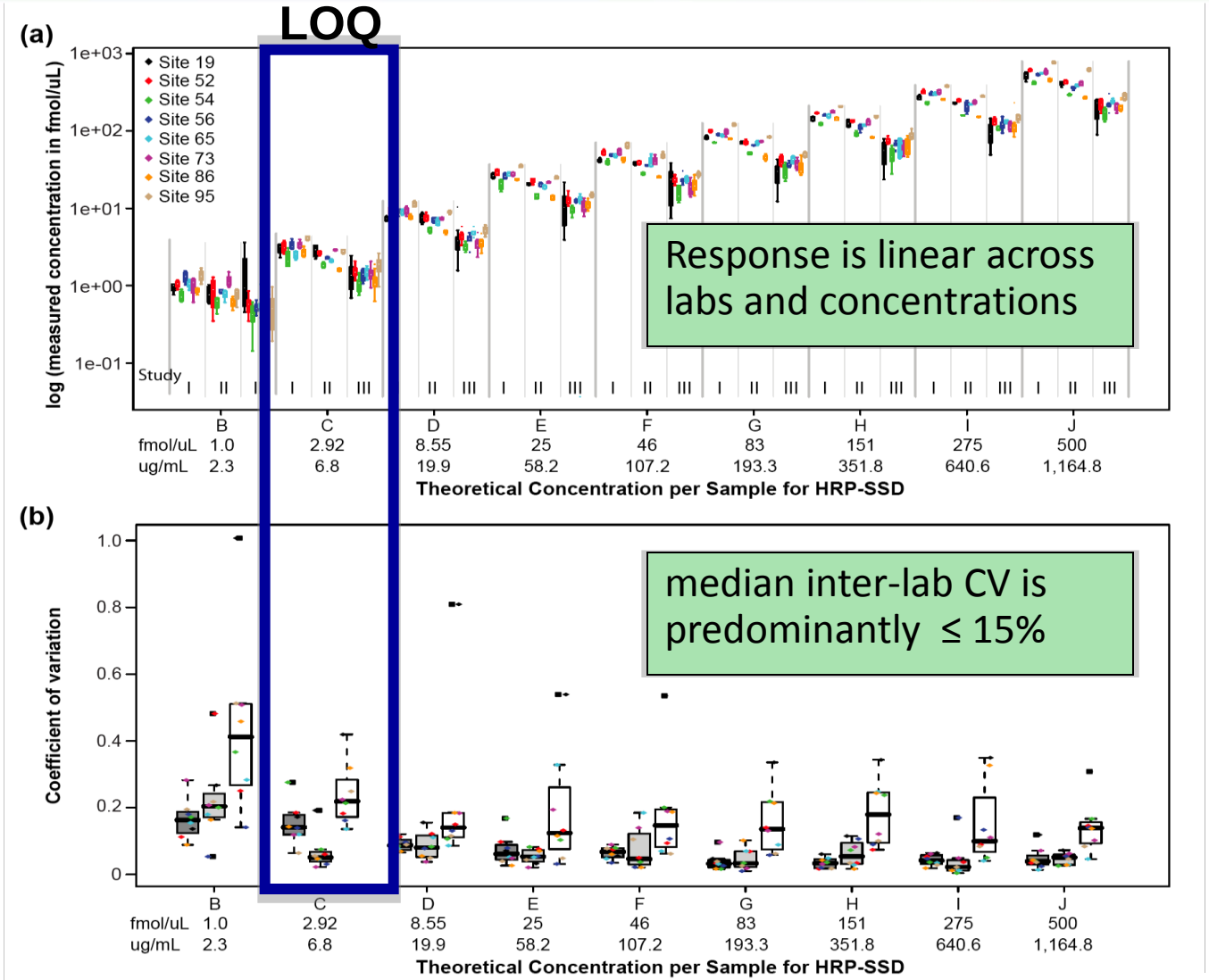
Study II: 5.5%

Study III: 21.9%



Addona et al., Nature Biotechnol., 2009, in press

Intra-lab and Inter-lab reproducibility across the 3 studies at all 9 concentrations



