Reproducibility, Stability Testing, Repeatability

Tuesday, May 2 – 11:00am session

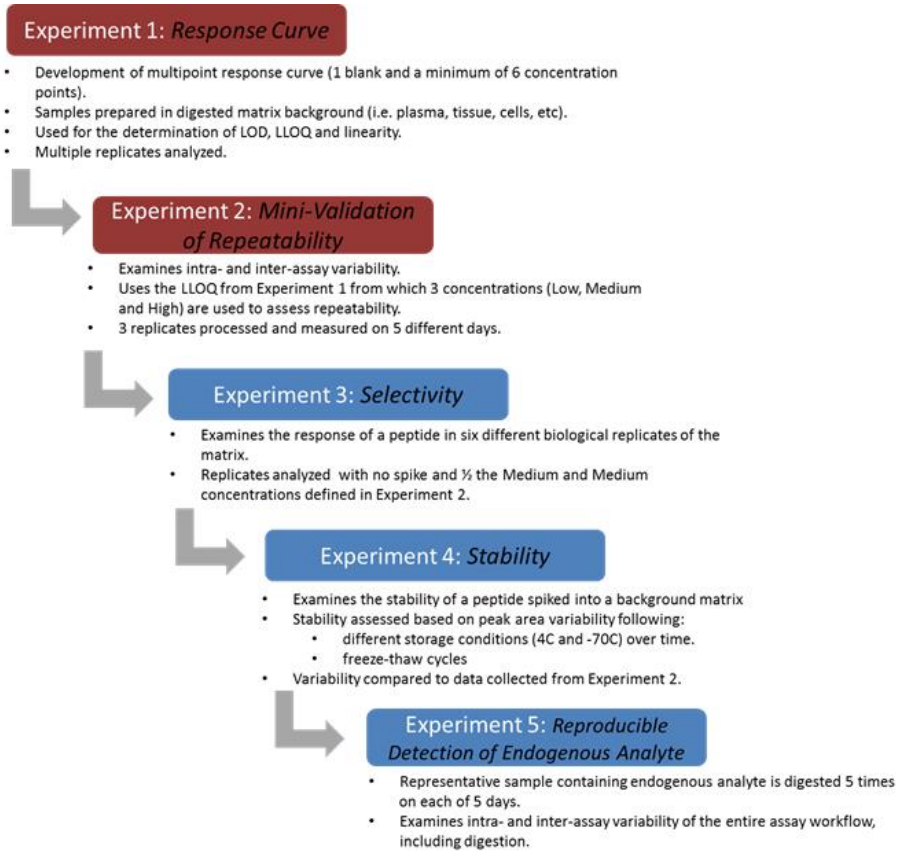
Targeted Proteomics with Skyline

objectives

- Assess sources of variation in your specific experiment, and consider how to account for them
- Identify appropriate validation experiments for your project

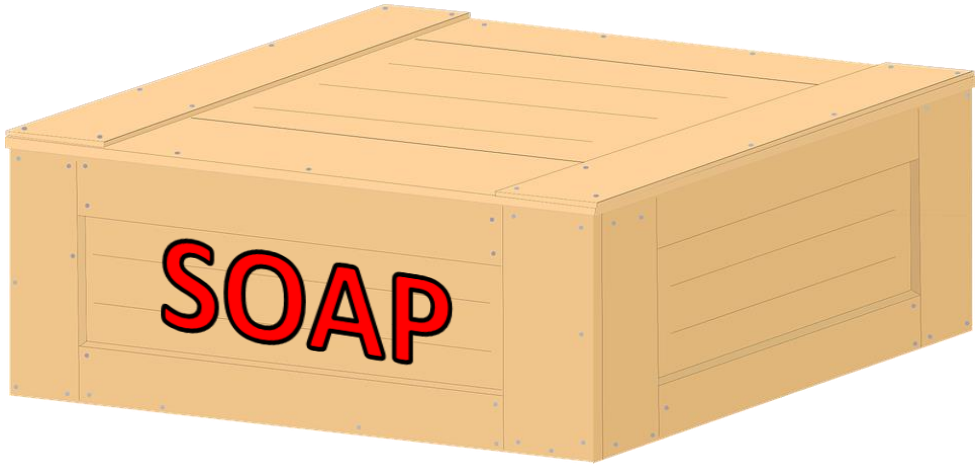
Agenda

- **Validation recommendations**
- Assay Portal validation requirements



Before we continue, a quick PSA!

- In this section, we emphasize that quantitative proteomics is not possible without extensive quality control
  - We will teach you ways that have worked for us.
- We emphasize when a measurement is and is not quantitative.
  - Not everyone will agree on this.
- You may hear contradictory things from different instructors.



