Generating Quantitative Assays for Biomarker Development

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Reliable assays are available for <5% of human proteins.

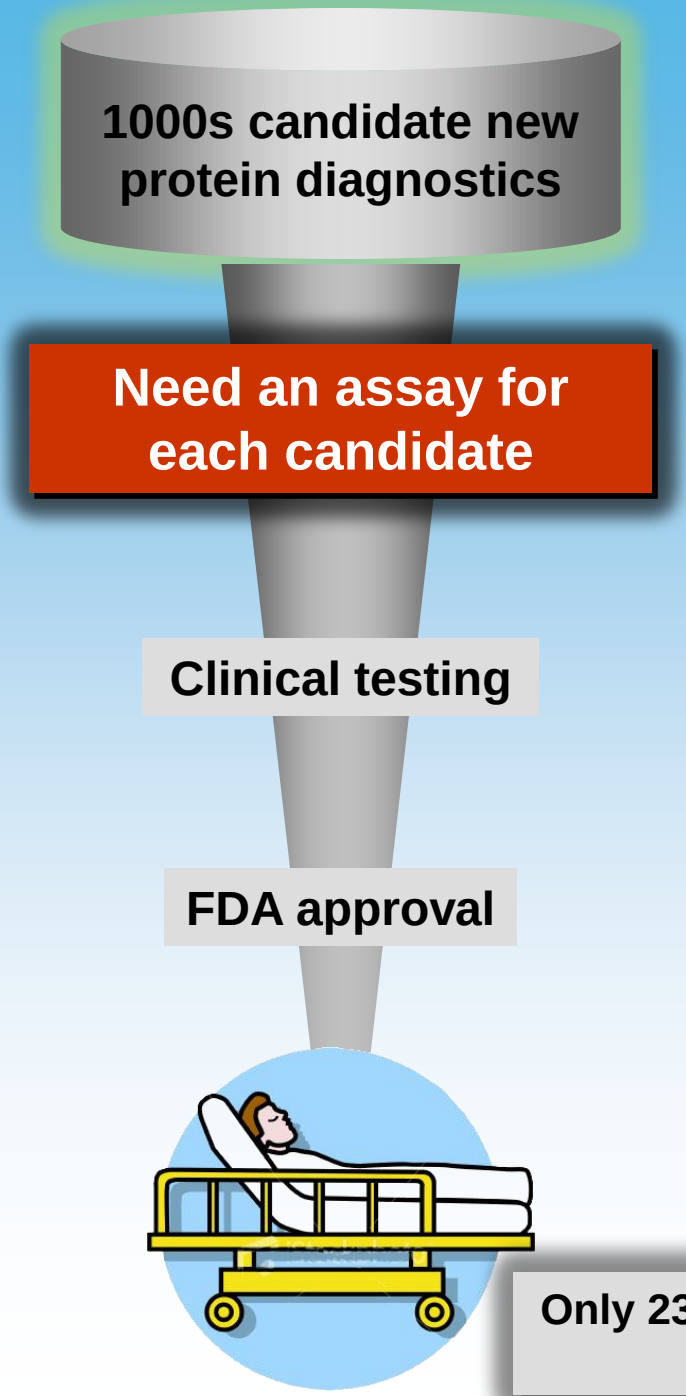
1. Assay development is largely commercially-driven.

- Every company focuses on the same “popular” proteins; there are many “orphan” proteins.
- There’s no coordinated global divide-and-conquer effort.
- Assay validation is prohibitively expensive, and many bad assays go to market.

2. Existing technologies are not readily scaled.

- High cost ($100k - $2 million per protein assay)
- Long development lead time (1-2 years)
- High rate of failure
- Poor performance characteristics

Most of the human proteome is clinically not accessible!



The lack of "assays" to human proteins prevents potential new protein diagnostics from ever being tested.

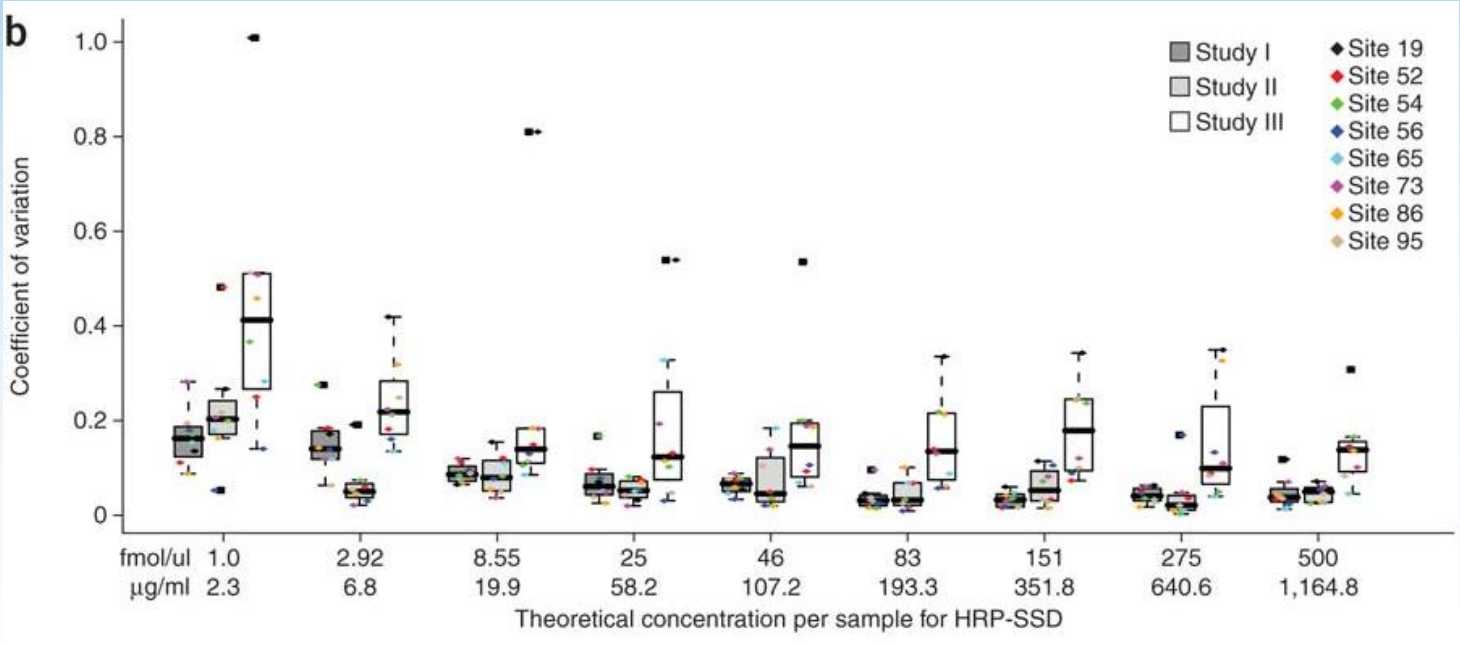
- There are no assays for most human proteins. We desperately search commercial sources for assays to test candidates, but **few assays are available**.
- *De novo* assay generation is prohibitively expensive and requires expertise.
- Most candidates have no clinical utility, and we can't yet predict which will.
- Very few candidates are tested, and almost none achieves clinical validation.

