Absolute quantification and method validation

Tuesday, May 2 - 9:00 am session
Targeted Proteomics with Skyline

objectives

- Relate validation criteria to your own experimental needs
- Prioritize requirements of absolute quantitation and necessary validation assays

Agenda

- **Absolute quantitation**
- Method validations and the “Fit for Purpose” approach
- *Bonus!* Introduction to absolute quantitation with data independent acquisition (DIA)
- CPTAC Assay Portal

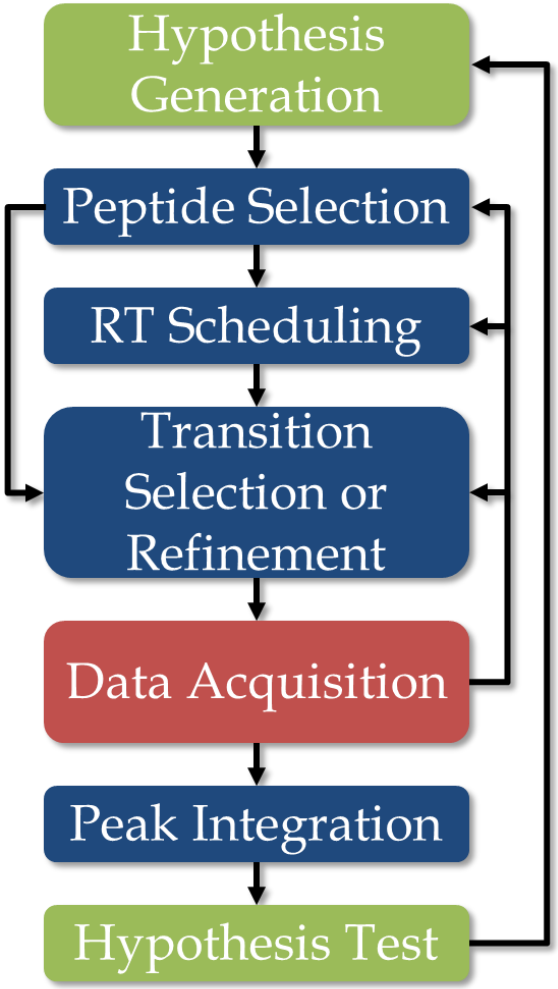
Review: Setting up a targeted MS method

Mass Spectrometer Method

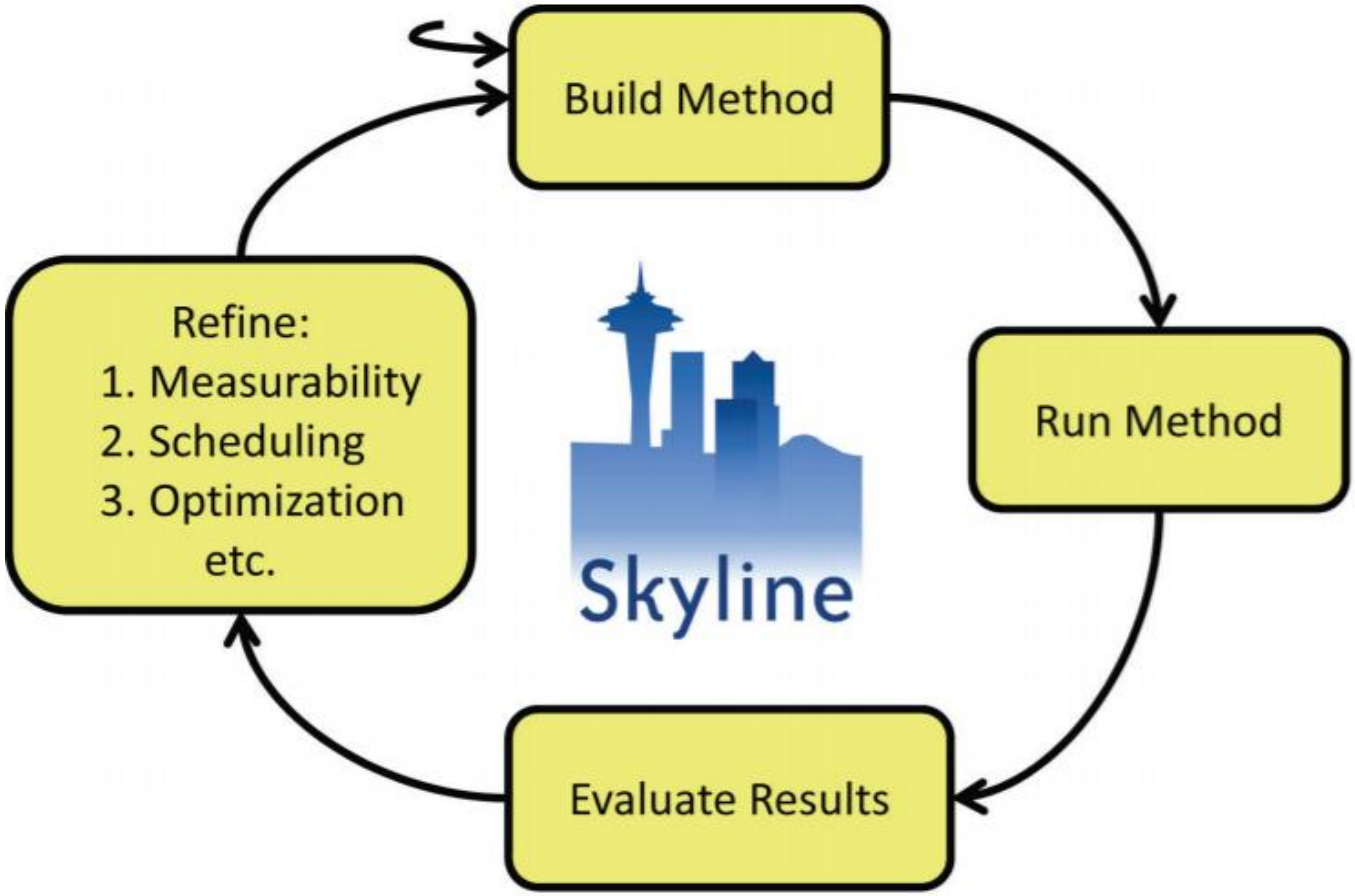
1. Targets:
   a) Peptides (precursor ions)
   b) Transitions (product ions)
2. Collision energy
3. *Retention time*

Liquid Chromatography Method

4. Separating/Analytical
   a) Starting conditions
   b) Duration, slope of gradient
5. Washing and equilibration
6. *Trapping*



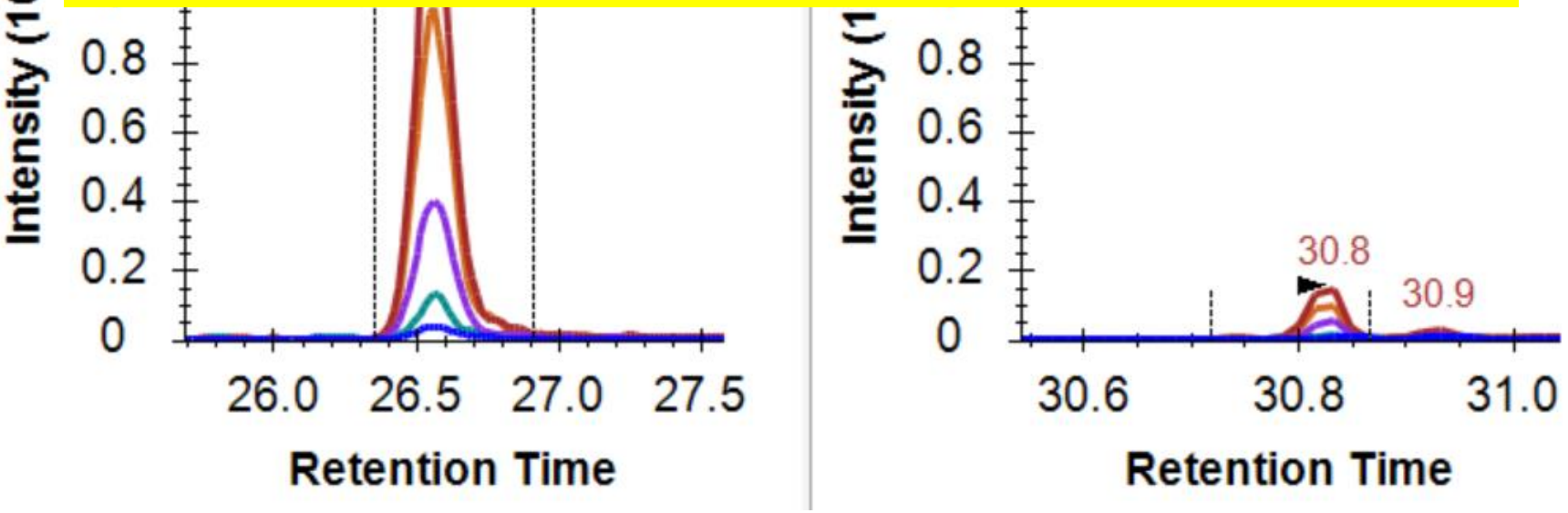
So you built a targeted method… now what?



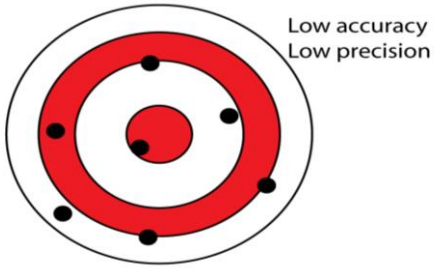
MS signal is not inherently quantitative

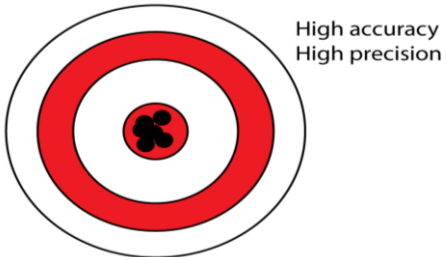
How do we turn these peak areas into absolute abundances?

Calibrate and validate!



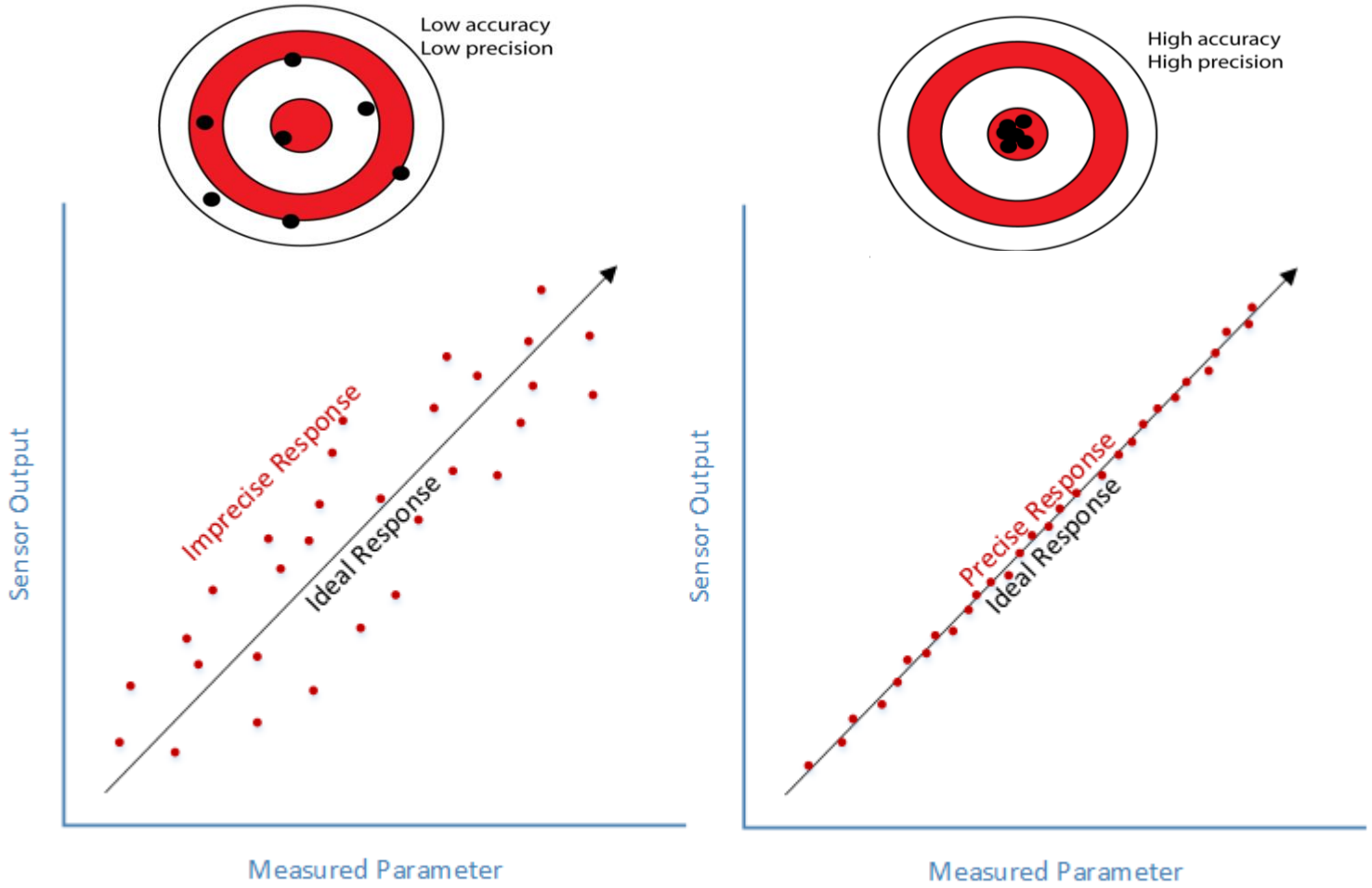
Precision is important when converting peak areas