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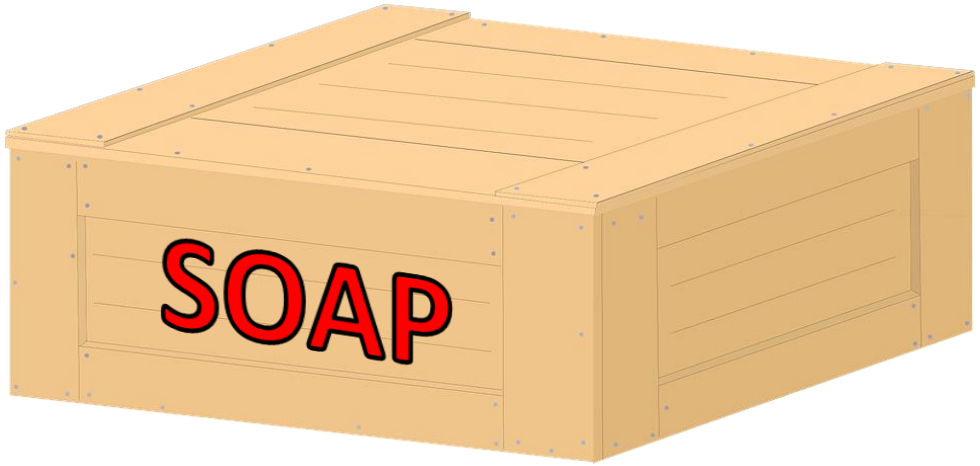
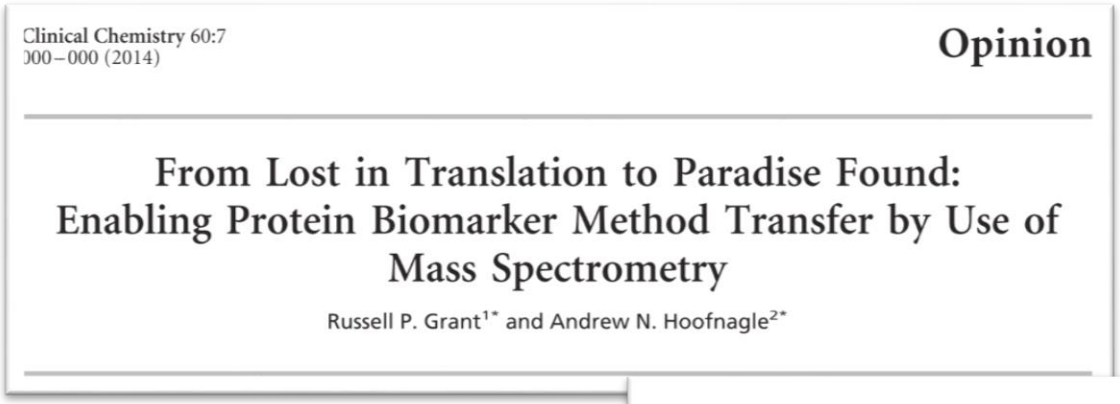
All in service of meaningful, reproducible science



Meaningful, reproducible quantitative MS proteomics

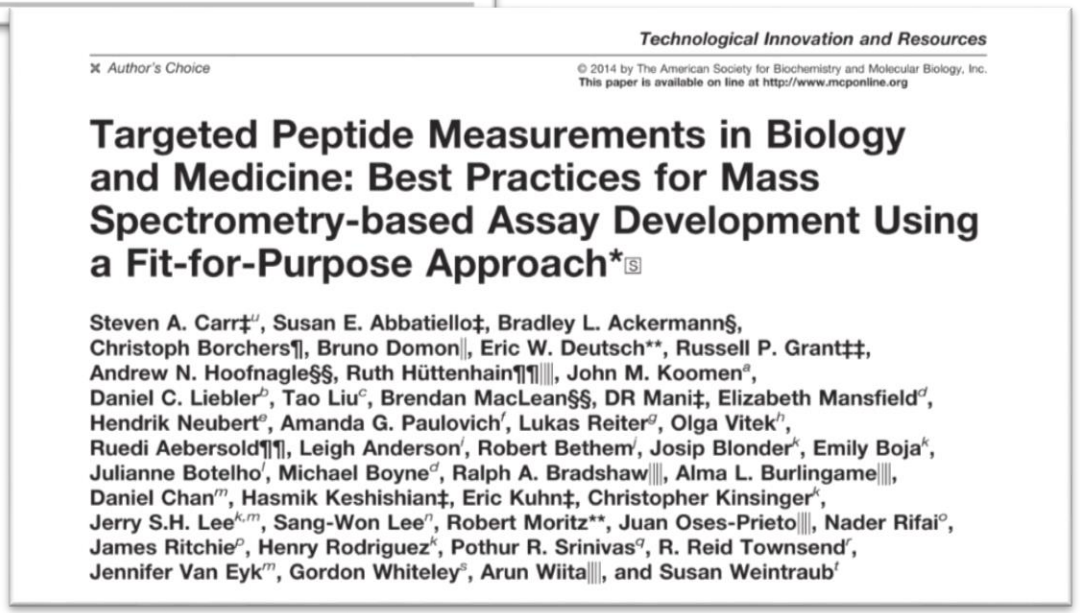


Clinical Chemistry 60:7
000–000 (2014)

Opinion

From Lost in Translation to Paradise Found: Enabling Protein Biomarker Method Transfer by Use of Mass Spectrometry

Russell P. Grant[1*] and Andrew N. Hoofnagle[2*]



Technological Innovation and Resources

Author's Choice

Targeted Peptide Measurements in Biology and Medicine: Best Practices for Mass Spectrometry-based Assay Development Using a Fit-for-Purpose Approach*

Steven A. Carr‡[u], Susan E. Abbatiello‡, Bradley L. Ackermann§, Christoph Borchers¶, Bruno Domon‖, Eric W. Deutsch**, Russell P. Grant‡‡, Andrew N. Hoofnagle§§, Ruth Hüttenhain¶¶‖‖, John M. Koomen[a], Daniel C. Liebler[b], Tao Liu[c], Brendan MacLean§§, DR Mani‡, Elizabeth Mansfield[d], Hendrik Neubert[e], Amanda G. Paulovich[f], Lukas Reiter[g], Olga Vitek[h], Ruedi Aebersold¶¶, Leigh Anderson[i], Robert Bethem[j], Josip Blonder[k], Emily Boja[k], Julianne Botelho[l], Michael Boyne[d], Ralph A. Bradshaw‖‖, Alma L. Burlingame‖‖, Daniel Chan[m], Hasmik Keshishian‡, Eric Kuhn‡, Christopher Kinsinger[k], Jerry S.H. Lee[k,m], Sang-Won Lee[n], Robert Moritz**, Juan Oses-Prieto‖‖, Nader Rifai[o], James Ritchie[p], Henry Rodriguez[k], Pothur R. Srinivas[q], R. Reid Townsend[r], Jennifer Van Eyk[m], Gordon Whiteley[s], Arun Wiita‖‖, and Susan Weintraub[t]