Only 23 protein biomarkers have cleared the FDA since 2003.

SRM/MRM assays have the potential to dramatically impact protein biomarkers.

Advantages of SRM/MRM

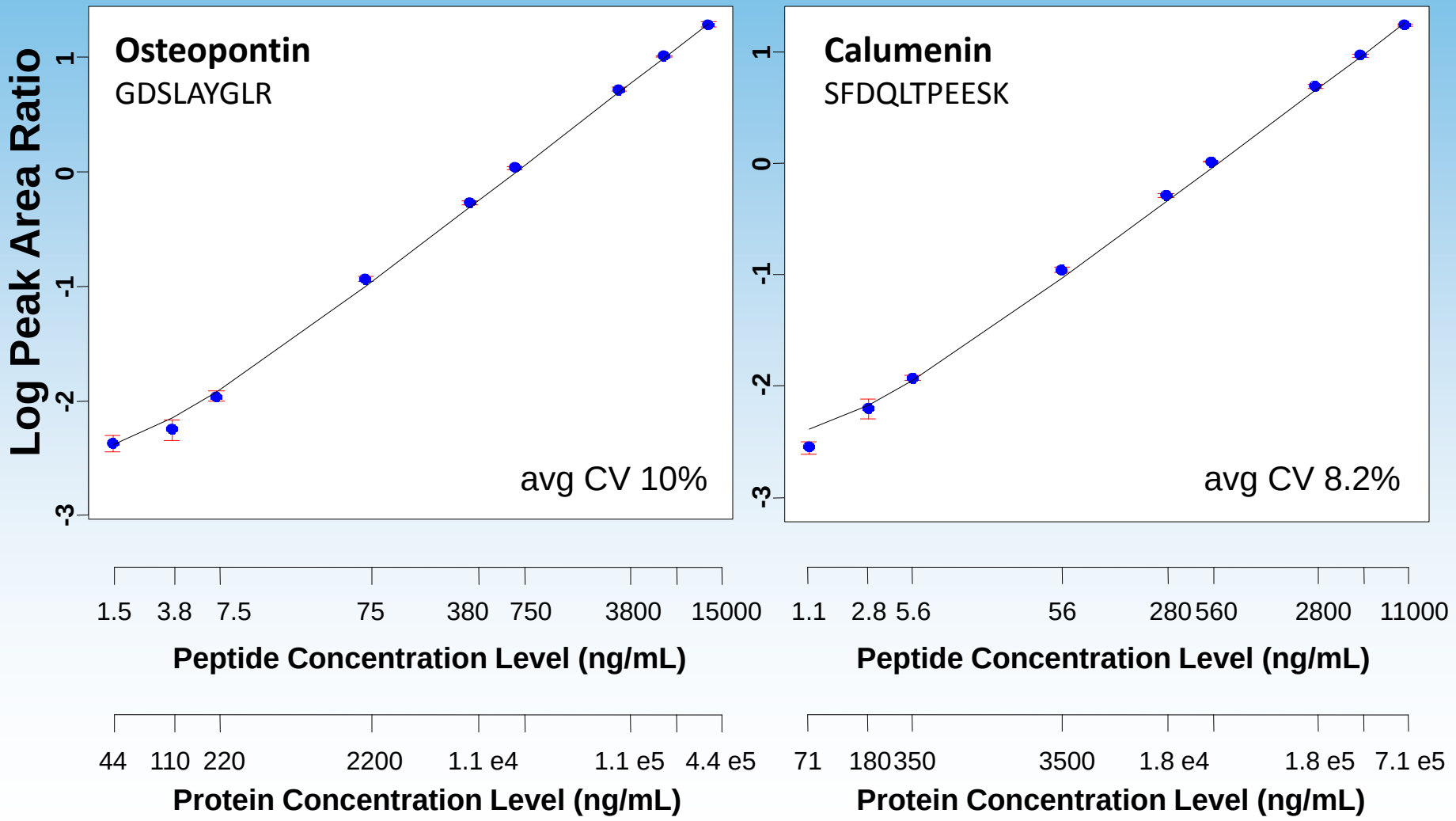
- Robust
- Portable
- Reproducible
- Quantifiable reference standard
- Relatively less expensive
- Specific
- Multiplexable



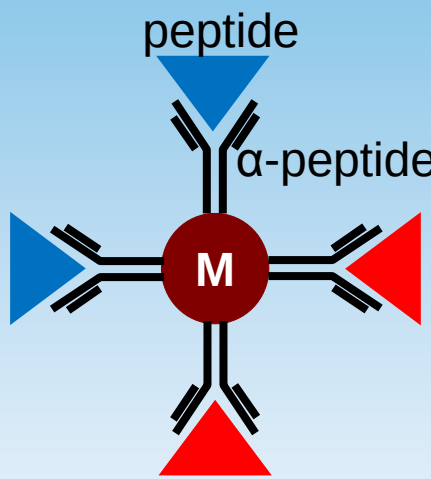
Nat Biotechnol. 2009 27(7):633-41.
Clin Chem. 2010 56(2):161-164.

Analytical performance of MRM-based assays is robust, but sensitivity is an issue.