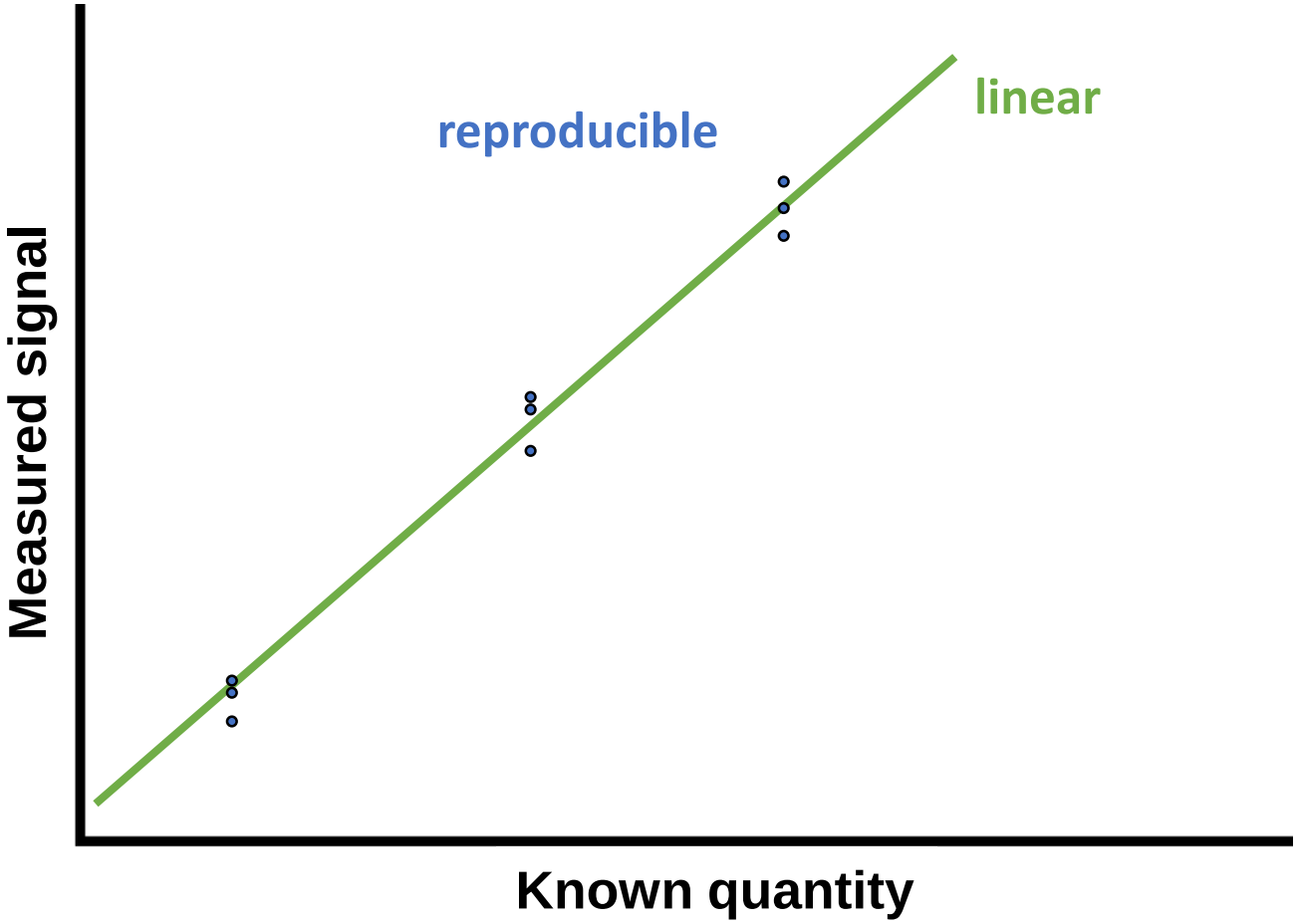
https://sites.google.com/a/apaches.k12.in.us/mr-evans-science-website/accuracy-vs-precision

Precision is important when converting peak areas

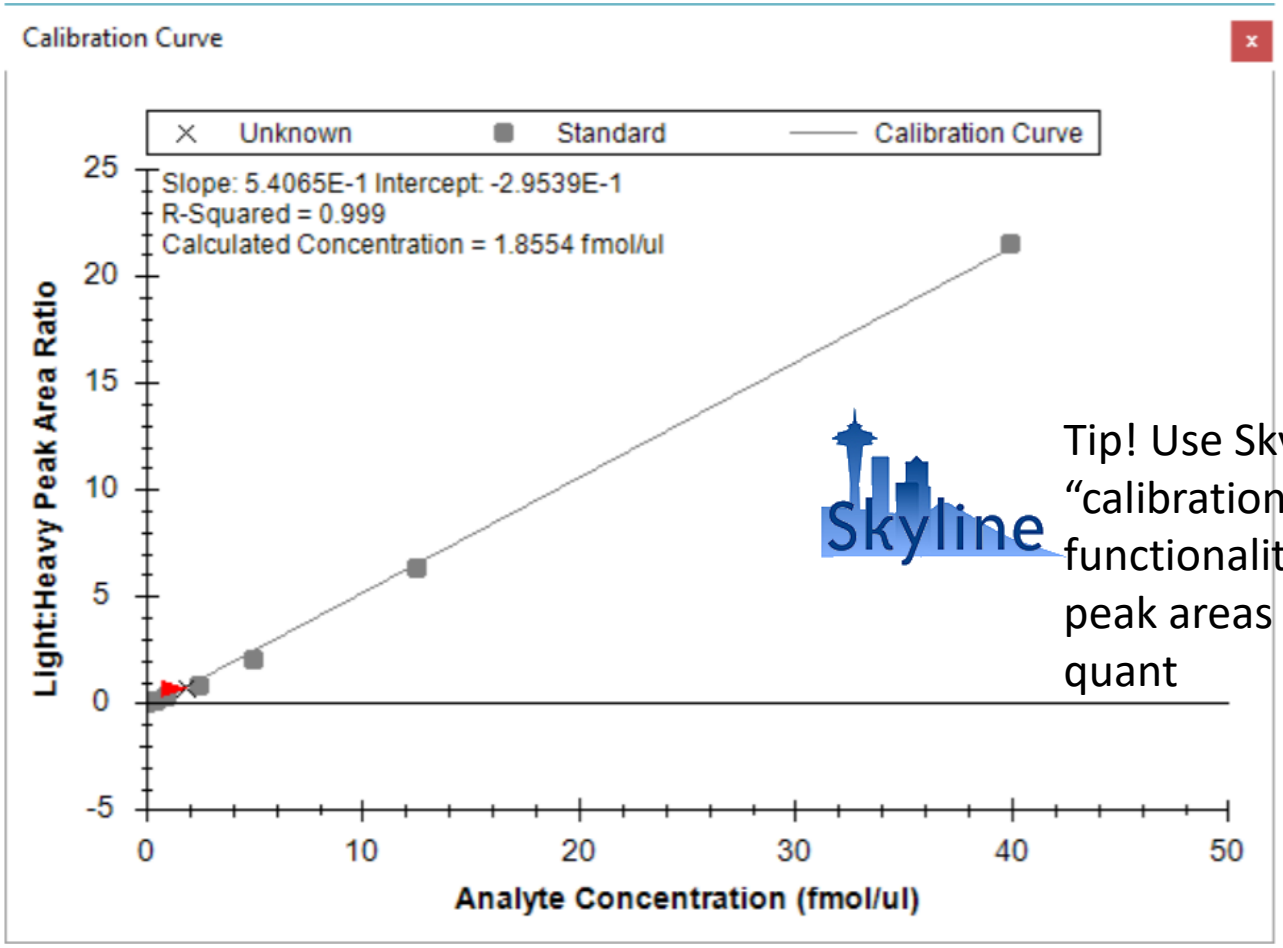


https://sites.google.com/a/apaches.k12.in.us/mr-evans-science-website/accuracy-vs-precision
https://learn.adafruit.com/calibrating-sensors/why-calibrate

Measured MS signal should be linear to input

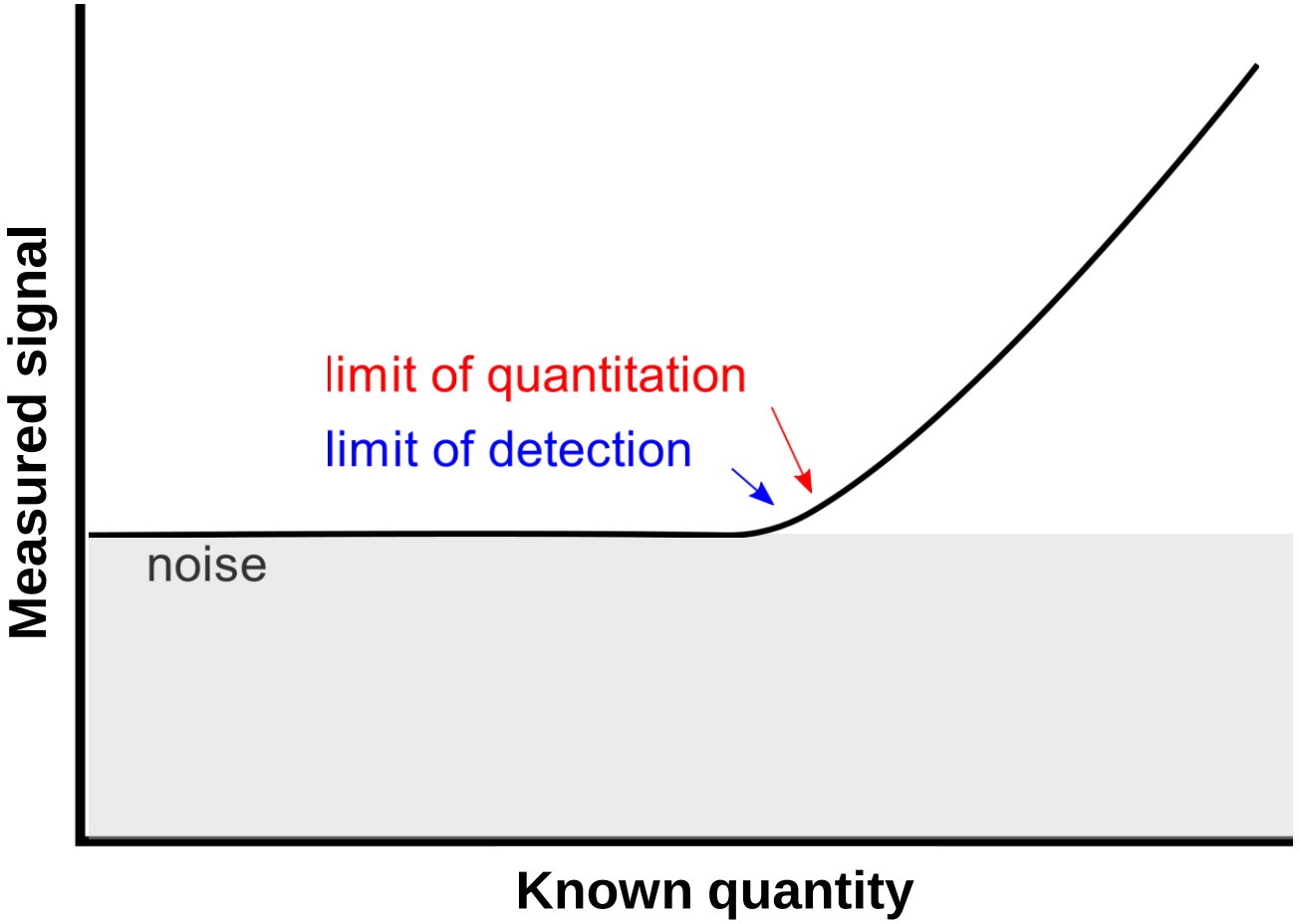


Unknown peak areas are then converted to an absolute quant



Tip! Use Skyline's "calibration curve" functionality to calibrate peak areas to absolute quant