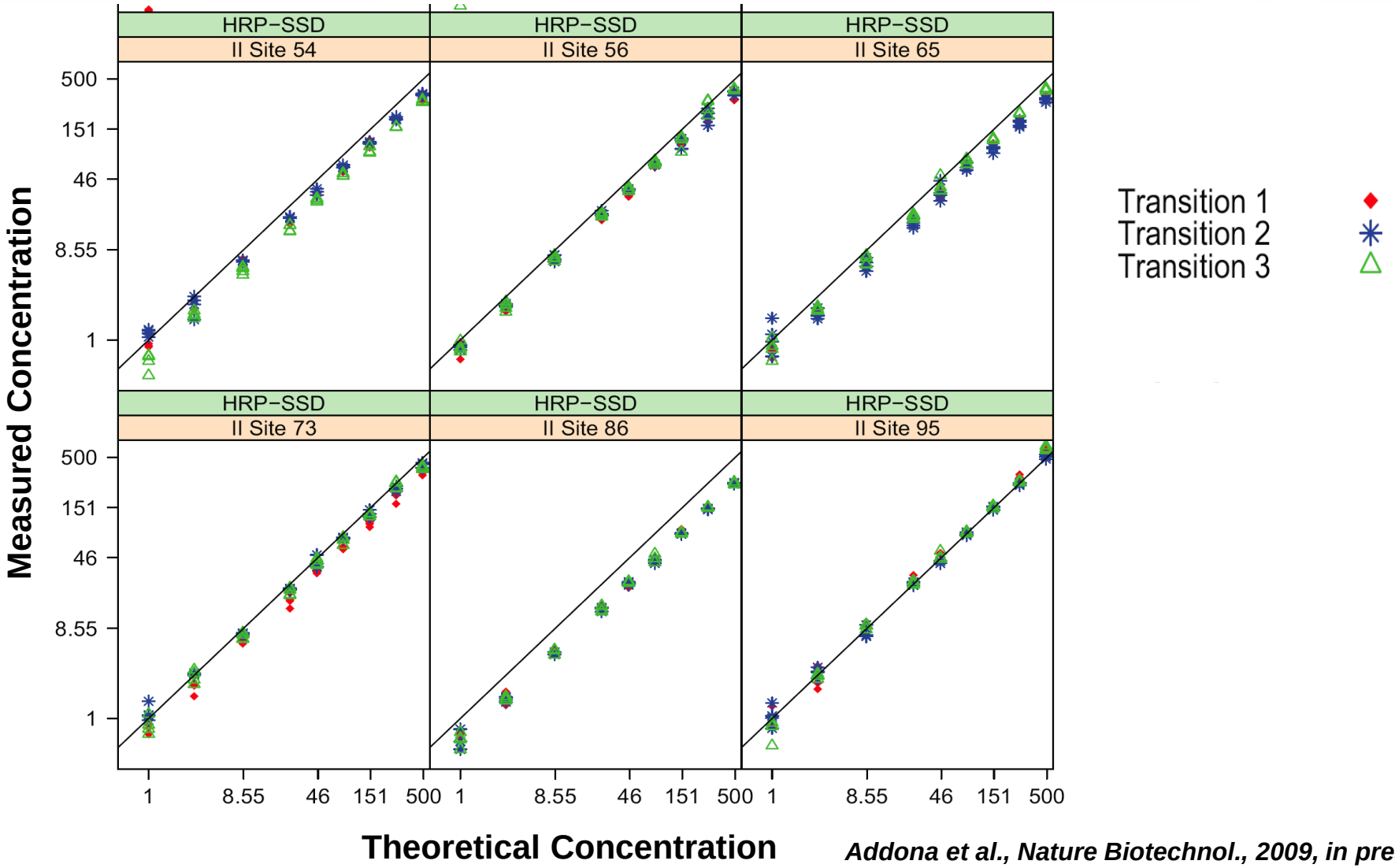
Key

- Site 19
- Site 52
- Site 54
- Site 56
- Site 65
- Site 73
- Site 86
- Site 95

- Study I
- Study II
- Study III

Addona et al., Nature Biotechnol., 2009, in press

MRM-MS concentration response curves are linear and reproducible between labs



Addona et al., Nature Biotechnol., 2009, in press

Major achievements of the CPTAC Verification Work Group

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Assessed MRM-MS performance in complex biological matrices

- first large-scale evaluation of MRM-MS for quantitative measurement of biomarker candidates in plasma
  - precisely measure large numbers of proteins simultaneously
  - highly specificity
  - can be rapidly and robustly configured, deployed across labs
  - achieves near-clinical assay reproducibility (intra, inter-lab)

Developed reagents, methods, datasets as community resource

- enable other labs to benchmark their performance in measuring proteins in plasma
- Aid acceptance and adoption of MRM-MS by proteomics and clinical communities

Addona et al., Nature Biotechnol., 2009, in press

Potential Impact of the CPTAC Verification Work Group Studies

First critical step in evaluation of MS-based assays for verification of novel protein biomarker candidates in plasma

- MRM-MS technology has potential as critical filter to assess protein candidate performance without immunoassays

Provides a critical component for a systematic biomarker pipeline, bridging Discovery to Clinical Validation

Addona et al., Nature Biotechnol., 2009, in press

Important questions and future directions

Can MRM-MS be made useful in the clinic lab?