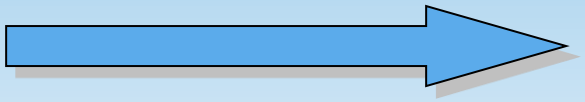
The limit of quantification of SRM in neat plasma is 100-1000s of ng protein / mL.



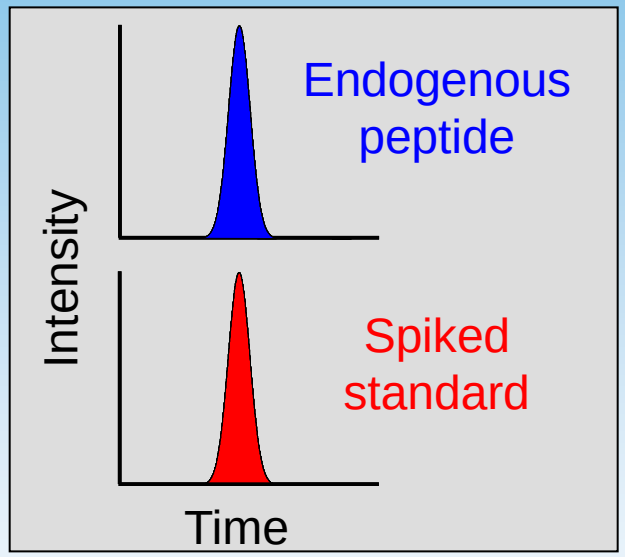
Immuno-MRM assays couple immuno-enrichment of peptide analytes to targeted mass spectrometry.



SISCAPA uses an α-peptide antibody to enrich endogenous and spiked standard peptides

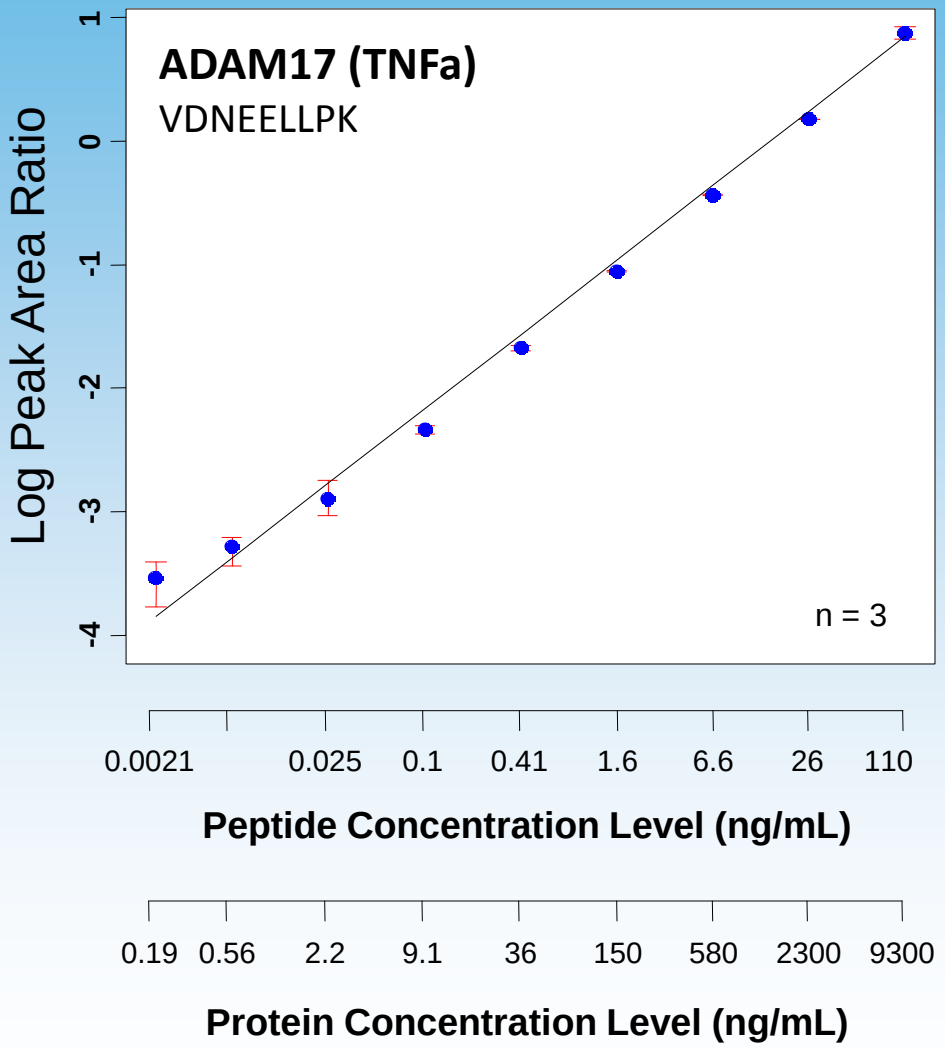


Measure



Anderson *et. al*., J Proteome Res. 2004, 3(2):235-44.

Immuno-MRM assays are sensitive and precise, and high-affinity monoclonals can be isolated.



$LOD^*_{protein}$ = 2.7 ng/mL

$LOQ^*_{protein}$ = 6.3 ng/mL

Average %CV = 13.7%

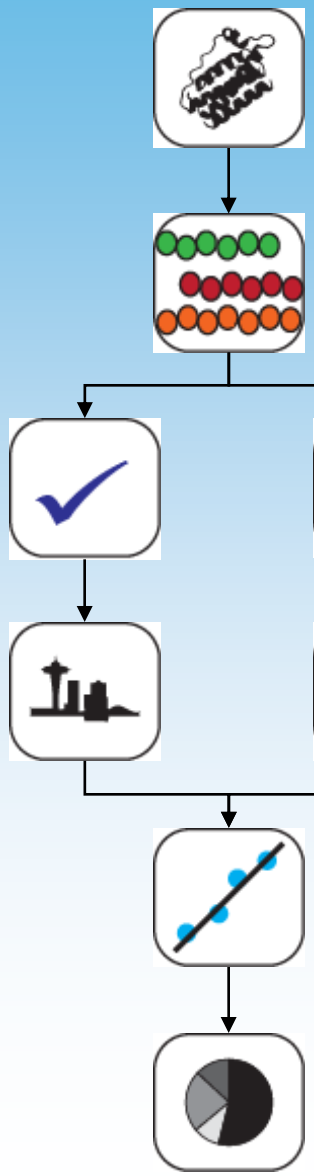
* 10 microliters plasma capture; achieve low pg/mL from 1 mL plasma
* assumes complete trypsin digestion

Schoenherr *et. al. J Immunol Methods*. 2010, 353:49-61.
Whiteaker *et. al. MCP.* 2010, 9:184-96.