Measurements should be reproducible, linear, and validated



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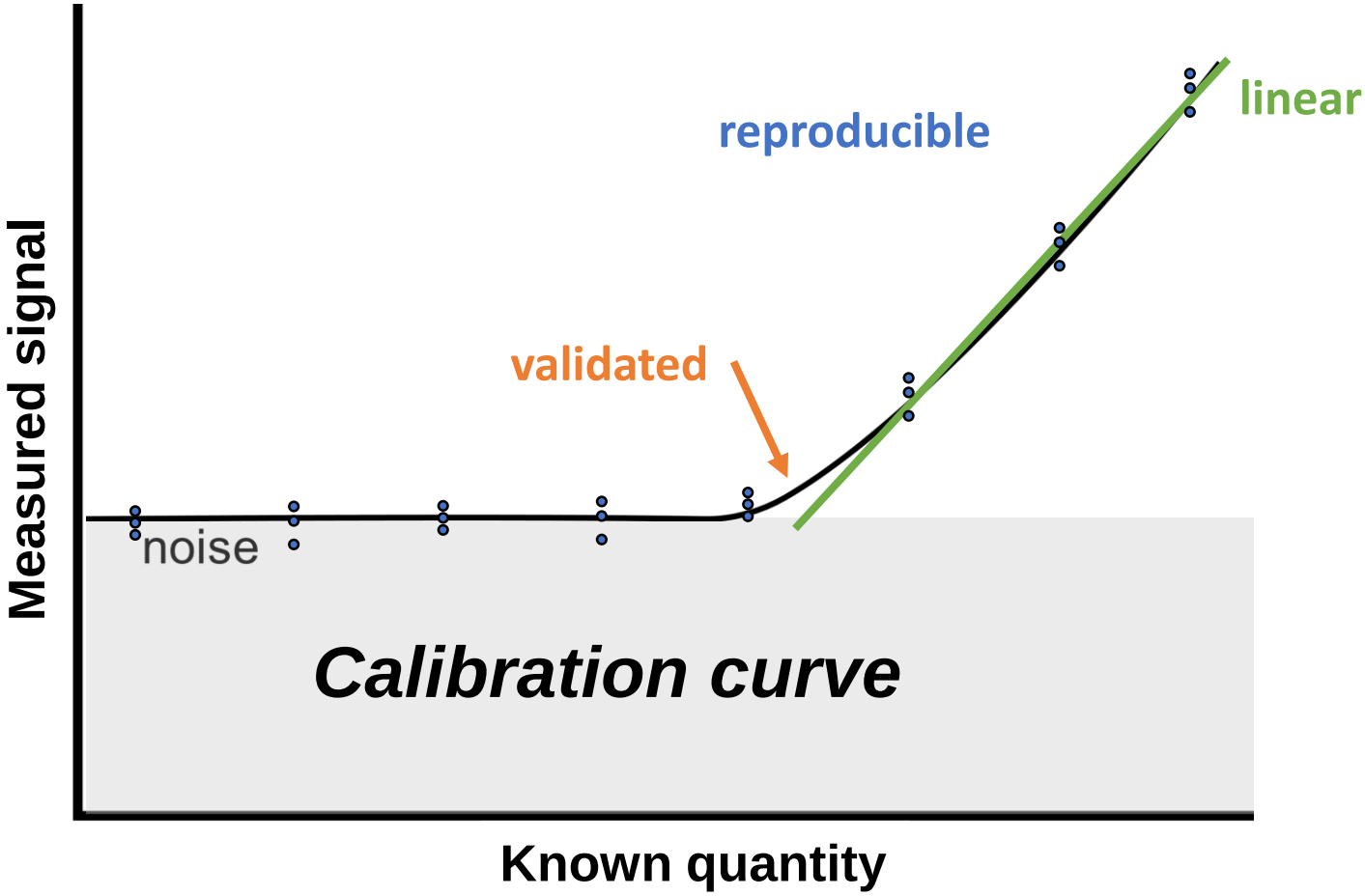
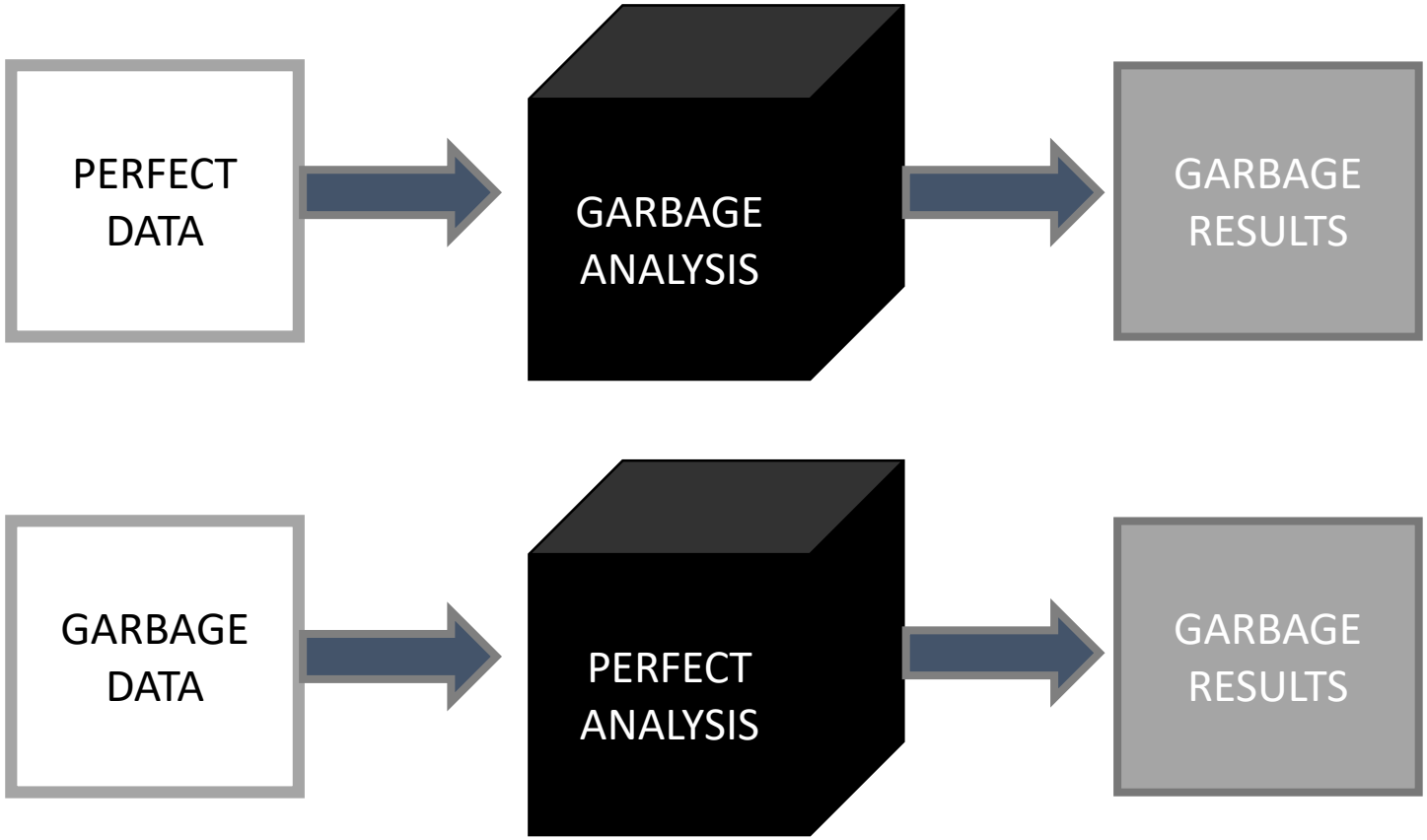


Agenda

- Absolute quantitation
- **Method validations and the “Fit for Purpose” approach**
- *Bonus!* Introduction to quantitative data independent acquisition (DIA)
- CPTAC Assay Portal

Why validate?
Garbage in, garbage out



REPORT

Establishing the Fitness for Purpose of Mass Spectrometric Methods

Robert Bethem
Alta Analytical Laboratory, El Dorado Hills, California, USA

Joe Boison
Canadian Food Inspection Agency, Saskatoon, Saskatchewan, Canada

Jane Gale
Bristol-Myers Squibb, New Brunswick, New Jersey, USA

David Heller
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Pfizer Global Research and Development, Ann Arbor, Michigan, USA

Steven Musser
FDA Center for Food Safety and Applied Nutrition, Washington, D.C., USA

Phil Price
Dow Chemical, South Charleston, West Virginia, USA

Stephen Stein
National Institute of Standards and Technology, Gaithersburg, Maryland, USA

This report is submitted by a working group sponsored by the ASMS Measurements and Standards Committee. The group responded to a 1998 opinion piece dealing with mass spectrometry in trace analysis (Bethem, R. A.; Boyd, R. K. *J. Am. Soc. Mass Spectrom.* **1998,** *9*, 643–648) which proposed that the concept of *fitness for purpose* addresses the needs of a wide range of analytical problems. There is a need to define *fitness for purpose* within the current context of mass spectrometry and to recommend processes for developing and evaluating

possible solutions.

Executive Summary of Recommendations

The unifying principles underlying this report are:

- **Analysts should use methods which are Fit for Purpose.**
- **Analysts should be able to show that their methods are Fit for Purpose.**

Analysts need to work with basic principles of Fitness for Purpose because in most cases we bear [illegible] of defending our methods and choices. No [illegible] dations or guidance from any agency, advi[illegible]

or professional society can fully remove this burden. Indeed, our own fitness as experts in our own field is dependent on familiarity with the issues described in this report (Appendix III, *Purpose*).