Validation Recommendation 1: "Fit for purpose" tiers of validation

TABLE I

Three Tiers of Targeted MS Measurements; experimental design parameters and assay characteristics are listed for each tier

Tier and Areas of Application	Degree of Analytical Validation	Labeled Internal Standards	Reference Standards	Specificity	Precision	Quantitative Accuracy	Repeat-ability	Comments and Suggested References
Tier 1 **Clinical bioanalysis/ diagnostic laboratory test; single analyte or small numbers of analytes**	High, including batch-to-batch QC	Yes, for every analyte	Yes	High	High (typically <20-25% CV achieved)	Defining accuracy is a goal; true accuracy difficult to demonstrate.	High	Precise, quantitative assays; established, high performance; may need comply with FDA and CLIA guidance depending on use of assay Refs. 30, 41, 42, 53
Tier 2 **Research use assays for quantifying proteins, peptides, and post-translational modifications; 10's to 100's of analytes**	Moderate-to-high	Yes, for every analyte	Limited use	High	Moderate-to-high (typically <20-35% CV achieved)	Not applicable	High	Precise, relative quantitative assays; established performance; suitable for verification Refs. 30, 31, 36, 37, 40, 51, 70, 71
Tier 3 **Exploratory studies; 10's to 100's of analytes**	Low-to-moderate	None-to-limited	No	Moderate-to-high	Low-to-moderate: similar to label-free discovery	Not applicable	Moderate-to-high	Discovery in a targeted mode; performance not defined; results require further verification using quantitative techniques Refs. 36, 37, 86-89

Carr 2014

Validation Recommendation 2: Minimal set of experiments for quantification

Table 1. List of minimal experiments for assay validation of LC-MS/MS protein quantification.

Experiment	Description	Determination	Best practice[a]
Reproducibility	Healthy and disease pools are analyzed 5 times on each of 5 days.	CV_{intra} and CV_{Inter}, CV_{total} as the sum of squares.	CV_{intra} and CV_{Inter} <20%
Peptide stability	Internal standard peptides are spiked before and after digestion to both pools.	Bias and CV of triplicate samples when IS added predigestion vs postdigestion.	Bias, CV <20%
Linearity	Healthy and disease pools are admixed 3:1, 1:1, and 1:3.	Bias and CV of triplicate admixed samples compared to extrapolated values from $inter_{mc}$ determinations.	Bias, CV <20%
Lower limit of quantification	Healthy pool is diluted with an analyte-free surrogate matrix or matrix from another species.	Bias and CV of triplicate diluted samples compared to expected values from $inter_{mc}$ determinations incorporating dilution factor.	Bias, CV <25%
Interferences	Clinically relevant potential interferents are added to the healthy pool.	CV of triplicate spiked samples. Bias when accounting for dilution of spiking (5%–50% dilution depending on interferent solution) compared to expected values from $inter_{mc}$ determination.	Bias, CV <20%
Stability	Healthy and disease pools are stressed before and after sample preparation.	Bias and CV of triplicate samples compared to expected values from $inter_{mc}$ determinations.	Bias, CV <20%

[a] Best practice acceptance criterion as defined by Lee et al. *(7)*, acknowledged as a hybrid of immunoassays and LC-MS/MS validation criteria derived from DeSilva et al. *(8)*.

Grant & Hoofnagle 2014

Validation Recommendation 3: CPTAC guidelines for assay characterization

Experiment 1: *Response Curve*

- Development of multipoint response curve (1 blank and a minimum of 6 concentration points).
- Samples prepared in digested matrix background (i.e. plasma, tissue, cells, etc).
- Used for the determination of LOD, LLOQ and linearity.
- Multiple replicates analyzed.

Experiment 2: *Mini-Validation of Repeatability*

- Examines intra- and inter-assay variability.
- Uses the LLOQ from Experiment 1 from which 3 concentrations (Low, Medium and High) are used to assess repeatability.
- 3 replicates processed and measured on 5 different days.

Experiment 3: *Selectivity*

- Examines the response of a peptide in six different biological replicates of the matrix.
- Replicates analyzed with no spike and ½ the Medium and Medium concentrations defined in Experiment 2.

Experiment 4: *Stability*

- Examines the stability of a peptide spiked into a background matrix
- Stability assessed based on peak area variability following:
  - different storage conditions (4C and -70C) over time.
  - freeze-thaw cycles
- Variability compared to data collected from Experiment 2.

Experiment 5: *Reproducible Detection of Endogenous Analyte*

- Representative sample containing endogenous analyte is digested 5 times on each of 5 days.
- Examines intra- and inter-assay variability of the entire assay workflow, including digestion.