Characterizing the process of assay generation

- **What does it cost?**
- **How long does it take?**
- **What is the success rate?**
- **Are the assays multiplexable?**
- **Are they amenable to a verification study?**

Immuno-MRM assays have been characterized for ~300 target peptides.



Protein list

Identify proteotypic peptides

Multiplex immunogens

Affinity-purify antibodies

Assay performance
Spike peptides into plasma at 4 concentrations generate immuno-MRM response curves

Evaluate performance & success rate

Synthetic Peptide QC
- ✓ Peptide purity
- ✓ Peptide concentration

Develop methods
- ✓Skyline

- 7-8 months per tranche
- 100s per tranche

Success rates are high for generating immuno-MRM assays to proteotypic peptides.

Assay Grade	Approximate detection level	Number of assays	Percent of total
A	0.05 fmol/μL or <10 ng/mL	63	29%
B	0.5 fmol/μL or 10-100 ng/mL	53	25%
C	5 fmol/μL or 100 ng/mL	46	21%
D	50 fmol/μL or 1 μg/mL	32	15%
F	Not detected	22	10%
	Total	216	

per **protein** success rates for generating grades A-B assays

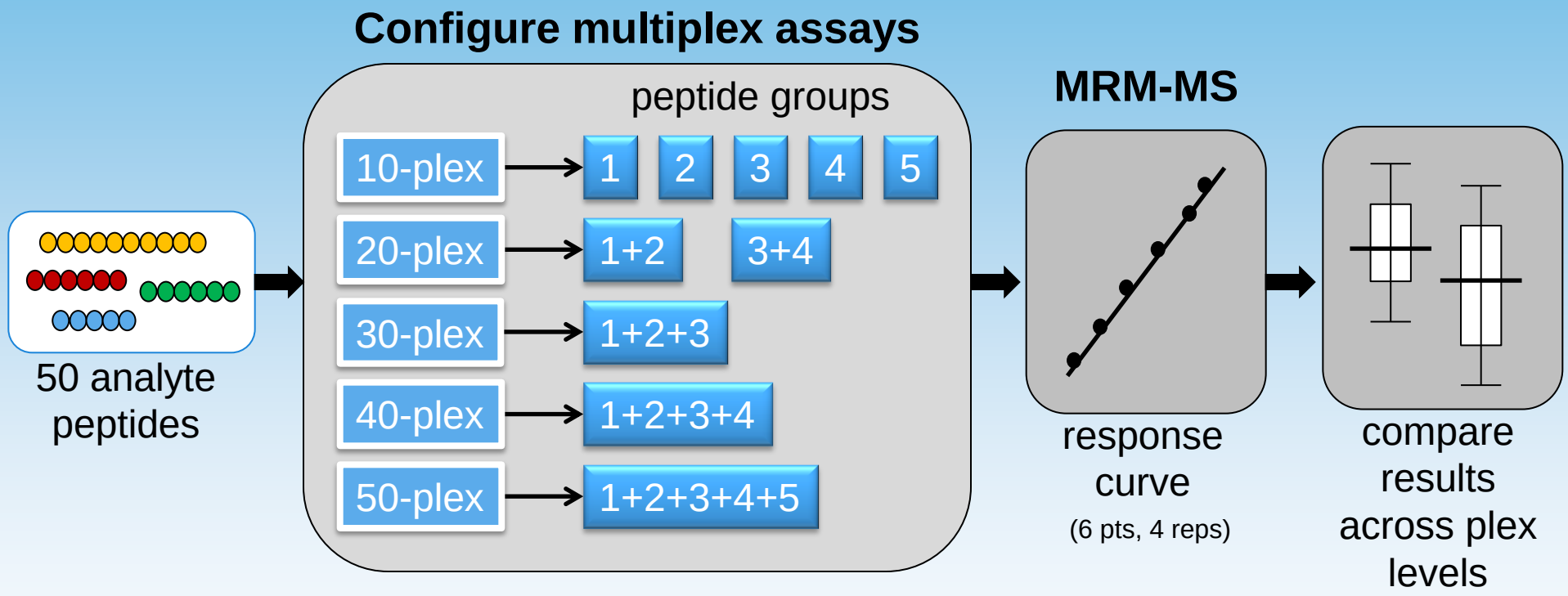
Number of antibodies that underwent affinity purification

Number immunogens multiplexed per animal	**1**	**2**	**3**
1	1/2		
2		2/3	
3		2/4	1/1
4		5/8	5/7
5	1/1	23/29	32/34

79% **94%**

Whiteaker *et. al. Molecular and Cellular Proteomics.* **2011**, Apr;10(4):M110.005645