- “mock” 510K process with FDA begins to define potential for clinical use of MRM-MS for protein assay

Can labs routinely achieve ng/mL LOQ and <25% assay CV for proteins in plasma?

- Inter-lab studies of protein enrichment and SISCAPA

Can plex-level be increased without loss in performance?

- Inter-lab studies of 100-plex using scheduled MRM
- blinded study of ~25 cancer-relevant proteins in plasma

BACKUPS

New approaches are needed to change candidates into potential biomarkers

- Discovery provides 100's of candidates with high (≥ 5-fold) differential expression between cases and controls
- Few samples are used for discovery, but data dimensionality is enormous
  - Most of these discoveries are due to biological or technical variability and are not disease-related
- Discovery "omics" yields biomarker candidates (hypotheses), not clinically useful biomarkers
- Immunoassay-grade Abs do not exist for vast majority of proteins and they will not be made unless market exists
- Need for entirely different technology that avoids need for immunoassay

MS/MS vs MRM-MS:
MRM-MS is faster, more sensitive, more specific

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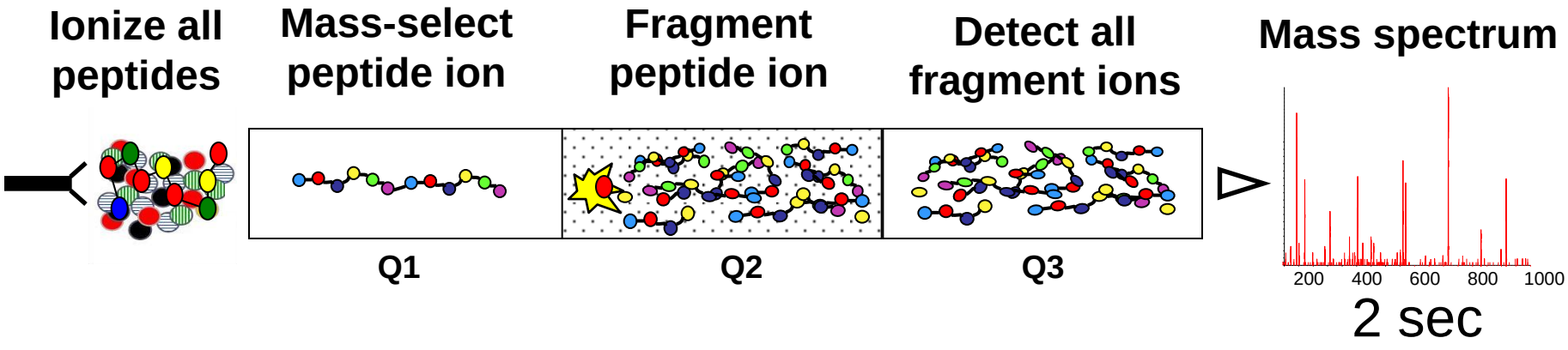
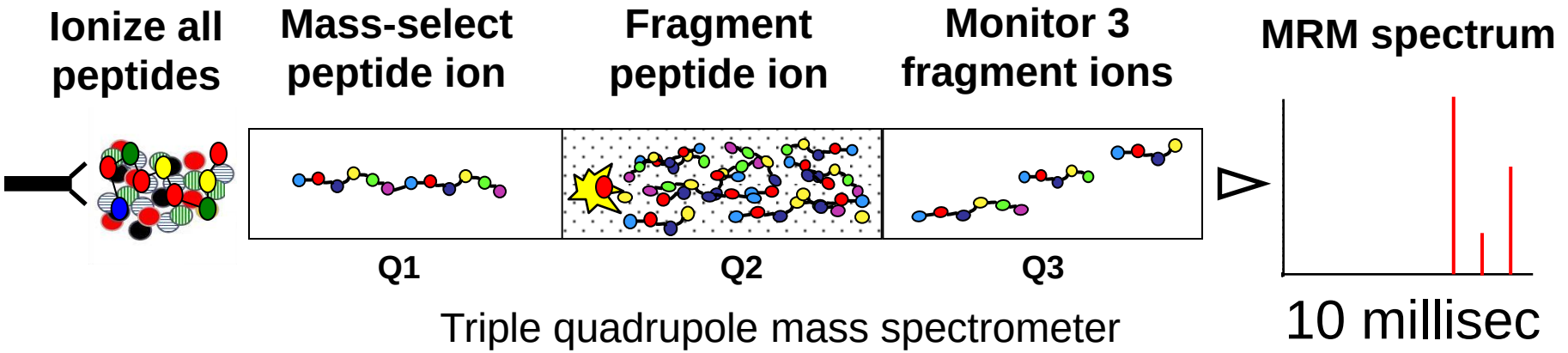
MS/MS Operating Mode