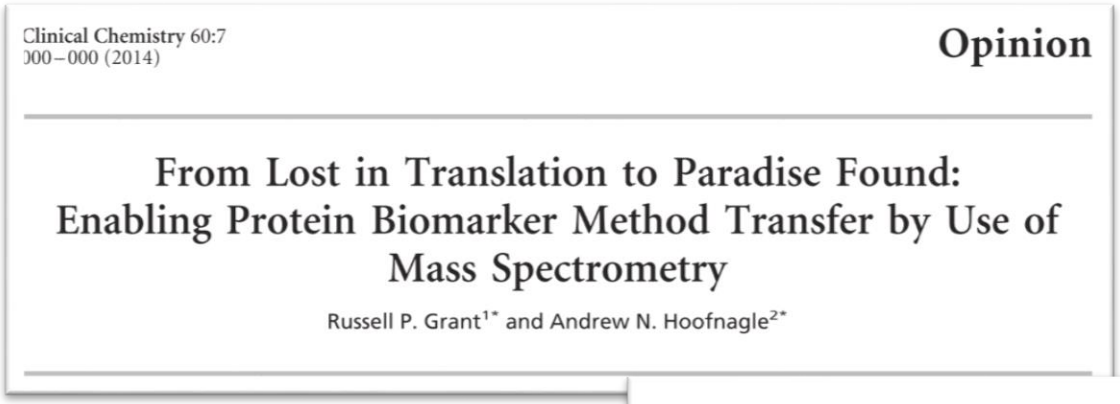
We advance the following definition:

- **Fitness for Purpose means that the uncertainty inherent in a given method is tolerable given the needs of the application area.**

Figure 1 is a flow chart showing a process for achieving and demonstrating method fitness. Basically the process consists of addressing the most important things [illegible]

Bethem 2003

Meaningful, reproducible quantitative MS proteomics

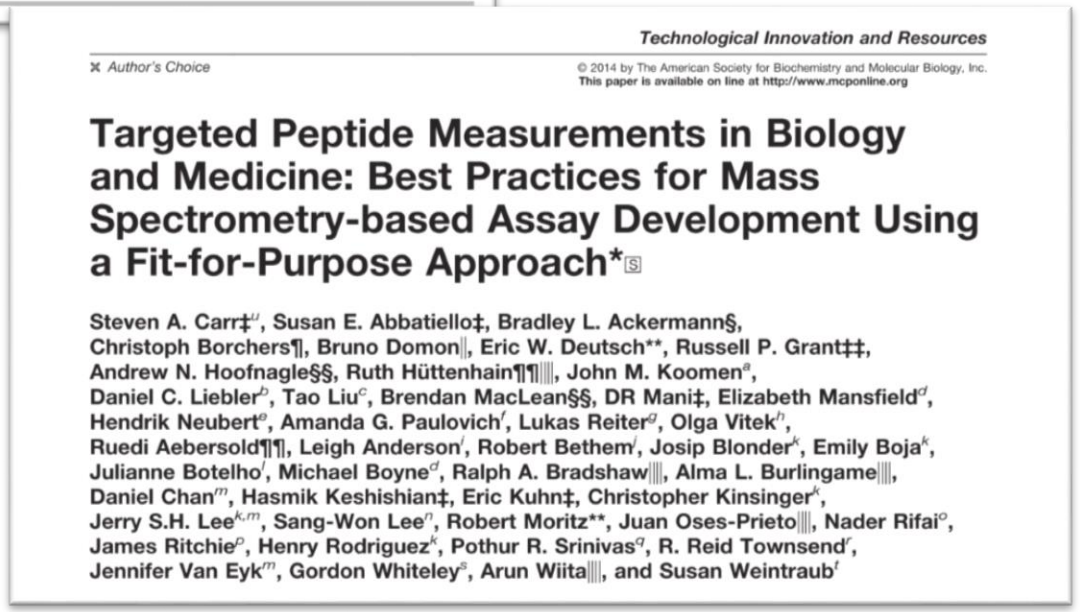


Clinical Chemistry 60:7
000–000 (2014)

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

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This paper is available on line at http://www.mcponline.org

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]

“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

Details for each assay validation experiment

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

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In depth coverage later this morning…

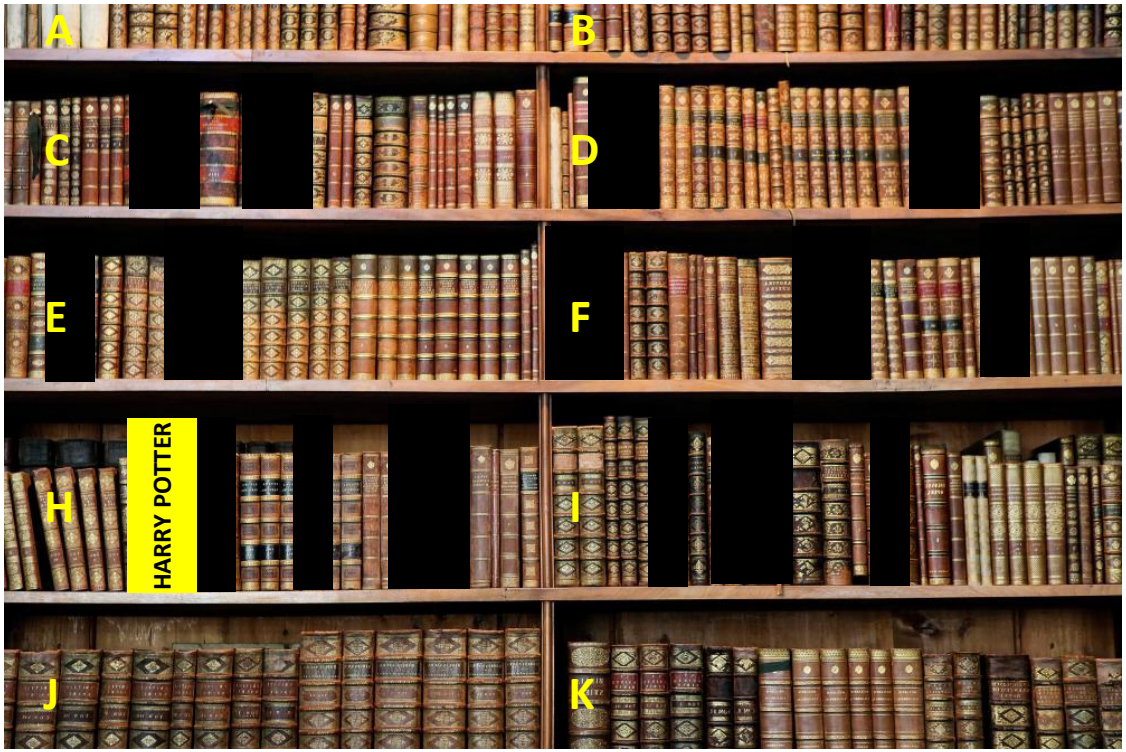
Grant & Hoofnagle 2014

Agenda

- Absolute quantitation
- Method validations and the “Fit for Purpose” approach
- ***Bonus!*** **Introduction to absolute quantitation with data independent acquisition (DIA)**
- CPTAC Assay Portal

What is data independent acquisition (DIA)?

Is Harry Potter in the library?



Moby Dick
Pride and Prejudice
War and Peace
Lord of the Rings
Catch-22
... no Harry Potter

Shamelessly stolen from Jarrett Egertson

What if you want to measure an entire genome?
Is it possible to target every protein?

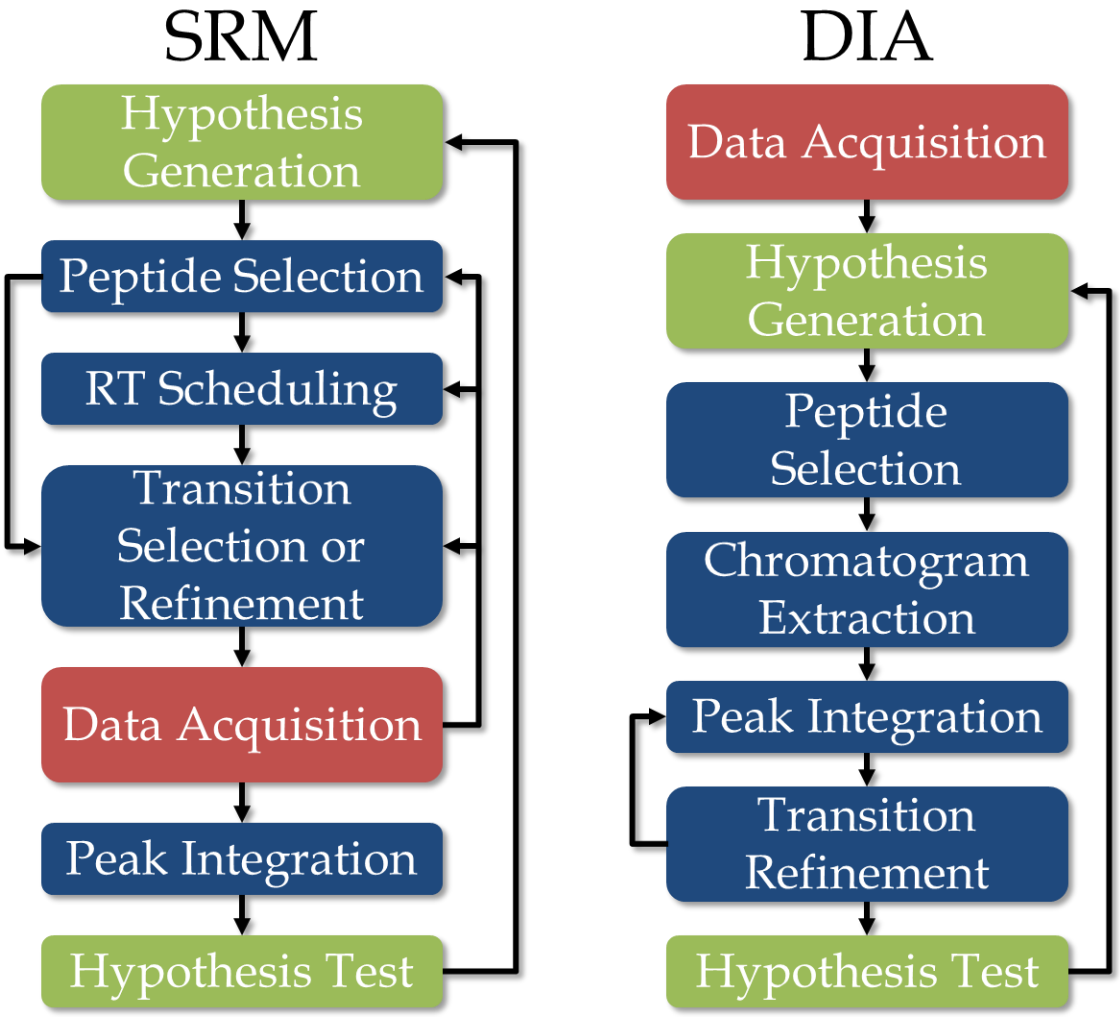
Possibly, but with some compromises…

PRM versus DIA versus DDA

- PRM:
  - Narrow, targeted windows, fragment quantitation
- DIA:
  - Wide, untargeted windows, fragment quantitation
- DDA:
  - Narrow, instrument targeted windows, precursor quantitation

	PRM	DIA	DDA
Comprehensive Detection	No	Compromise	Yes
Selective Quantitation	Yes	Compromise	No

Method development for DIA compared to SRM



Egertson (unpublished)

Refinement of DIA methods largely focuses on isolation window scheme

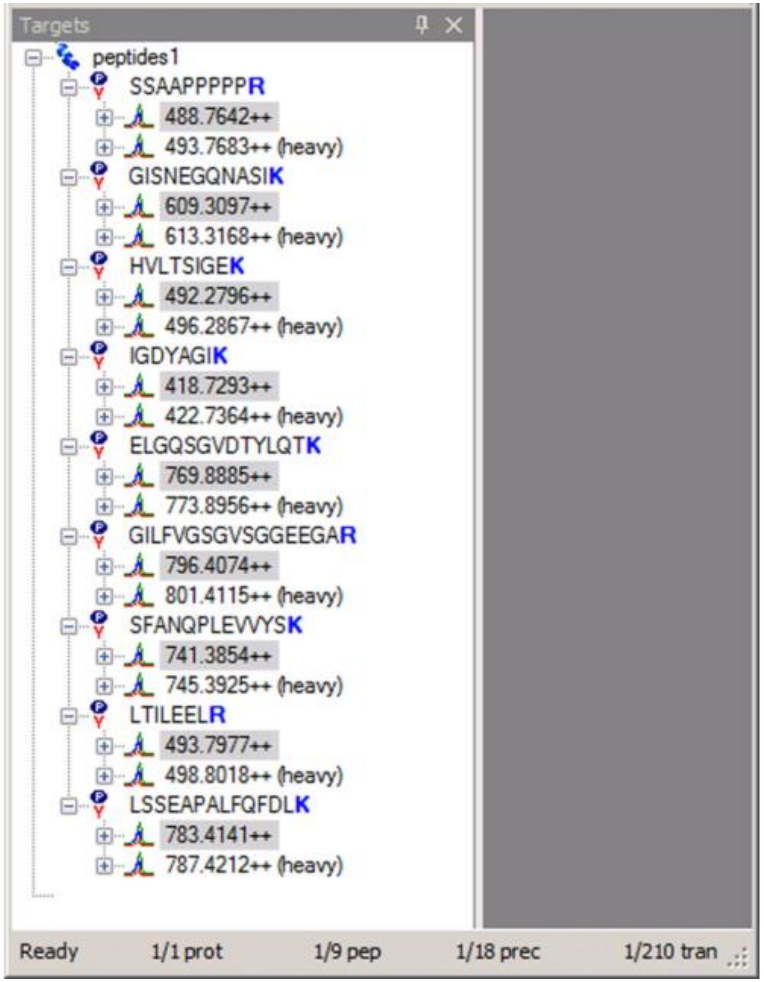
TABLE 1 | Guide to modifying the DIA method.