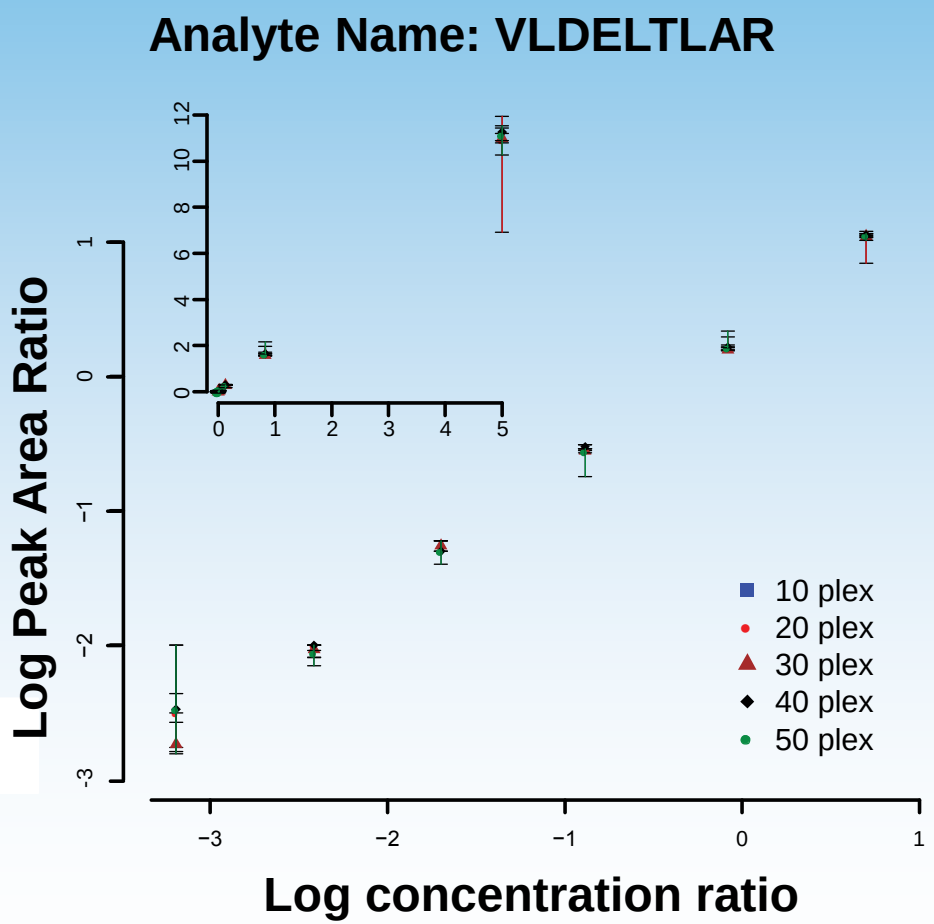
Immuno-MRM assays are readily multiplexed.



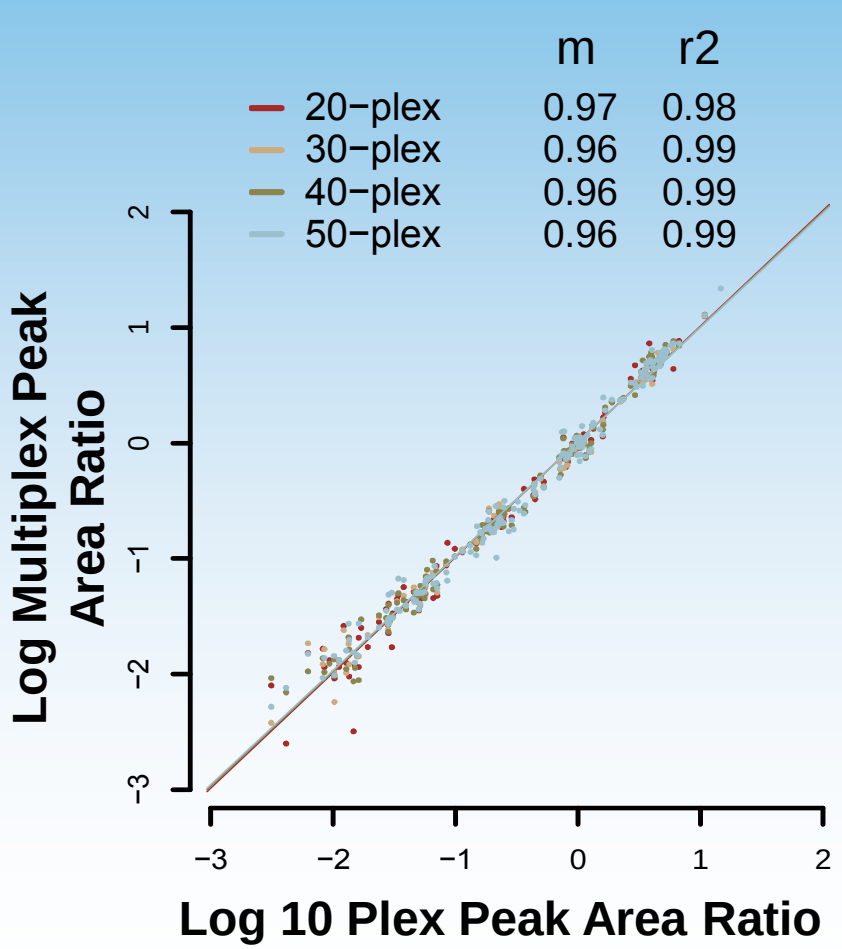
Whiteaker *et. al. Molecular and Cellular Proteomics.* 2011 Dec 27. [Epub ahead of print]

We observed a 98% success rate for configuring 48-plex immuno-SRM assays.

Equivalent performance at each mux level (98%)