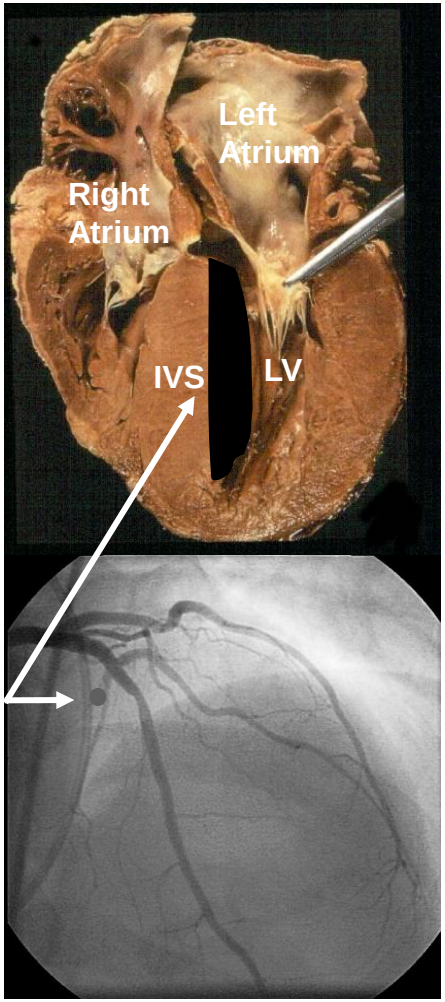


MRM-MS Operating Mode

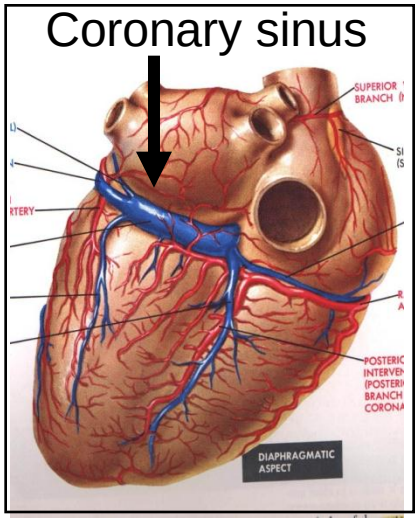


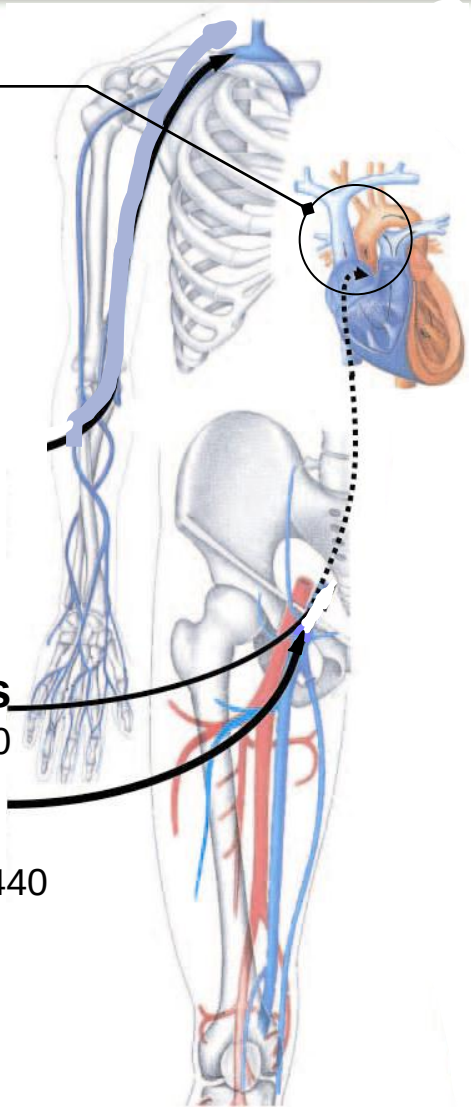
Human Model for Myocardial Injury

Hypertrophic Obstructive Cardiomyopathy (HOCM)