Goal	Selectivity	*m/z* range covered	Number of sample injections	Chromatographic sampling rate
Increase sensitivity				
Reduce isolation width	↑	↓		
Reduce isolation width and use multiple injections per sample	↑		↑	
Increase max ion inject time[a]				↓
Increase resolving power and max ion inject time[a]	↑			↓
Sample more peptides				
Use multiple injections per sample		↑	↑	
Increase the isolation width	↓	↑		
Improve chromatogram sampling				
Increase the isolation width	↓			↑
Reduce the *m/z* range covered		↓		↑

[a]Instruments with an ion-trap mass analyzer that use automatic gain control (e.g., Thermo Scientific LTQ, Velos, Orbitrap and Exactive series). The direction of the arrow below each metric indicates an increase (up) or decrease (down) in that metric.

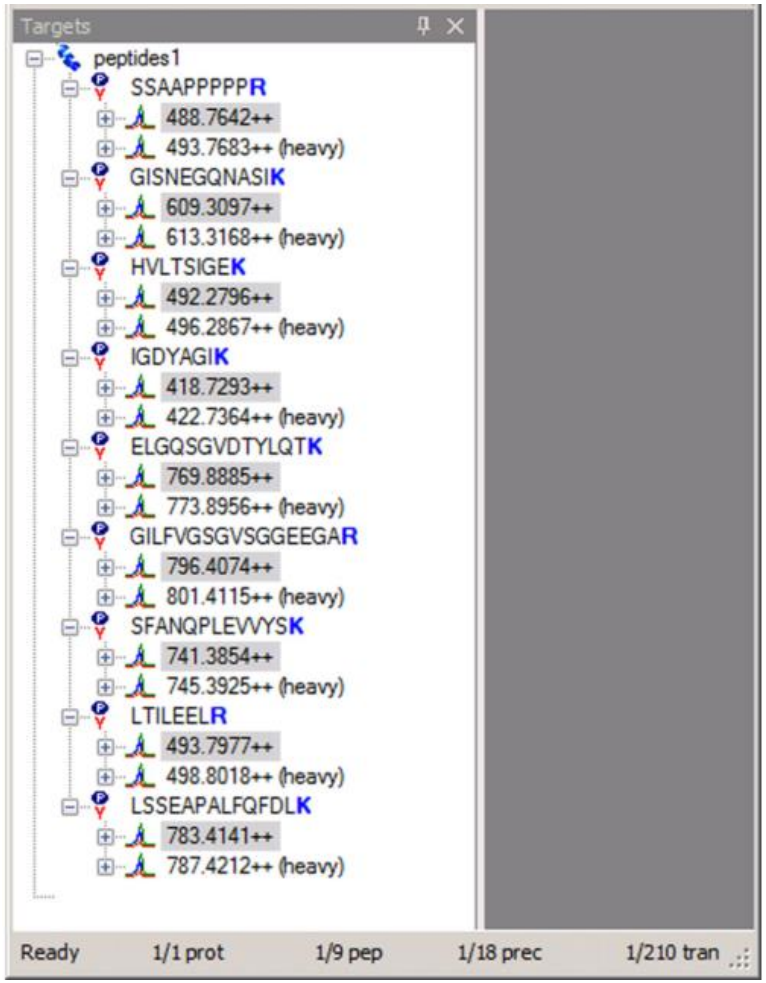
Egertson 2015

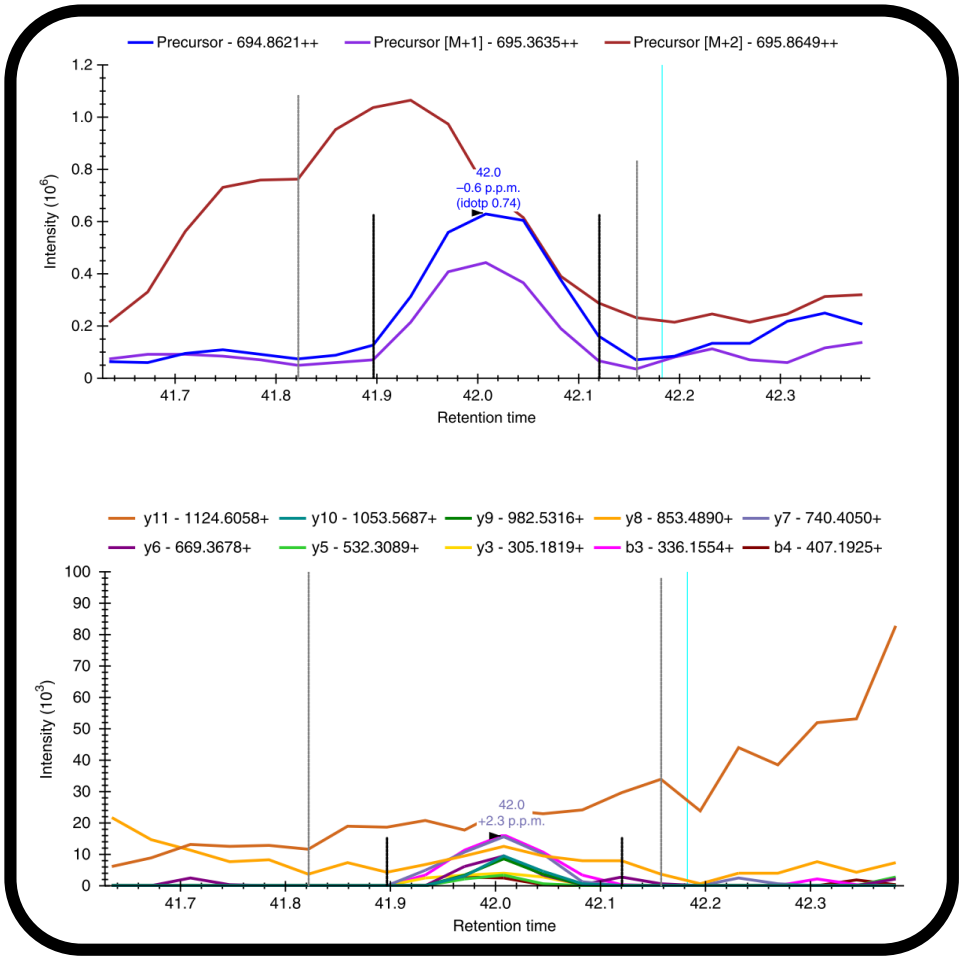
Quantitative analysis of DIA is mainly targeted



- Add protein, peptide targets post-acquisition
- Reanalyze by simply changing target list and re-importing the data

Quantitative analysis of DIA is mainly targeted



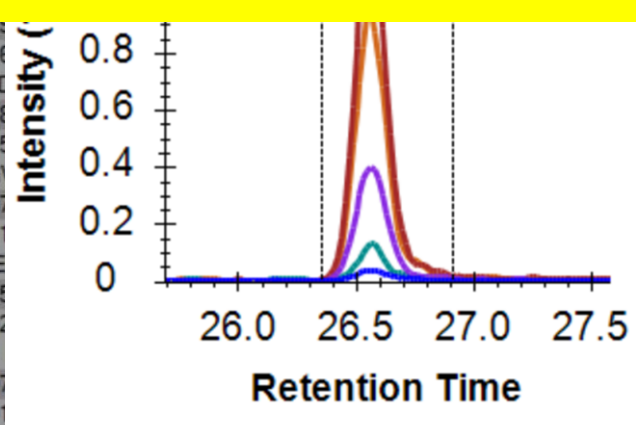


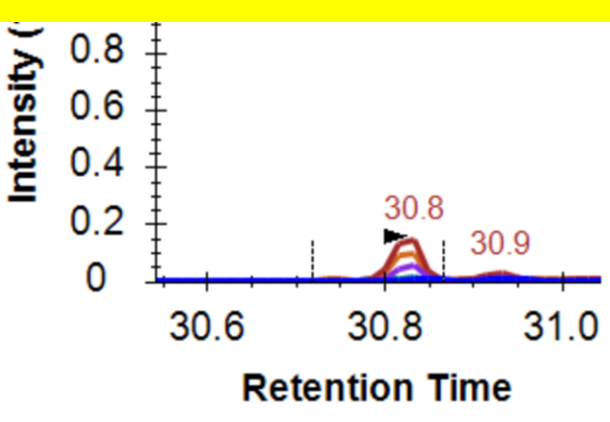
Quantitative analysis of DIA is mainly targeted

MS signal is not inherently quantitative

How do we turn these peak areas into absolute

Calibrate and validate!





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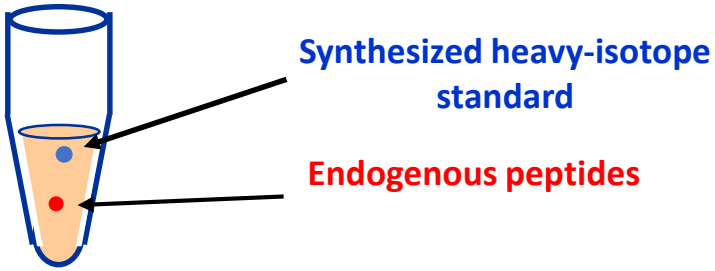
How to make a calibration curve for an entire proteome?

Targeted MS

Scale: 10's - 100's of peptides

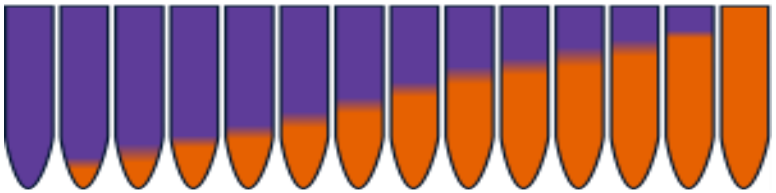
Method: Synthesize labeled isotope standards



DIA-MS

Scale: 1,000 – 10,000's of peptides

Method: Background proteome



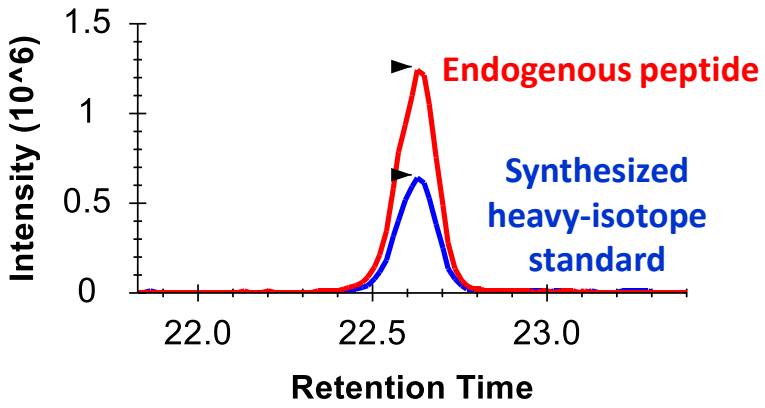
How to make a calibration curve for an entire proteome?