Step-by-step break down of CPTAC guideline assay characterization experiments

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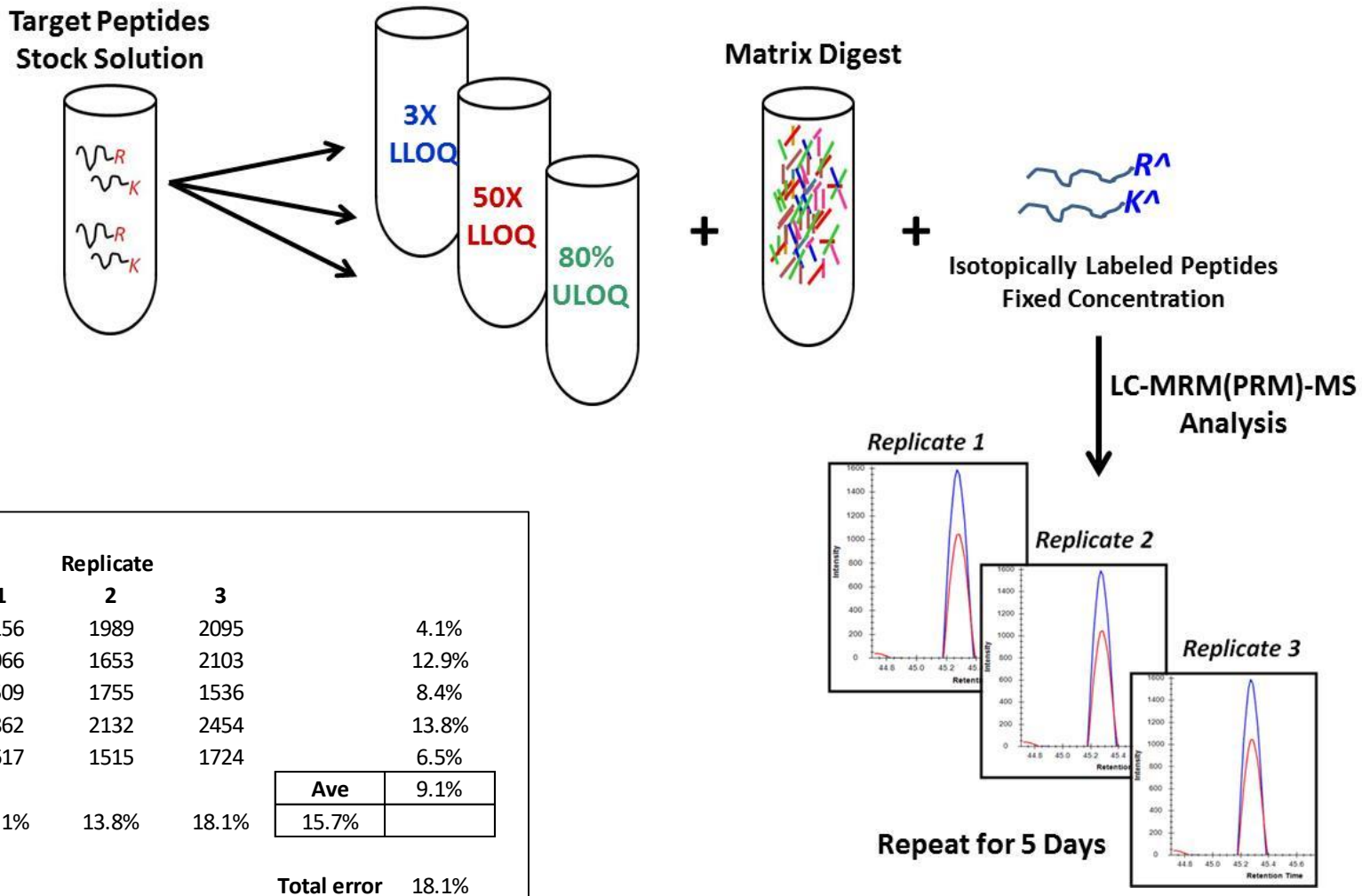

	Replicate				
Day	1	2	3		
1	2156	1989	2095		4.1%
2	2066	1653	2103		12.9%
3	1509	1755	1536		8.4%
4	1862	2132	2454		13.8%
5	1617	1515	1724		6.5%
				Ave	9.1%
	15.1%	13.8%	18.1%	15.7%	
				Total error	18.1%

Experiment 2: mini validation of repeatability (aka “the 3x5”)

KEY POINTS

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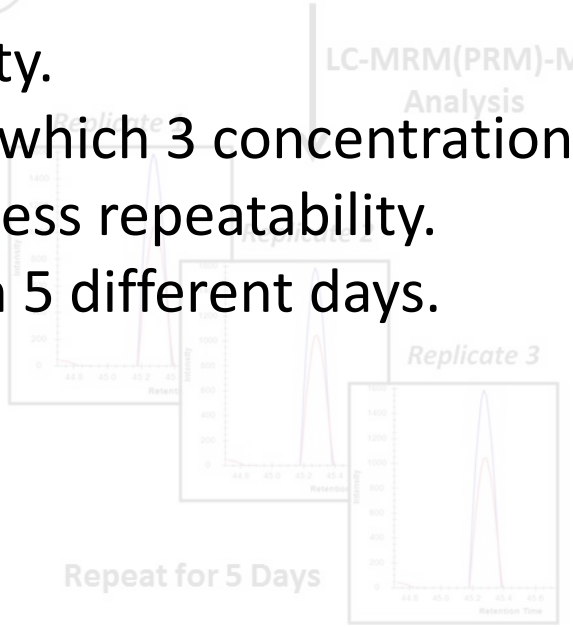