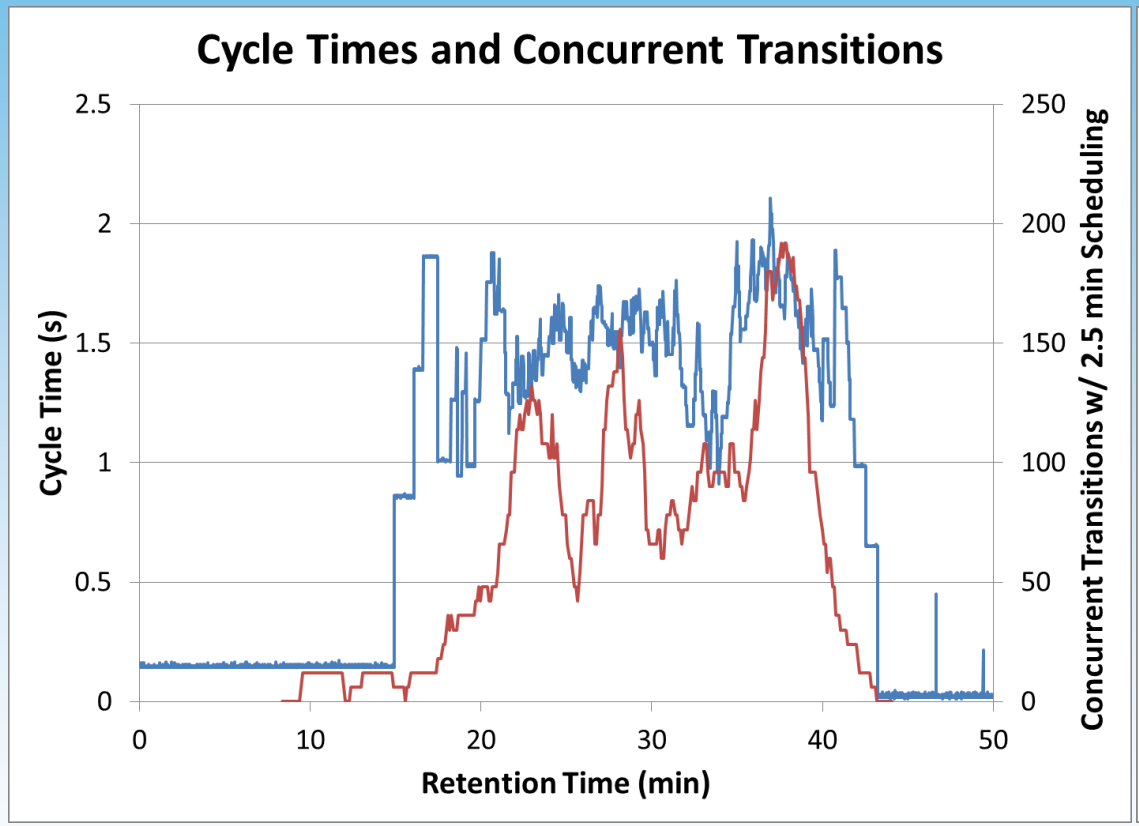


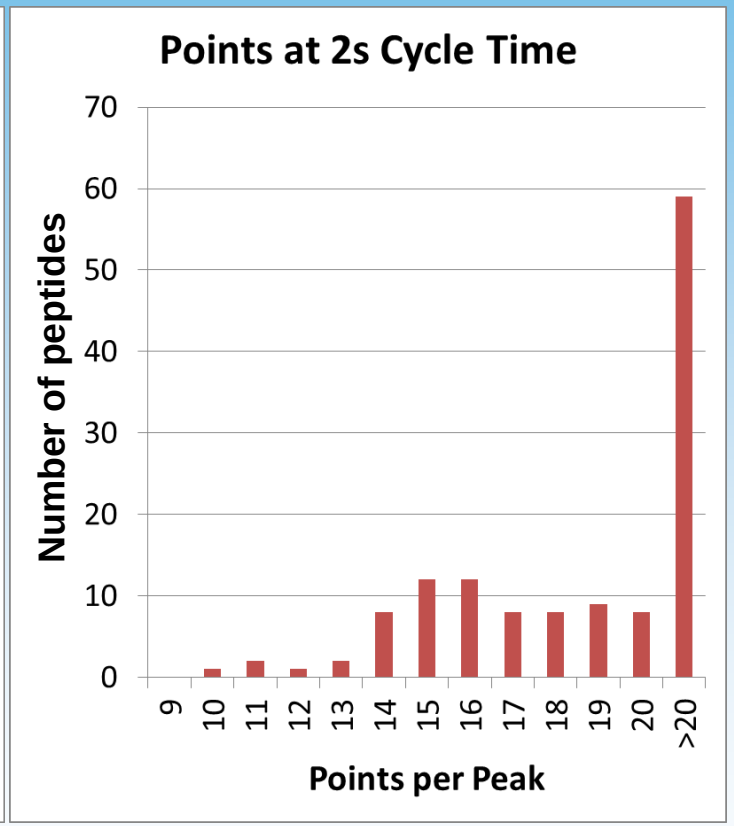
Response at all –plex levels are highly correlated