Planned therapeutic myocardial infarction by alcohol ablation

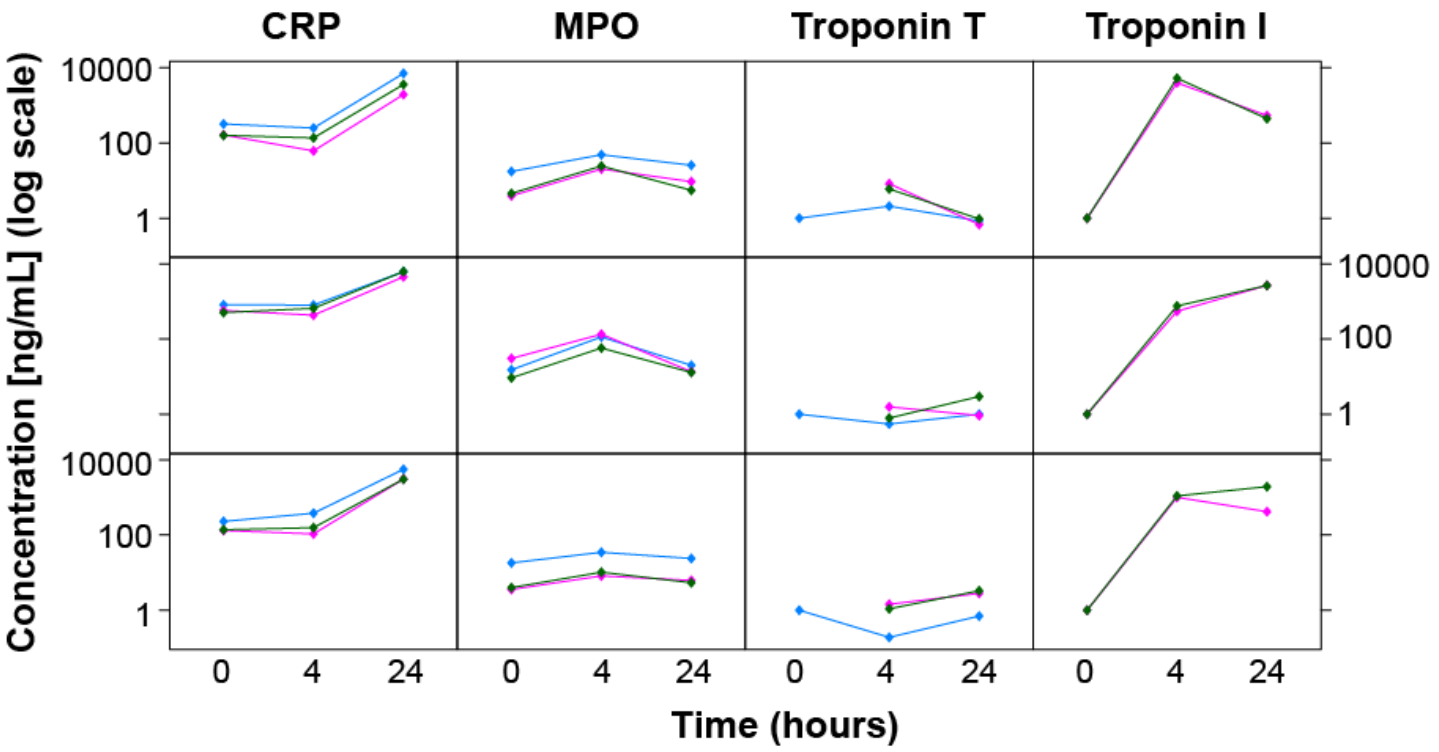




Coronary Sinus Samples
Time (min): Baseline, 10, 60

Femoral Vein Samples
Time (min): Baseline, 10, 60, 240, 1440

MI model provides proof-of-principle that MRM-MS can quantify low ng/mL levels of real biomarkers in plasma with trends consistent with ELISA



- **Inter-assay CV of the process replicates <25%**
- **temporal trends consistent with ELISA**

Keshishian et al., MCP in review

MRM-MS with Ab-capture of peptides increases sensitivity and assay robustness (SISCAPA*)

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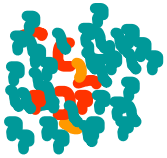
Plasma-derived peptides