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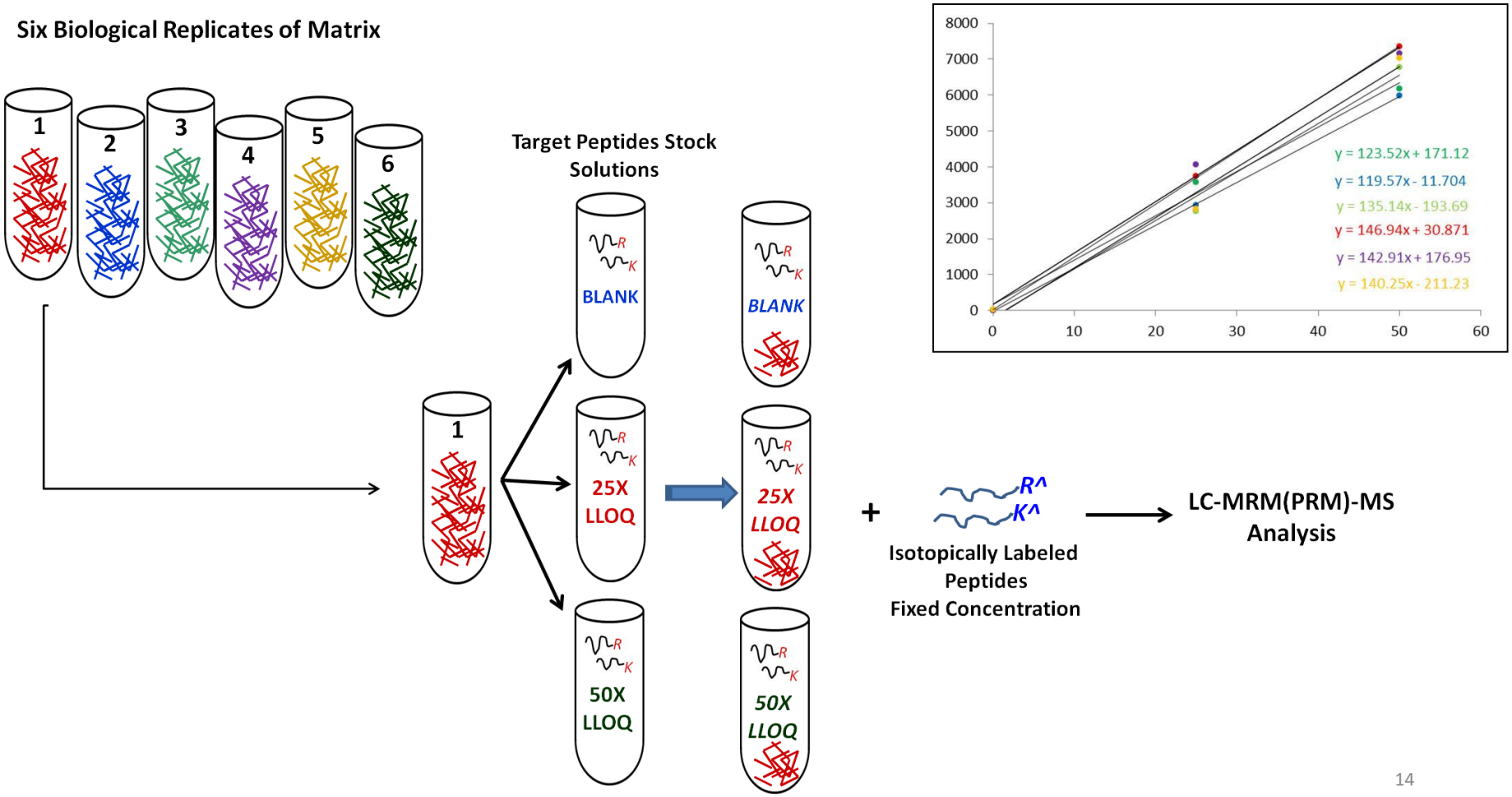

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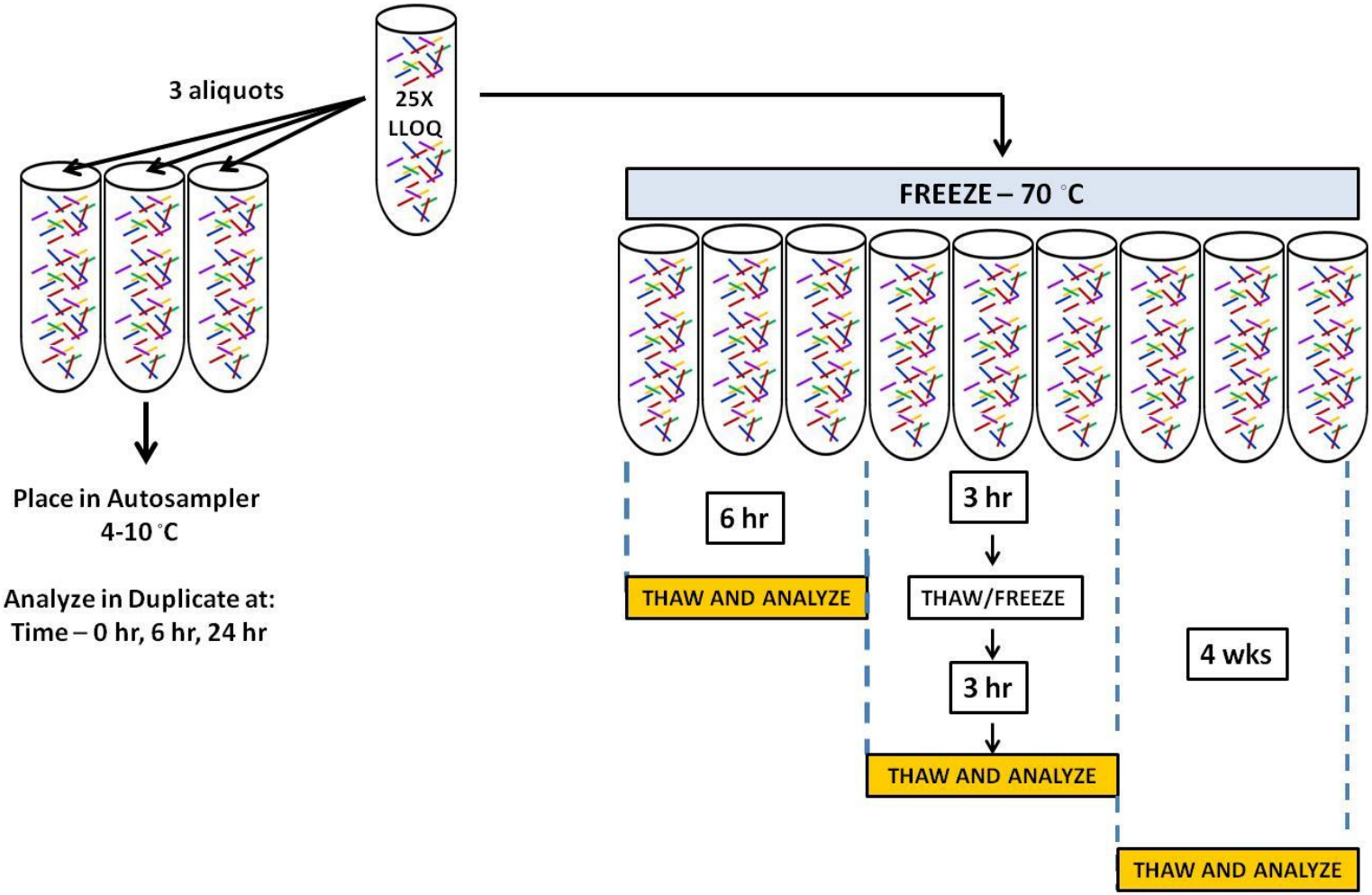