Expanding multiplexing using MRM

150 peptides, 900 transitions

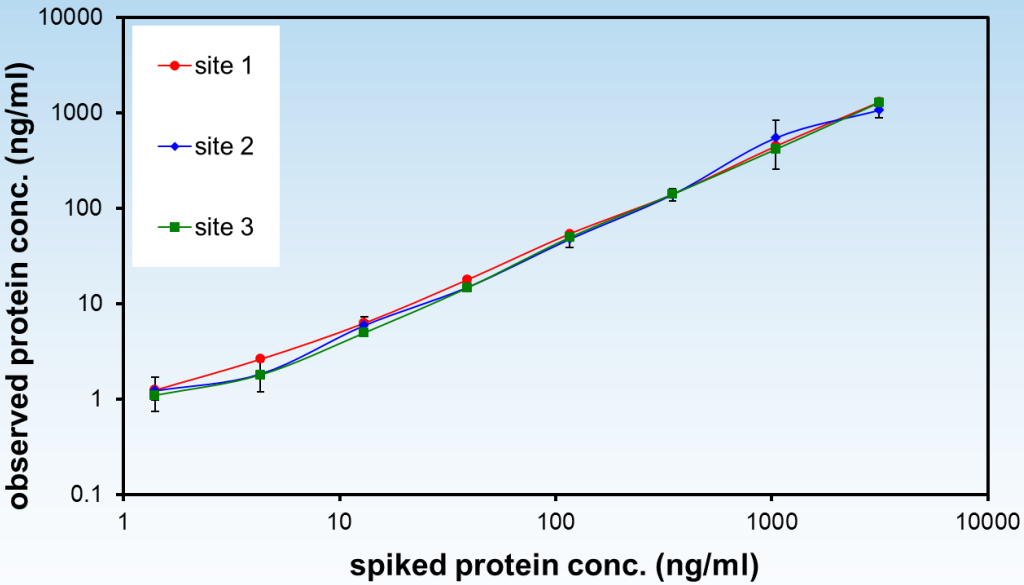




Jake Kennedy WOH, 3:50pm

Inter-laboratory reproducibility is high

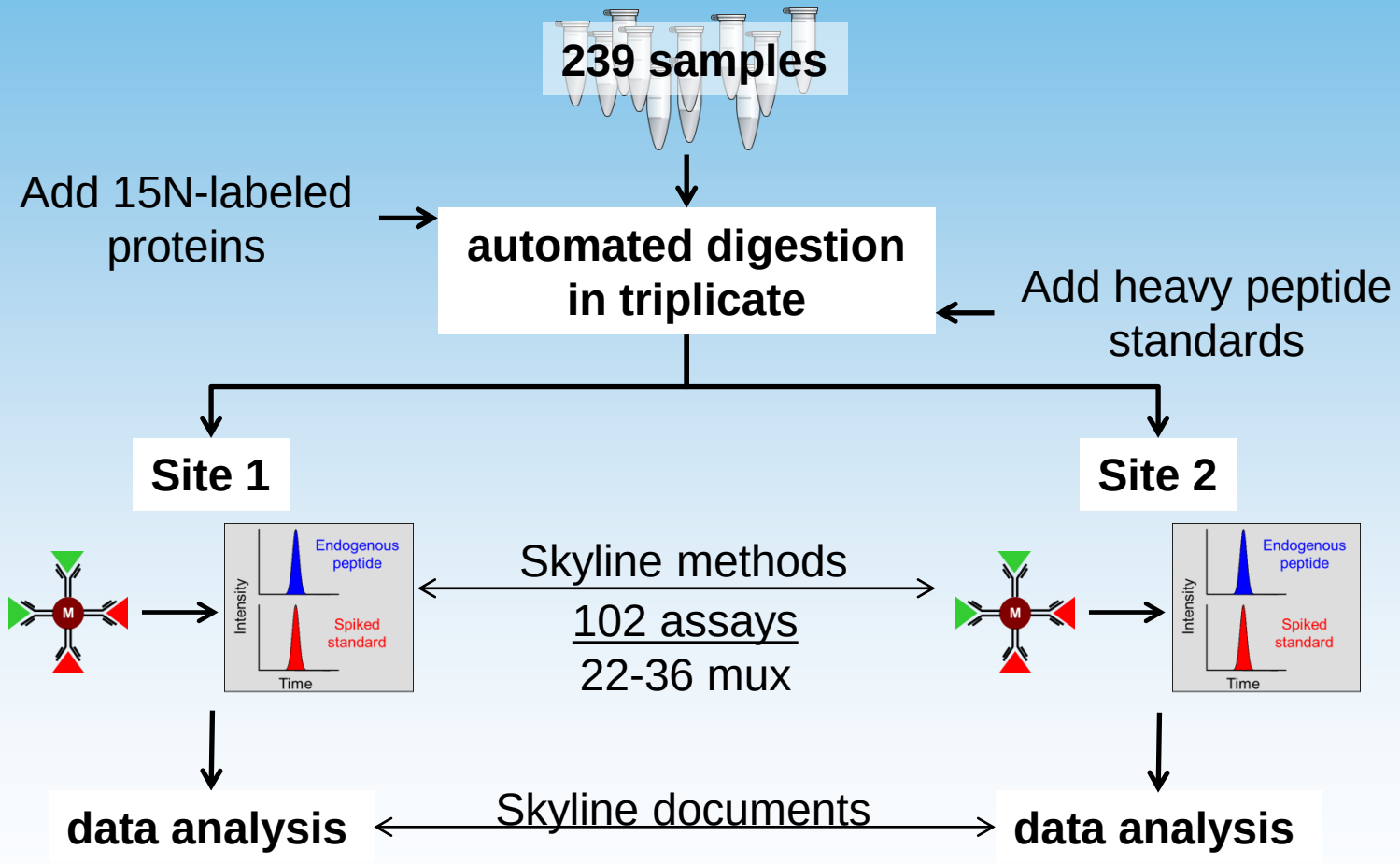
8-plex assay measured in three laboratories



Gene.Peptide	Interlab CV
S100A8.AMV	10.8%
S100B.ELI	14.2%
CSF3.IQG	6.7%
S100A8.ALN	5.5%
S100A12.GHF	10.2%
IL1RN.IDV	8.1%
S100A7.GTN	7.1%
S100A7.ENF	4.3%
median	*7.6%*

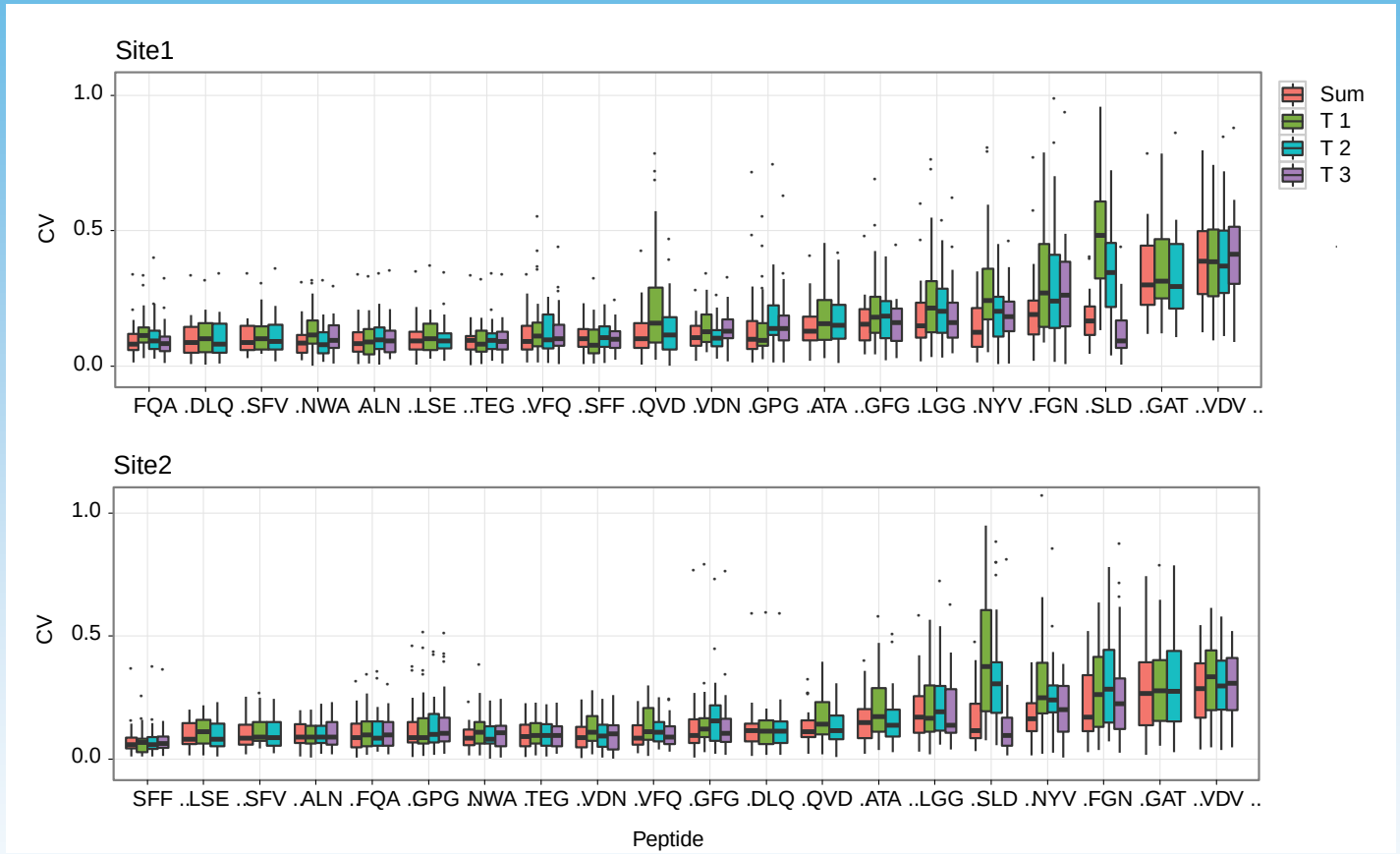
Kuhn *et. al. Molecular and Cellular Proteomics.* 2011 Dec 22. [Epub ahead of print]