Targeted MS

Scale: 10's - 100's of peptides

Method: Synthesize labeled isotope standards

Measure: peak area ratio of synthetic standard to endogenous

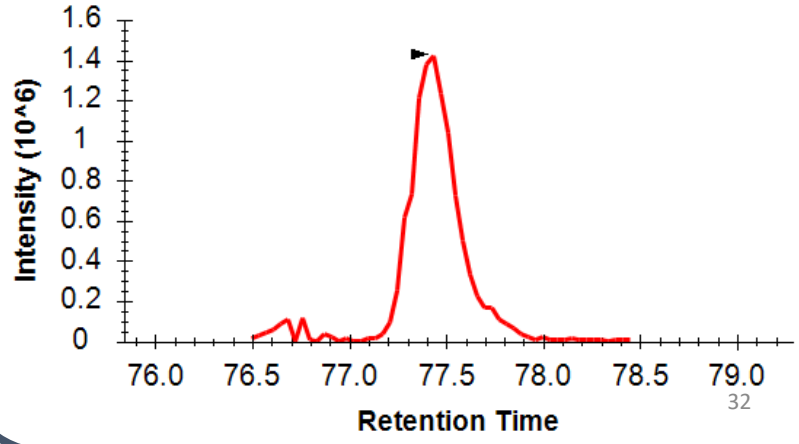


DIA-MS

Scale: 1,000 – 10,000's of peptides

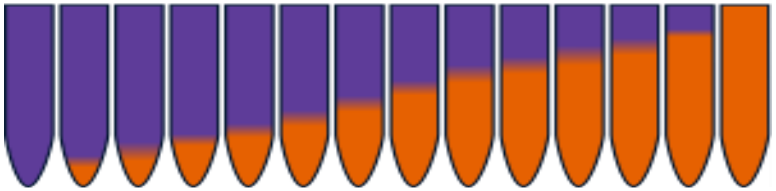
Method: Background proteome

Measure: label-free peak area of proteome of interest



Our method uses a “background proteome” to dilute a “proteome of interest”

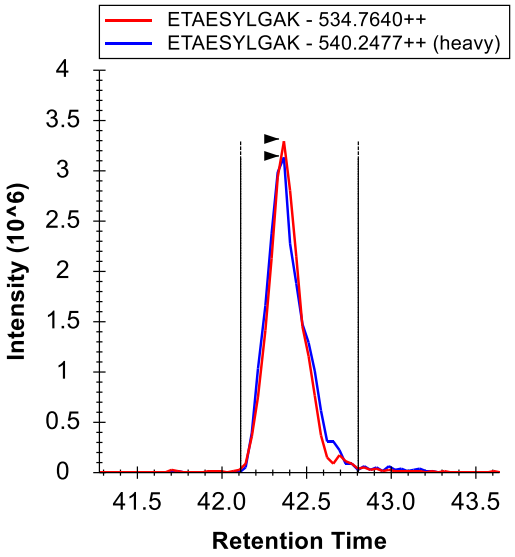
Background proteome



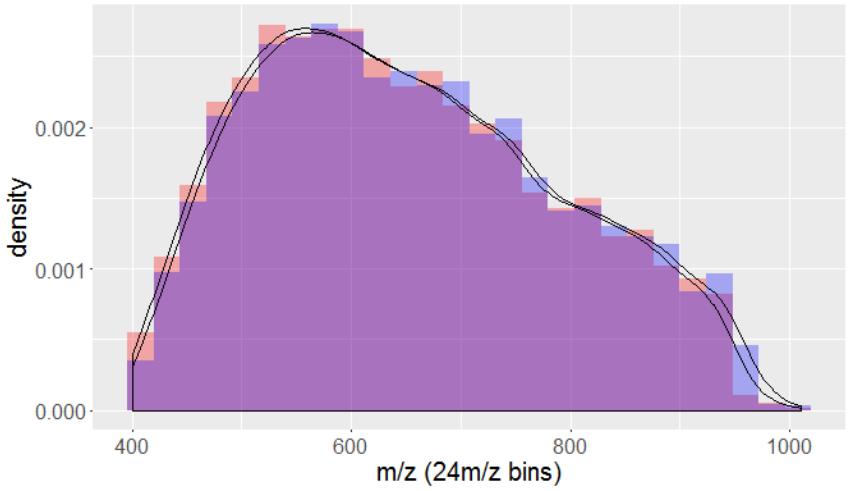
Proteome of interest

Experimental design should match proteomes for:

1. chromatographic elution profile

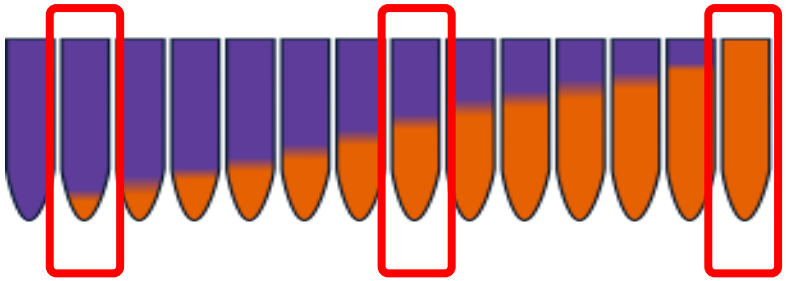


2. ion m/z distribution

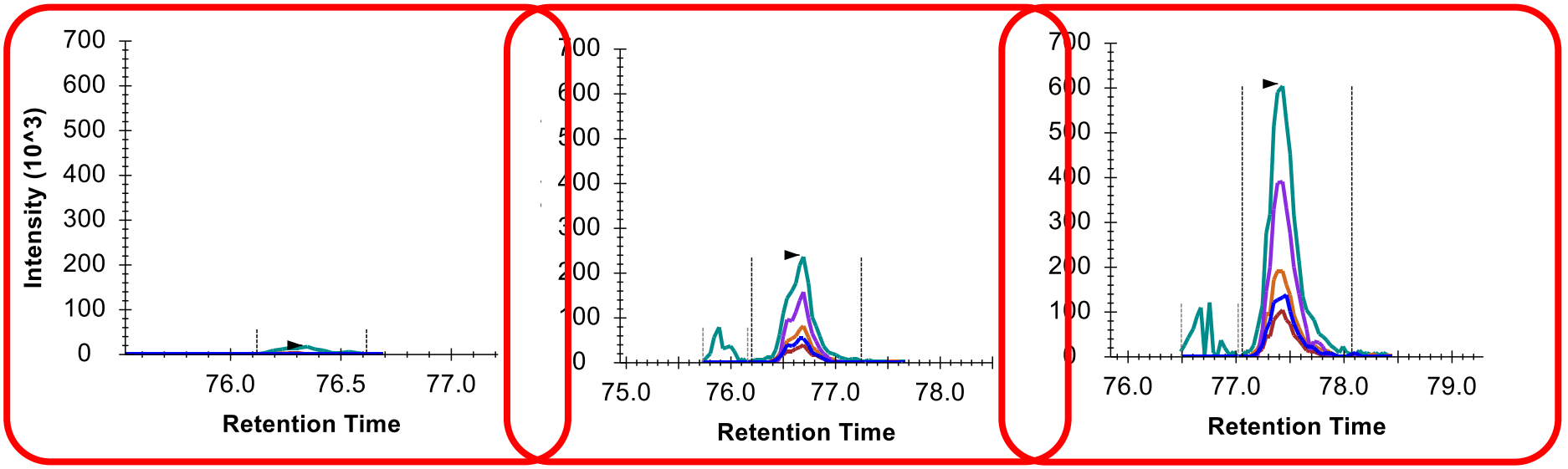


Matched proteome of interest and background proteome scales MS signal with proteome of interest amount