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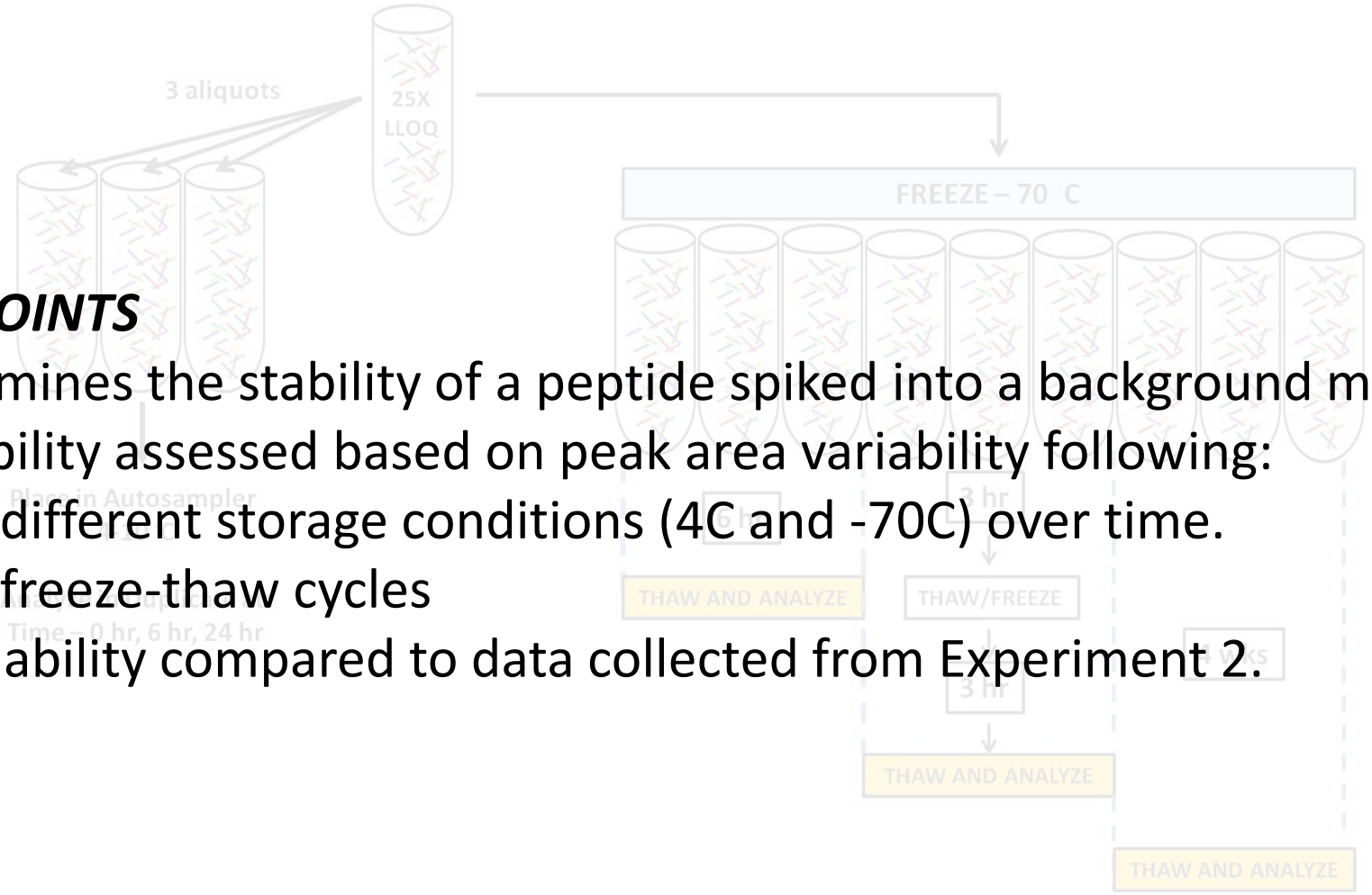