Implementing assays in a biomarker verification setting across multiple laboratories.



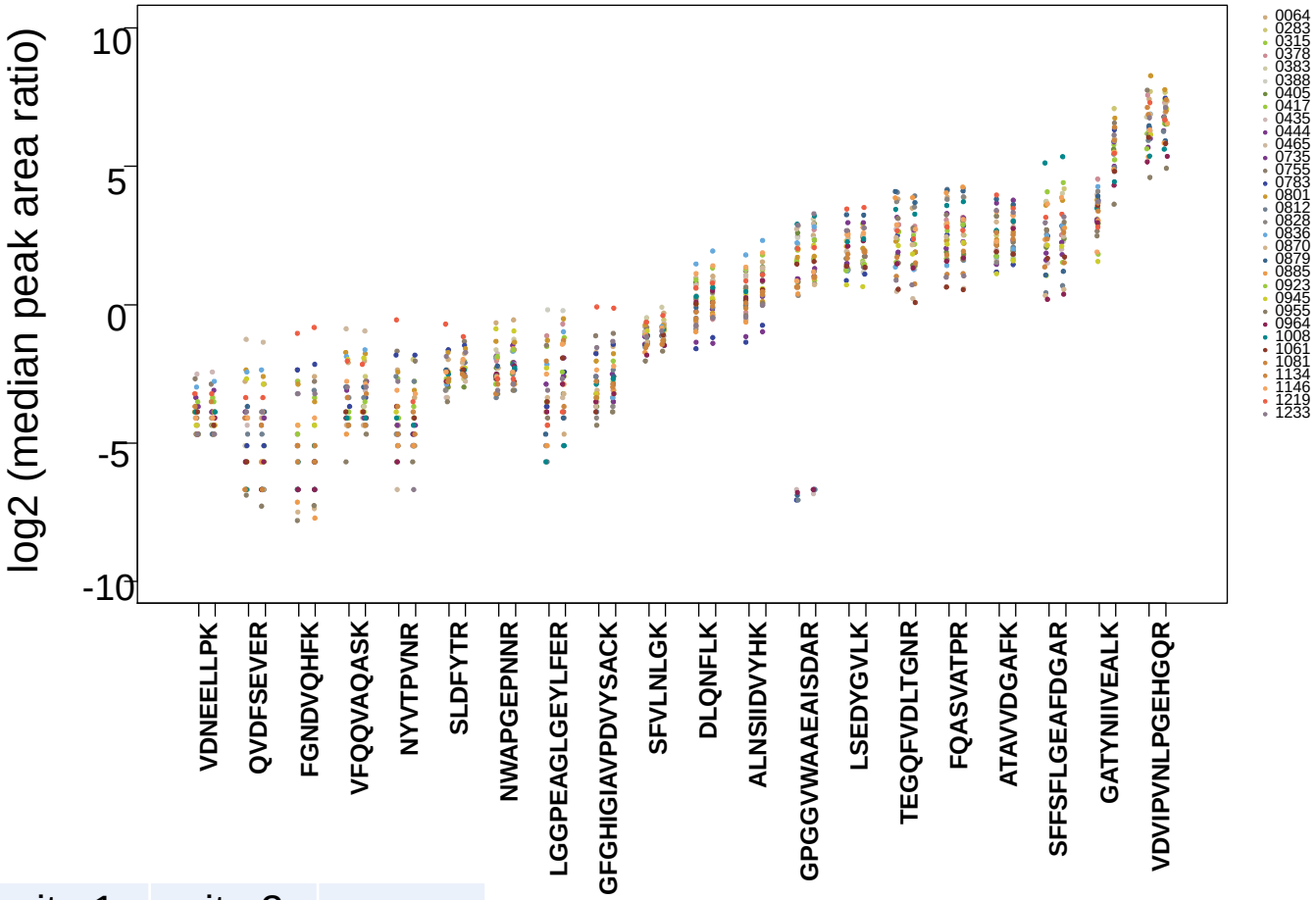
Manually checking integration and adding annotation

Analytical CVs are acceptable for the majority of assays



CV	site 1	site 2	
	0.081	0.058	min
	0.387	0.286	max
	0.101	0.094	median

Biological variation can be significantly higher.



	site 1	site 2	
Inter-individual CV	0.244	0.257	min
	1.521	1.482	max
	0.593	0.534	median

Conclusions

1. There is a protein assay technology that can be scaled for precise, specific, multiplex quantification of large suites of human proteins in large sample sets; this has the potential to have impact *across the biomedical sciences*.

2. The Skyline software behind our development efforts has truly been enabling
    1. Transition selection, evaluation, and optimization
    2. Standardization of methods across laboratories/platforms
    3. Ease of method and data sharing

FHCRC

Paulovich laboratory

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