Background proteome

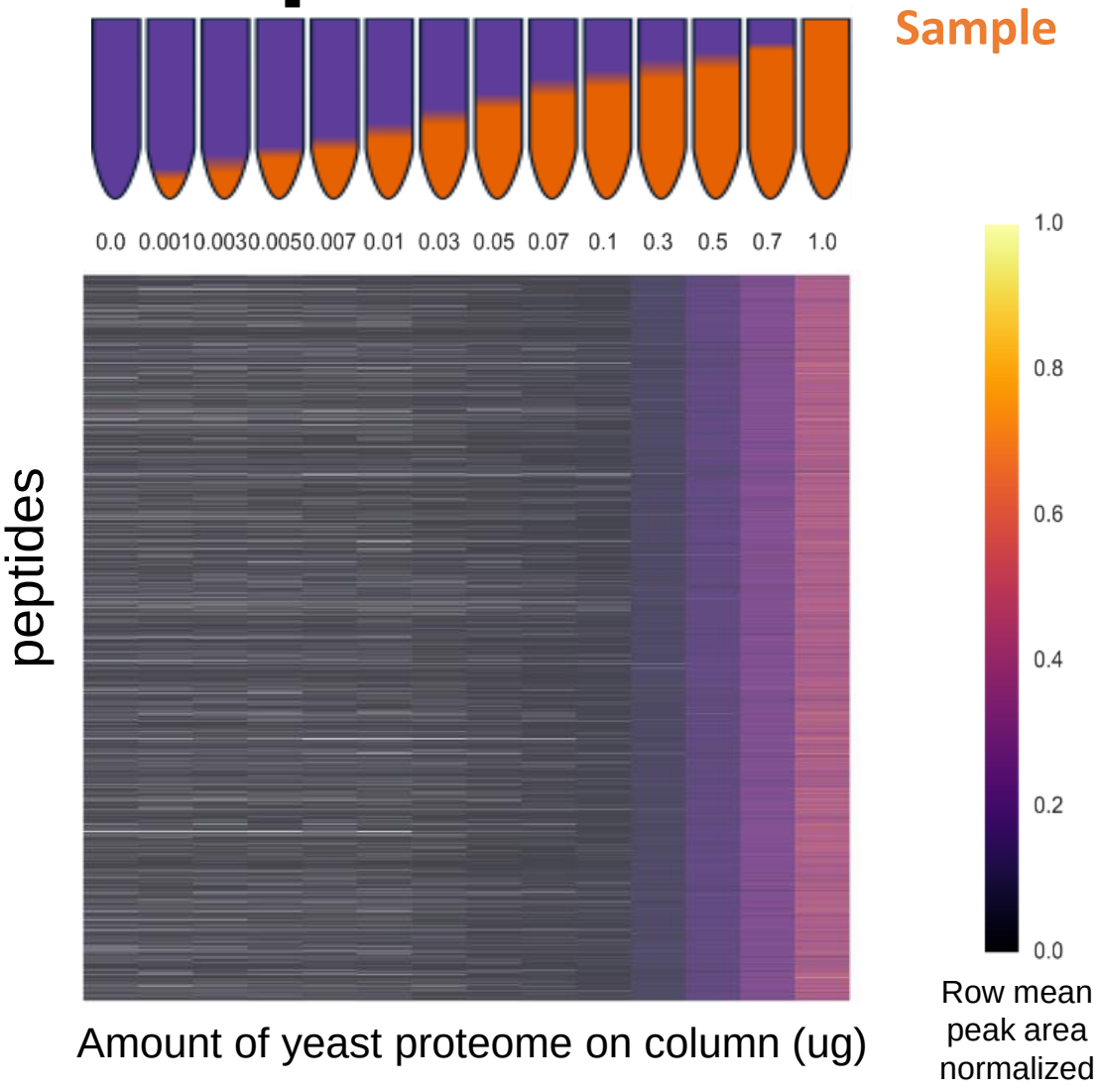


Proteome of interest

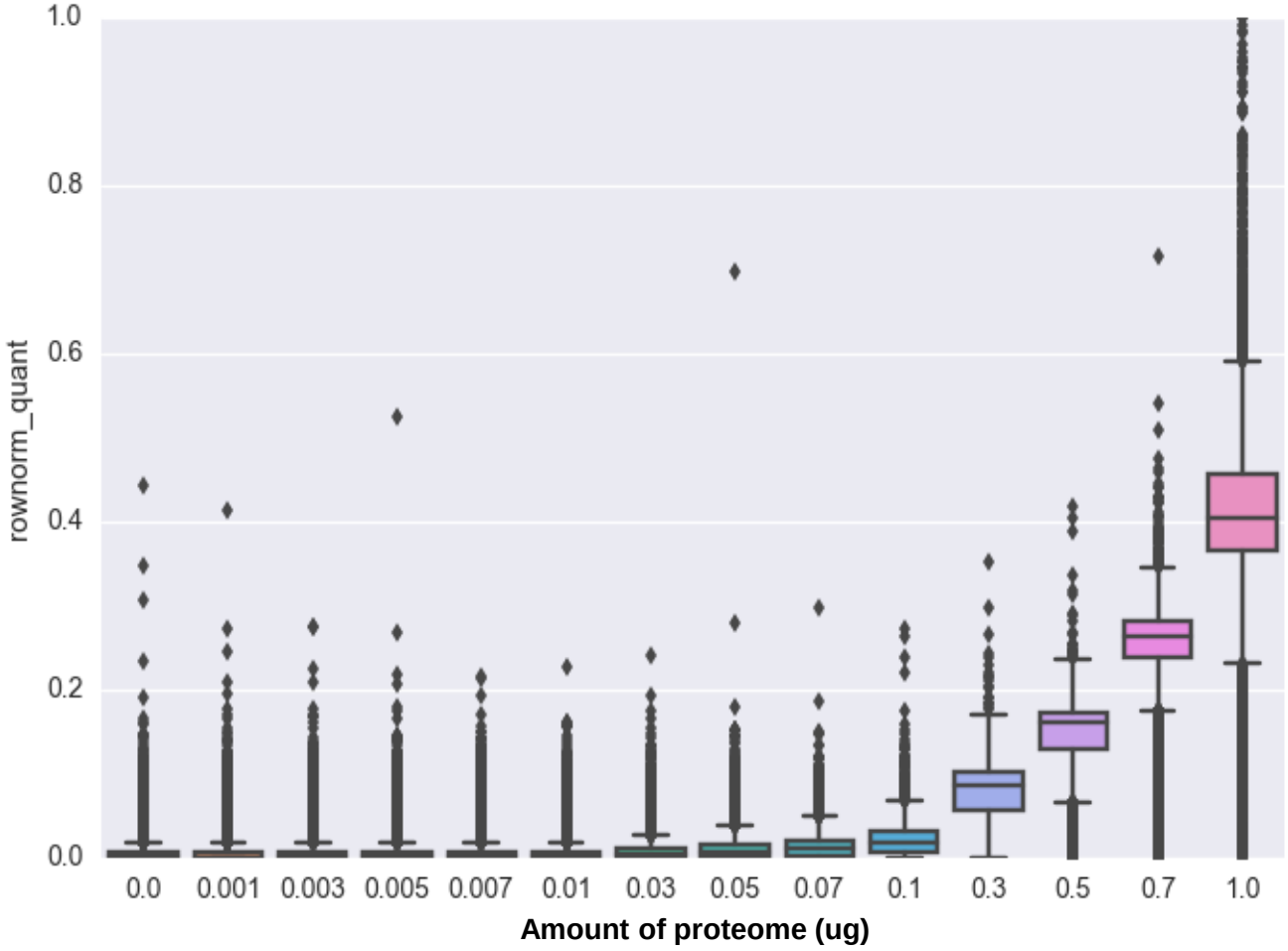


GVVIEGYPTIVLYPGGK++

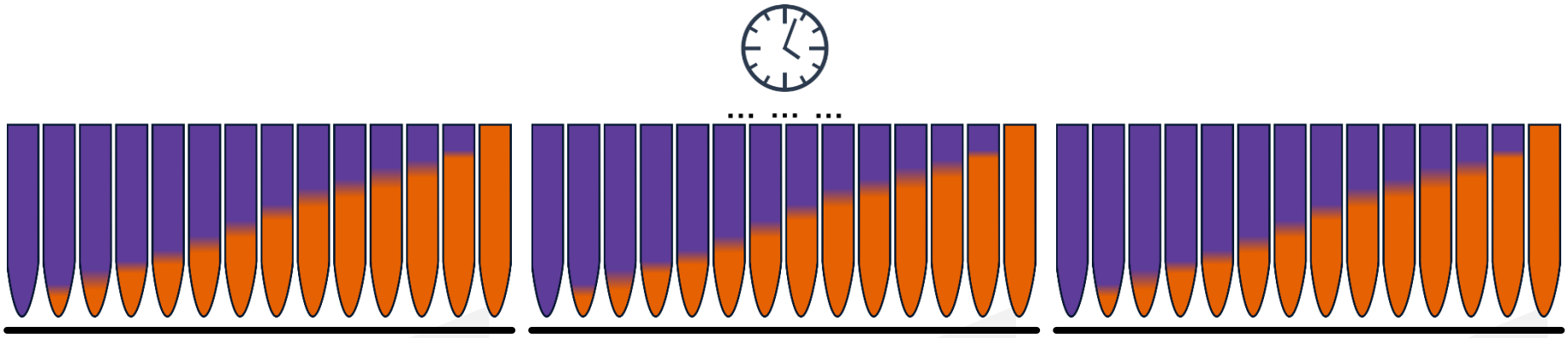
Case study: Calibration curves for global *S. cerevisiae* peptide quantification



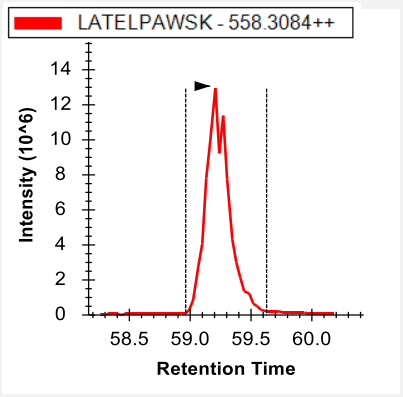
Whole-proteome calibration curve has a canonical “hockey stick shape” signal-to-input trend

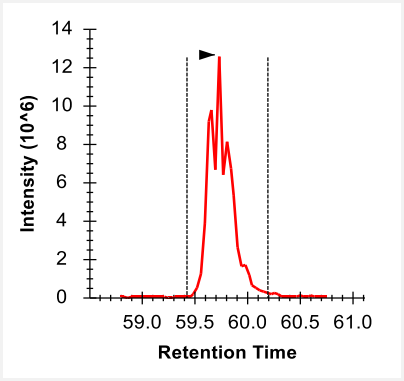


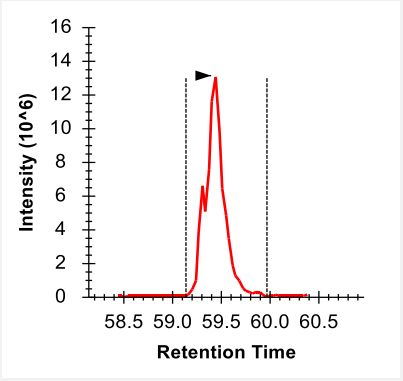
Measurement reproducibility (percent coefficient of variation) using the highest concentration point



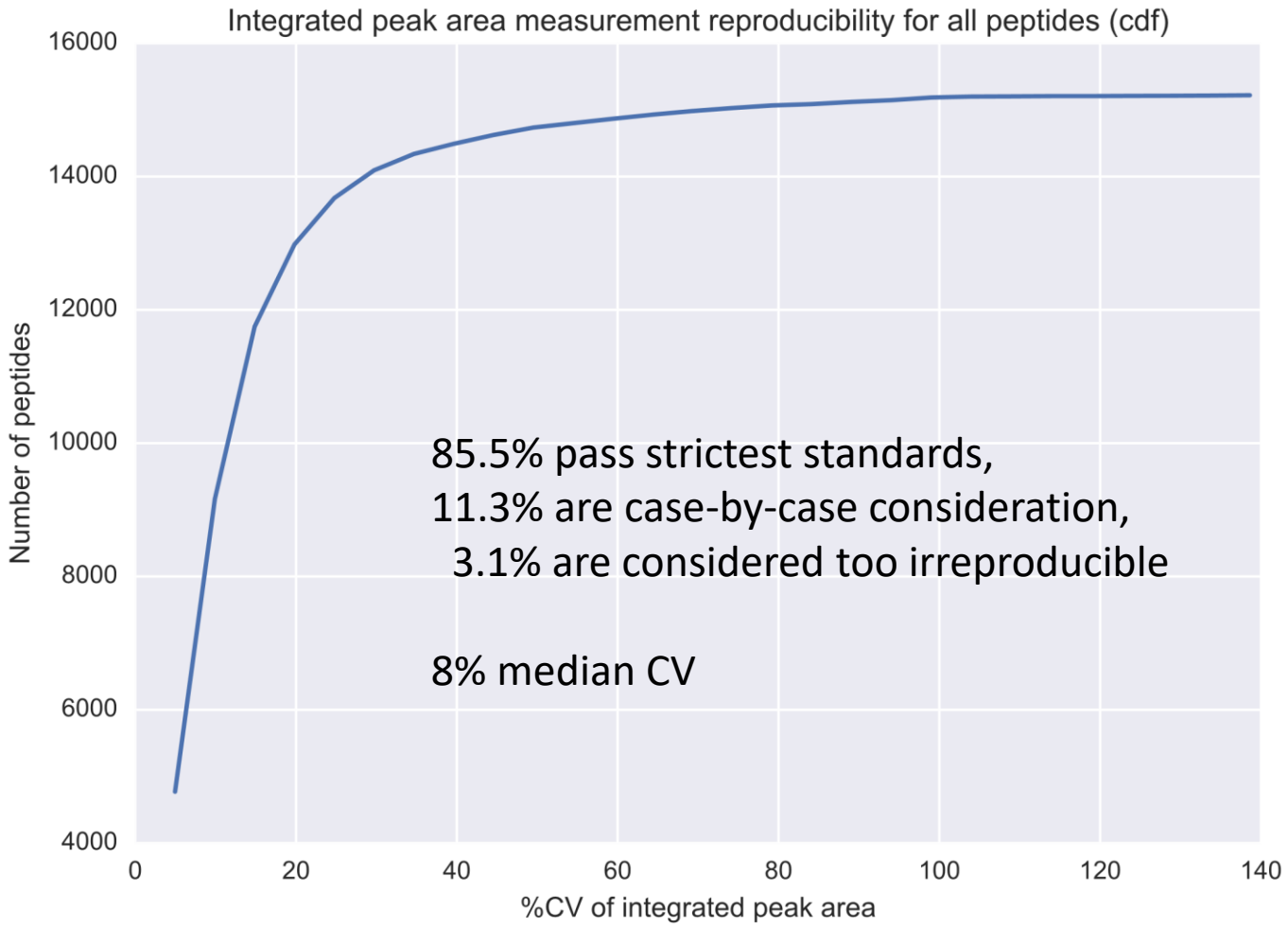
Replicate 1 | Replicate 2 | Replicate 3







Of >15000 candidate targets, over 85% are measured with highest reproducibility standards



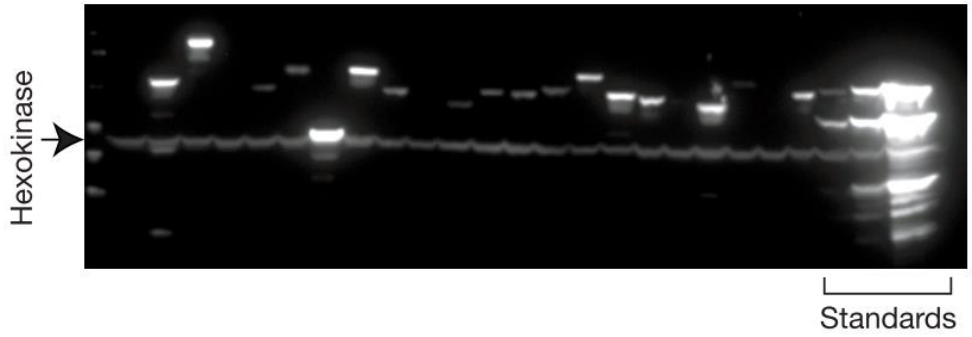
Comprehensiveness in context of other global protein expression profiling