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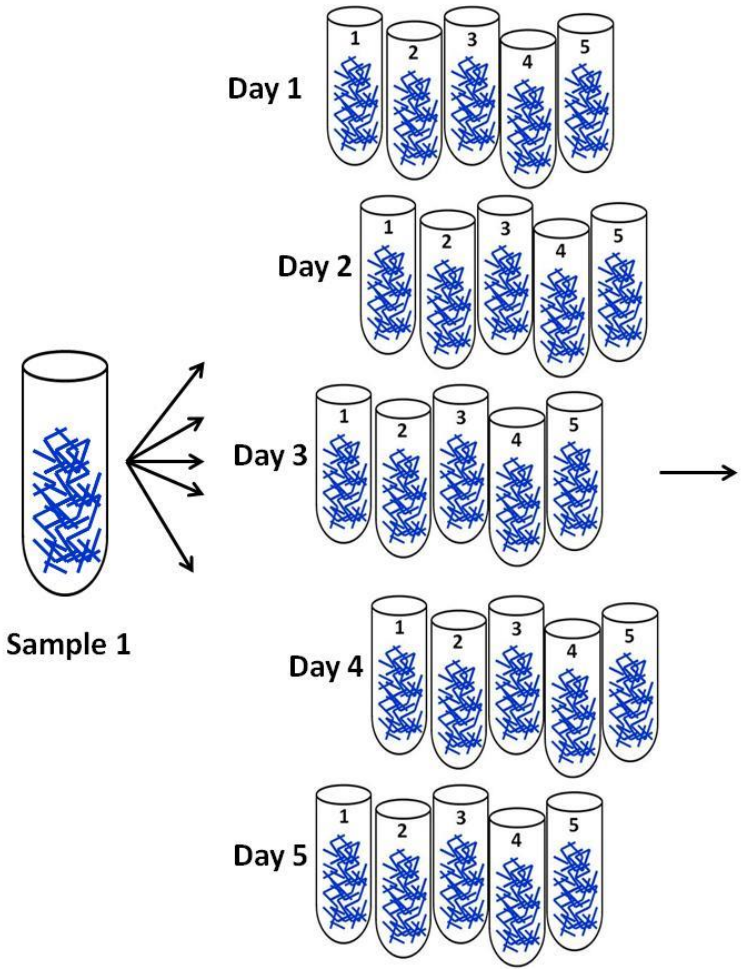

	Replicate					
Day	**1**	**2**	**3**	**4**	**5**	
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2	24686	21386	21000	23260	22226	6.6%
3	22121	26532	22050	20064	26730	12.7%
4	17280	18572	20295	14404	21384	14.8%
5	21881	19859	21450	16840	22113	10.7%
						Ave 10.6%
	13.4%	17.2%	9.0%	20.1%	16.5%	13.2%
						Total error 17.0%

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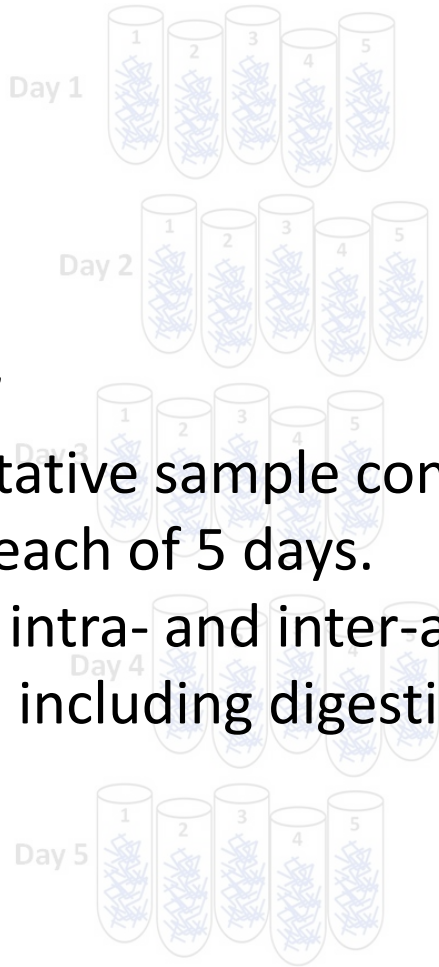

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2	24686	21386	21000	23260	22226	6.6%
3	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
4	17280	[illegible]	20295	17404	21584	14.8%
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KEY POINTS