1. Add ^{13}C-labeled signature peptide

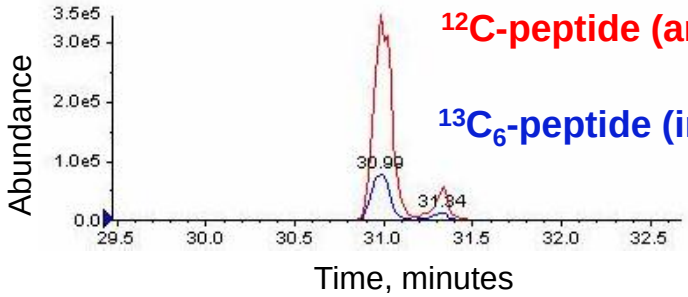
2. anti-peptide Ab capture

NFPSPVDAAFR

NFPSPVDAAFR

native (R = ^{12}C) and exogenous (R = ^{13}C) forms of peptide

Ratio ^{13}C-peptide to ^{12}C-peptide by SID-MRM-MS



^{12}C-peptide (analyte)

$^{13}C_6$-peptide (int. stnd.)

Advantages of SISCAPA

- Easy to obtain useful anti-peptide Abs
- Only requires 1 Ab
- Simpler sample handling prior to LC-MS/MS to reach ng/mL
- High potential to automate

*Anderson et al. (2004) J. Prot. Res.

Multiplexed SISCAPA MRM-MS assay for cTnI and IL-33 achieves LOQ of 1 ng/mL in 50 uL of trypsin digested patient plasma

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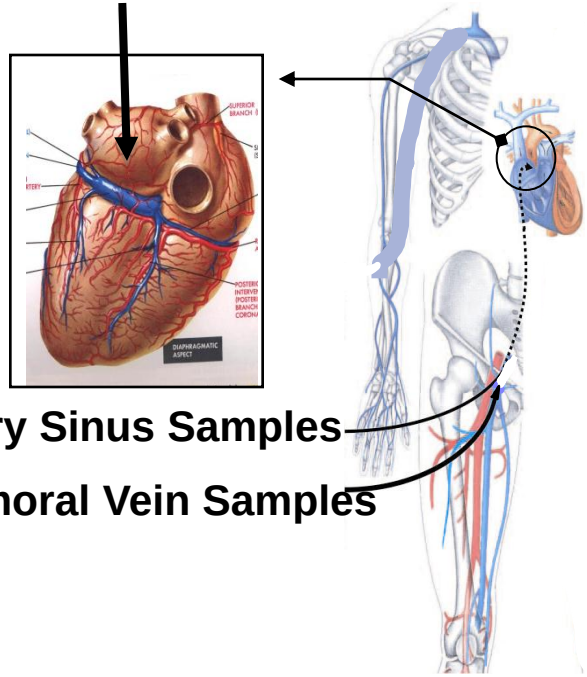
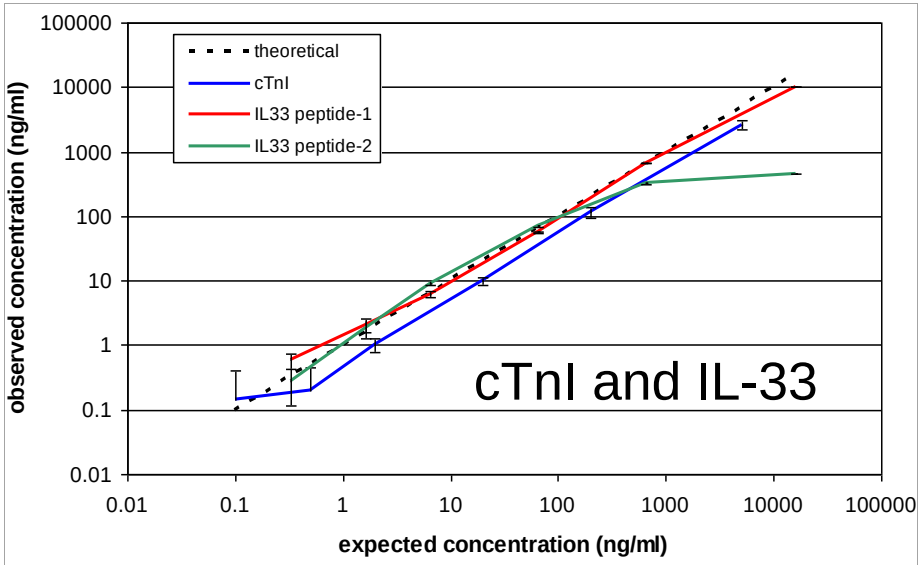
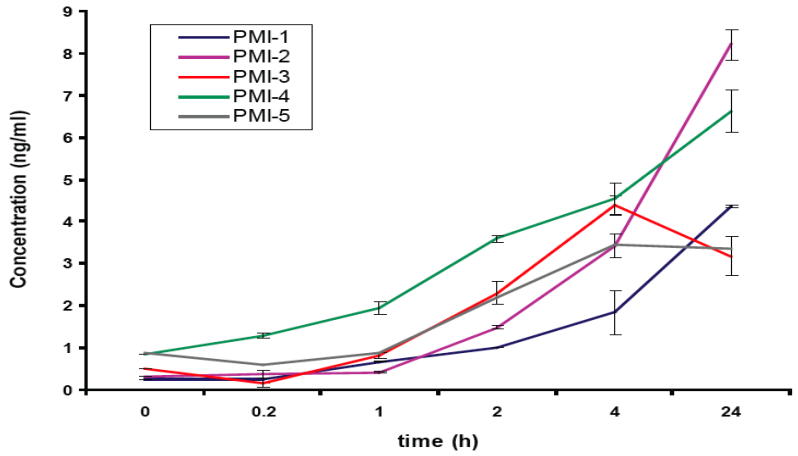
LOQ of ca. 1 ng/mL directly from 50 uL of plasma