4517 proteins

TAP-tag + Western

Individually tandem affinity purification (TAP)-tagged ORFs

+ quantitative western blot analyses



Ghaemmaghami 2003

Comprehensiveness in context of other global protein expression profiling

4517 proteins

TAP-tag + Western

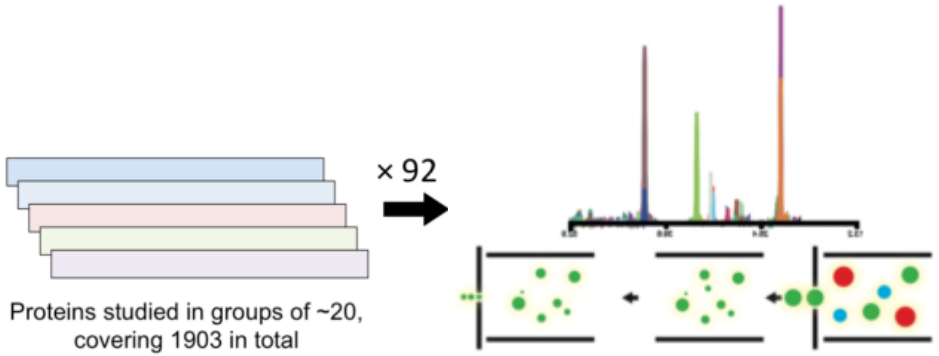
1169 proteins

Recombinant Protein

+ 92x SRM-MS

Design of >100 synthetic recombinant proteins

+ 92 hours worth of targeted (SRM-) MS



Lawless 2016

Comprehensiveness in context of other global protein expression profiling

4517 proteins

TAP-tag + Western

1169 proteins

Recombinant Protein
+ 92x SRM-MS

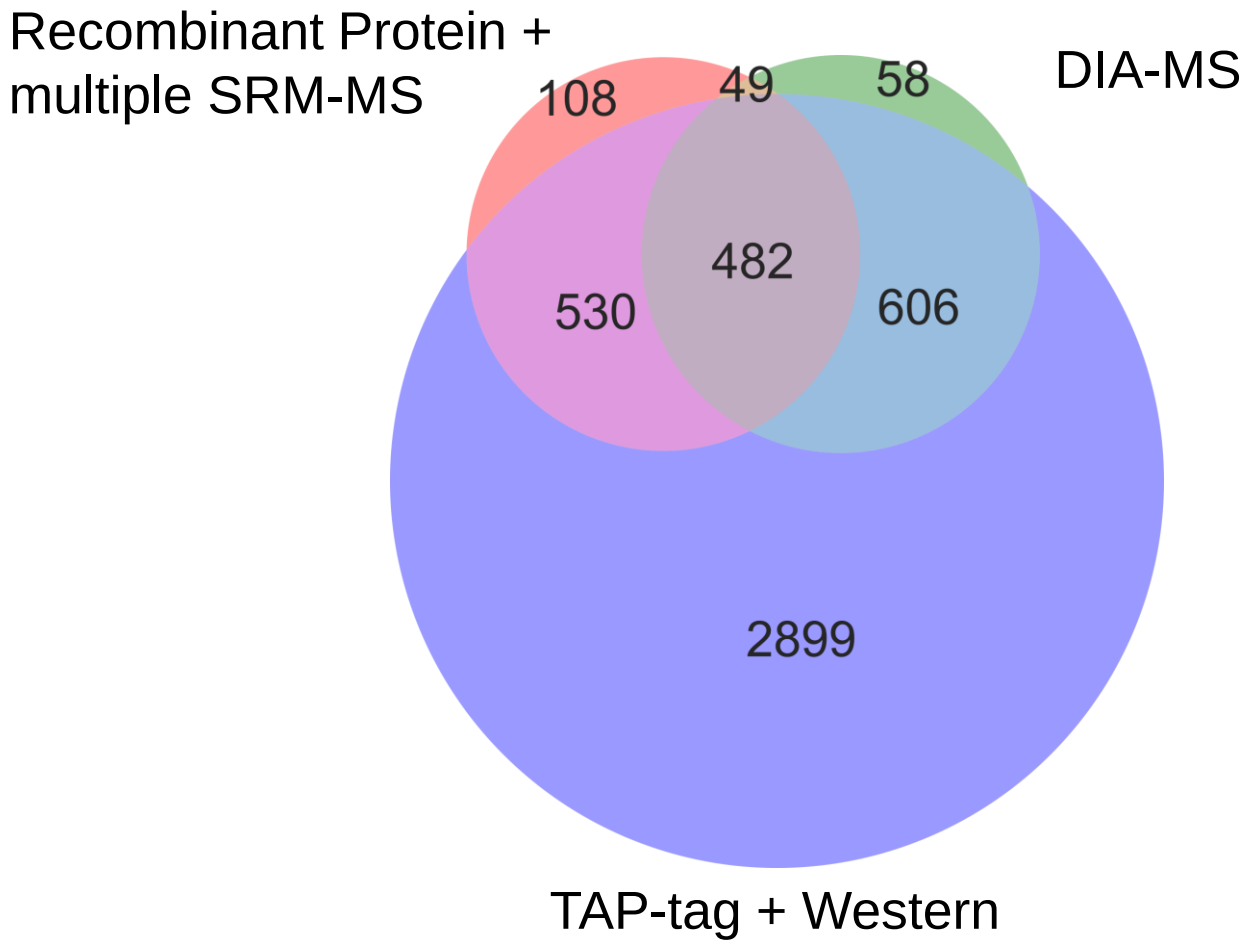
1195 proteins

DIA-MS

(Label-free)

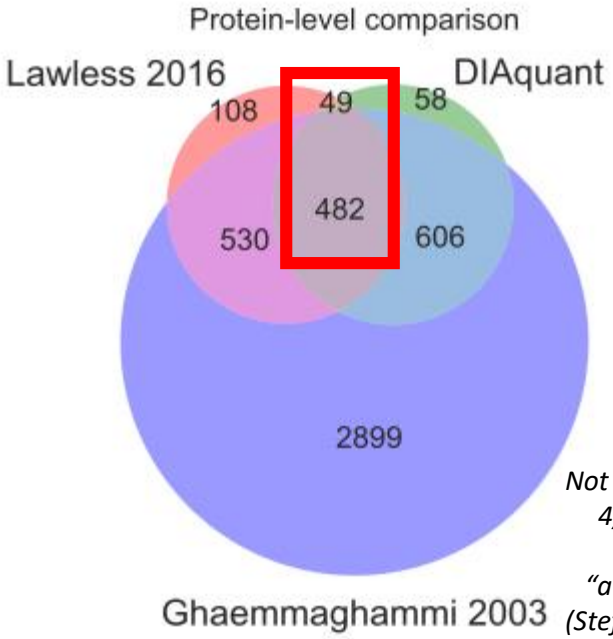
2 hours worth of DIA-MS

Faster, equally comprehensive proteome coverage as other MS-based expression profiling



Coverage: in context of other proteome-wide quantifications

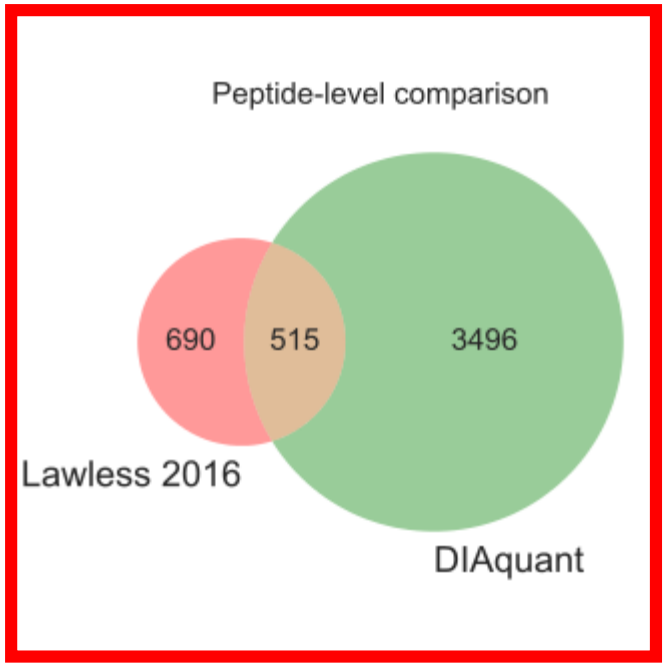
Proteins included



Not shown: DDA 4,040 proteins total (average 3,180), "at least 1 pep/pro" (Stefely 2016)

DIAquant: 2 pep/pro (unique)
Lawless 2016: at least 1 rank A/B peptide

Peptides from shared proteins (DIA-Quant, Lawless 2016)



DIAquant: 6,976 unique peptides
Lawless 2016: 3,835 A/B/C peptides

Agenda

- Absolute quantitation
- Method validations and the “Fit for Purpose” approach
- *Bonus!* Introduction to absolute quantitation with data independent acquisition (DIA)
- **CPTAC Assay Portal**

Recall from Monday morning…

Protein (peptide) Targets from Public Resources: CPTAC Assay Portal (https://assays.cancer.gov)

- Assays developed with internal standards
- Experimental details
- LOD/LOQ
- Precision
- Repeatability

https://assays.cancer.gov/CPTAC-795

Requirement checklist for the CPTAC Assay Portal

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.

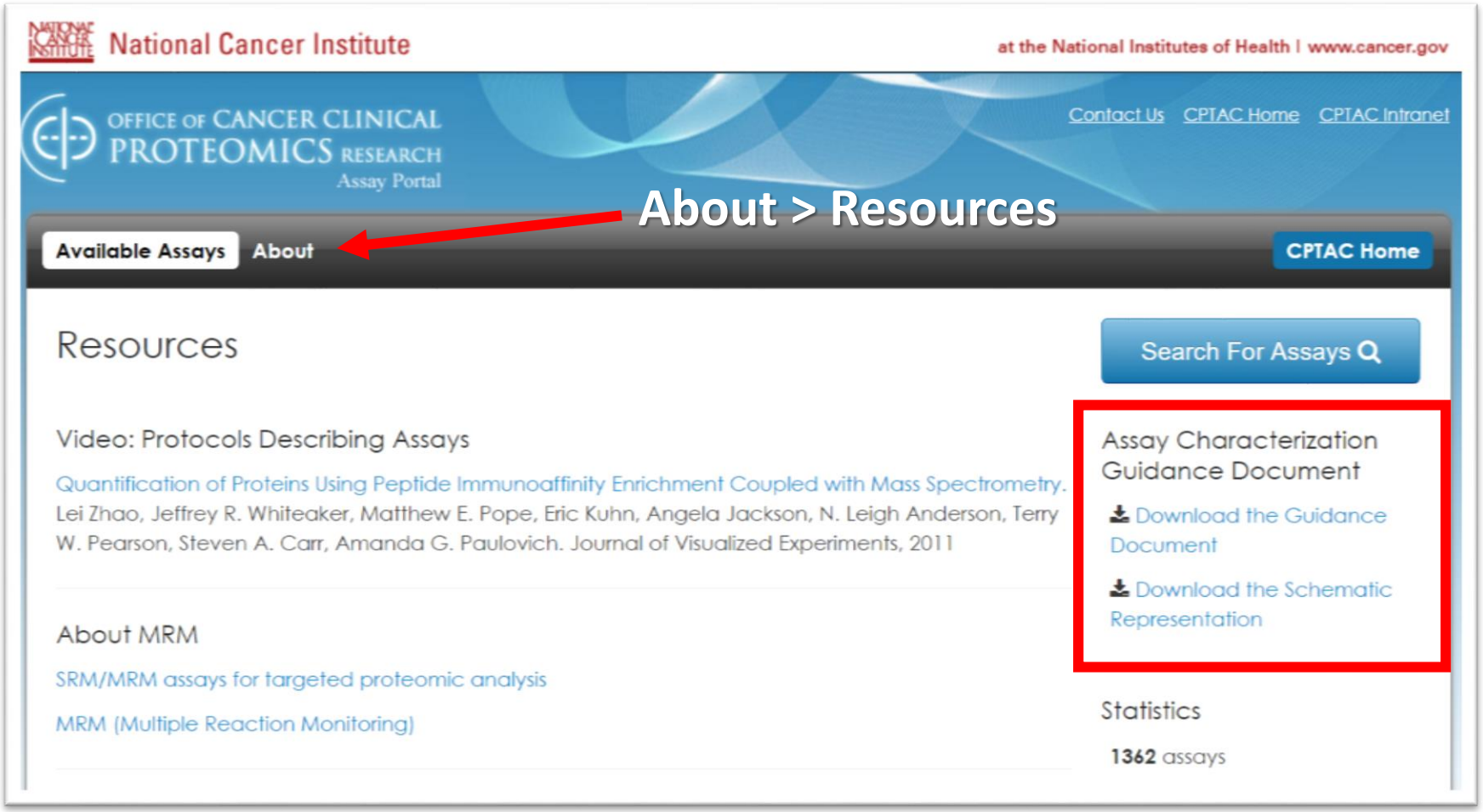
Experiment 4: *Stability*

- 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.

For detailed assay characterization guidance documentation



Conclusions

- Targeted methods should be calibrated and validated for reliable quantitation
- Rigor of validation depends on experimental goals (“fit for purpose” approach)
- Ideally, all targeted experiments should attempt the highest level of validation possible

For more information

- VIDEO: On serum protein quantification (Andy Hoofnagle, MD PhD)
  https://www.youtube.com/watch?v=czQUPfDsZ0s
- TUTORIAL: Setting up peak area calibration for absolute quant in Skyline
  https://skyline.ms/_webdav/home/software/Skyline/%40files/tutorials/AbsoluteQuant-3_5.pdf
- PROTOCOL: Developing a DIA method with Skyline
  http://www.nature.com/nprot/journal/v10/n6/abs/nprot.2015.055.html
- INFORMATION: DIA workflow using Skyline
  https://skyline.ms/wiki/home/software/Skyline/page.view?name=tutorial_dia