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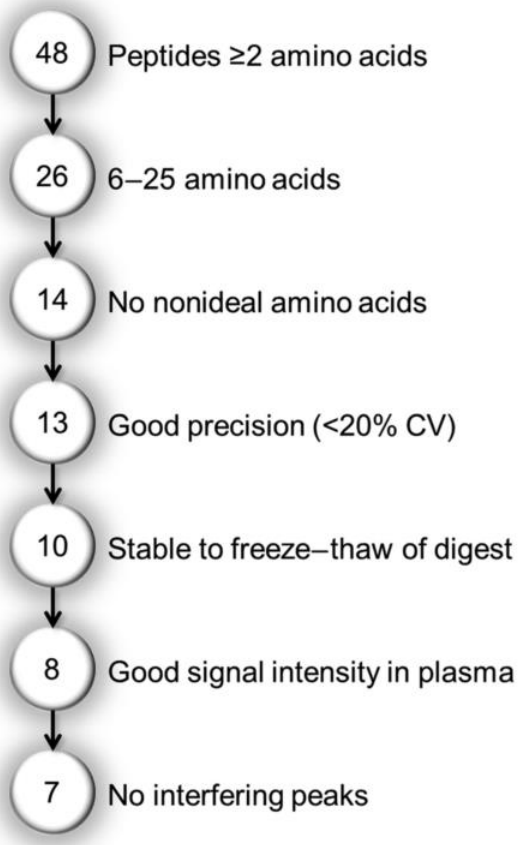
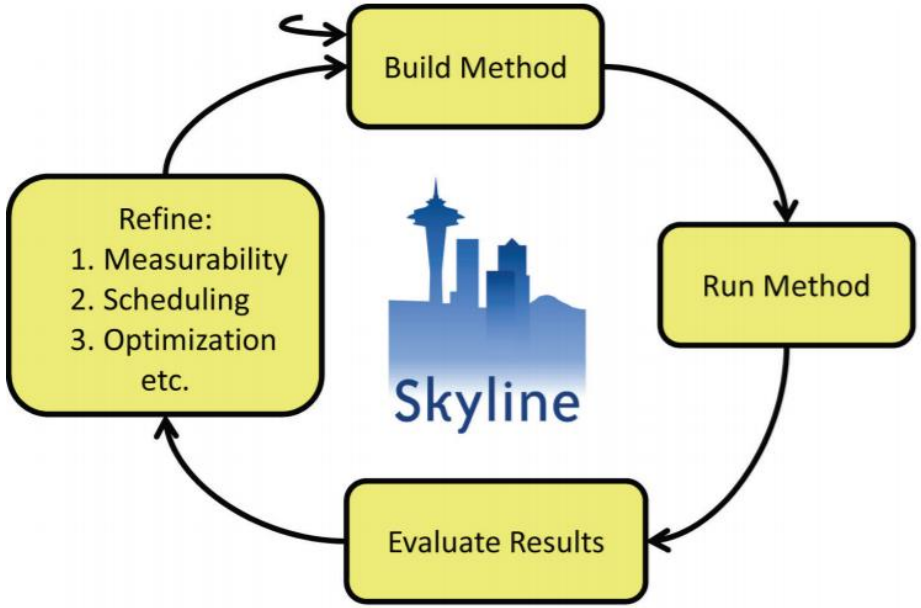
Assay development and validation can and should eliminate candidate targets





Henderson 2016

Hands on: "Measurement by a novel LC-MS/MS methodology reveals similar serum concentrations of Vitamin D-binding protein in blacks and whites" Henderson 2016

Clinical Chemistry 62:1
179–187 (2016)

Proteomics and Protein Markers

Measurement by a Novel LC-MS/MS Methodology Reveals Similar Serum Concentrations of Vitamin D–Binding Protein in Blacks and Whites

Clark M. Henderson,[1] Pamela L. Lutsey,[2] Jeffrey R. Misialek,[2] Thomas J. Laha,[1] Elizabeth Selvin,[3] John H. Eckfeldt,[4] and Andrew N. Hoofnagle[1,5*]

CONCLUSIONS: Validated mass spectrometric methods for the quantification of proteins in human samples can provide additional information beyond immunoassay.

Counter to prior observations by immunoassay, VDBG concentrations did not vary by race.

Hands on: “Measurement by a novel LC-MS/MS methodology reveals similar serum concentrations of Vitamin D-binding protein in blacks and whites” Henderson 2016

Bias

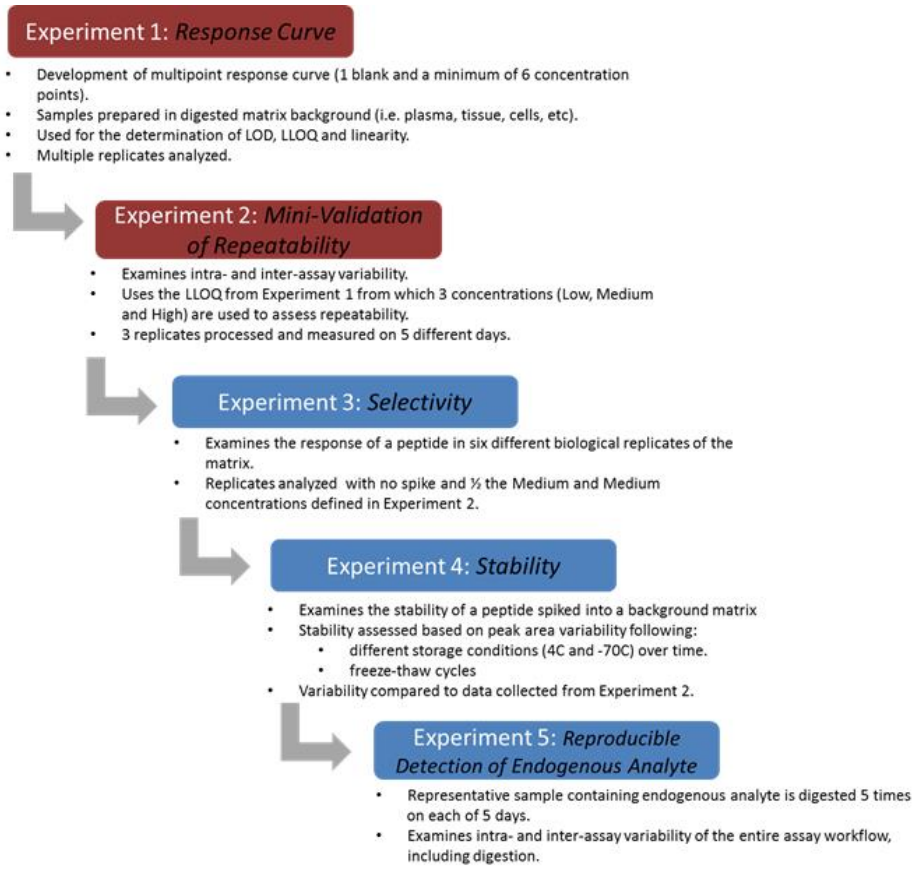
Interferences

Linearity

LLOQ

Precision

Stability



For more information…

- Literature
  - Grant & Hoofnalgle 2014 “paradise found”: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4315805/
  - Carr 2014 “fit for purpose”: https://www.ncbi.nlm.nih.gov/pubmed/24443746