Coronary sinus







Kuhn et al. Clinical Chemistry 2009, 55, 1108-1117

Limits of detection and quantitation are highly reproducible within and across labs

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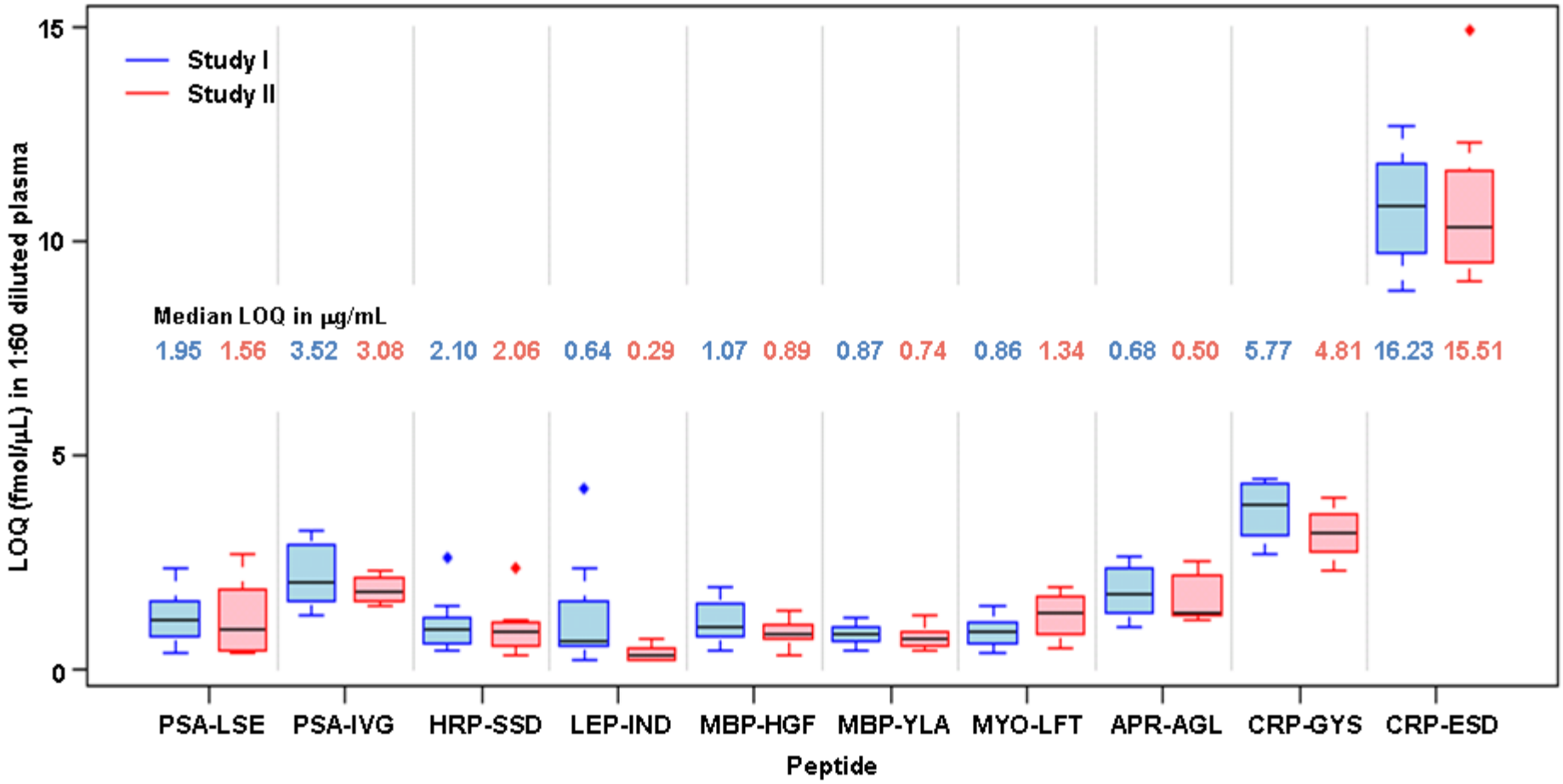
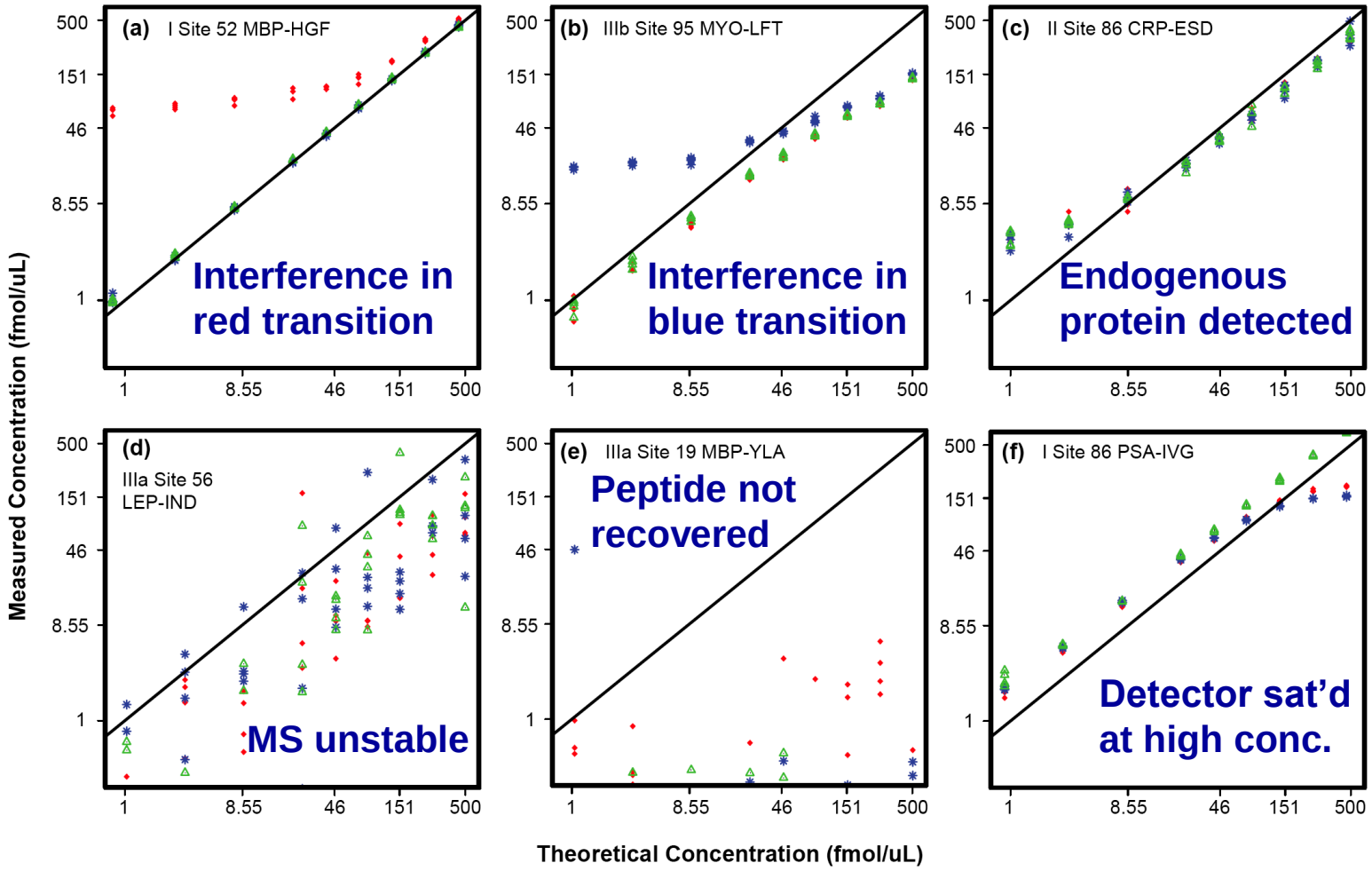


Problems do arise, but are readily detected and resolved



provides guidance to community on assay construction and use