Leiden University
Medical Center

Calibration and metrology for protein MS tests

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MSACL SALZBURG
26 SEPTEMBER 2019

Learning objectives

- Understand the concept of ‘trueness’
- Know the role of internal standardization and understand reasons to select certain types of internal standards
- Understand important properties of calibrators and calibration strategies for quantitative bottom-up proteomics
- Know the concepts of standardization and metrological traceability
- Able to explain the calibration strategy for the multiplex apolipoprotein method

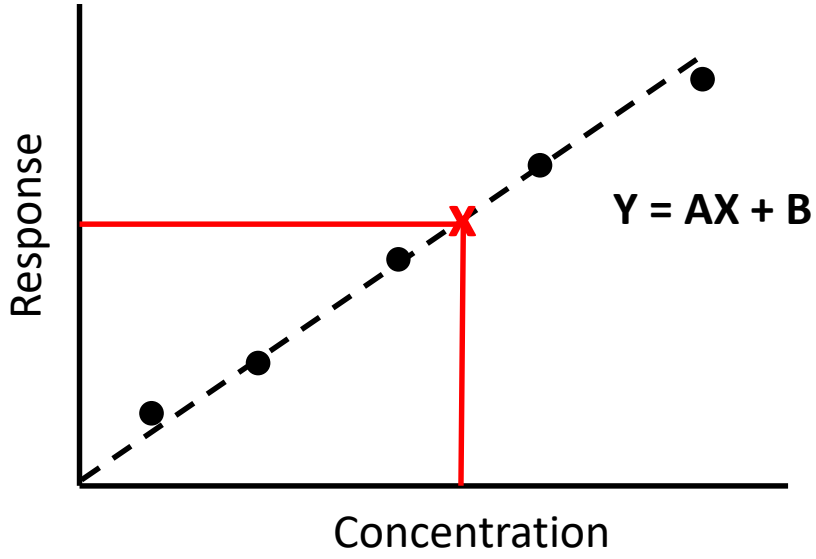
Overview

- Introduction to trueness and calibration
- Internal standardization
- Calibrators
- Metrological traceability and standardization

What is calibration?

VIM definition:

"Operation that, under specified conditions, in a first step, establishes a relation between the quantity values with measurement uncertainties provided by measurement standards and corresponding indications with associated measurement uncertainties and, in a second step, uses this information to establish a relation for obtaining a measurement result from an indication"



The lack of standardization of PSA

Test 1: calibrated based on first PSA test

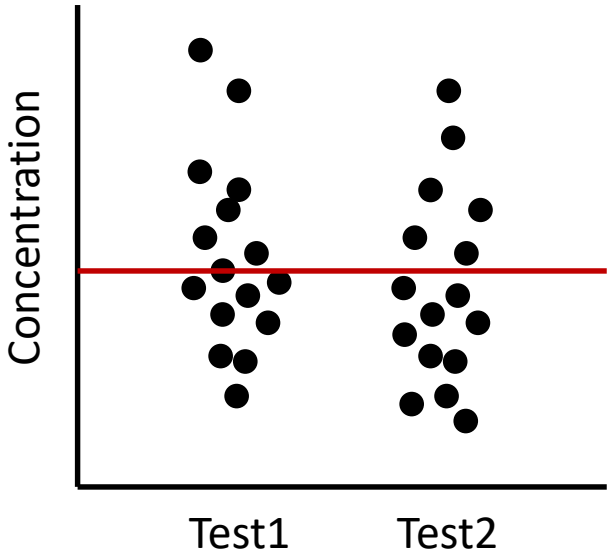
Test 2: calibrated based on WHO reference standard

Different results are obtained using the two tests

Both tests use the traditional cut-off of 4.0 µg/L

19% of patients would be candidates for biopsy in test 1, but not in test 2.

Result is adverse patient outcome, either due to too many biopsies or missed malignancies.



Armbruster and Miller, Clin Biochem Rev, 2007

How to achieve an accurate result?

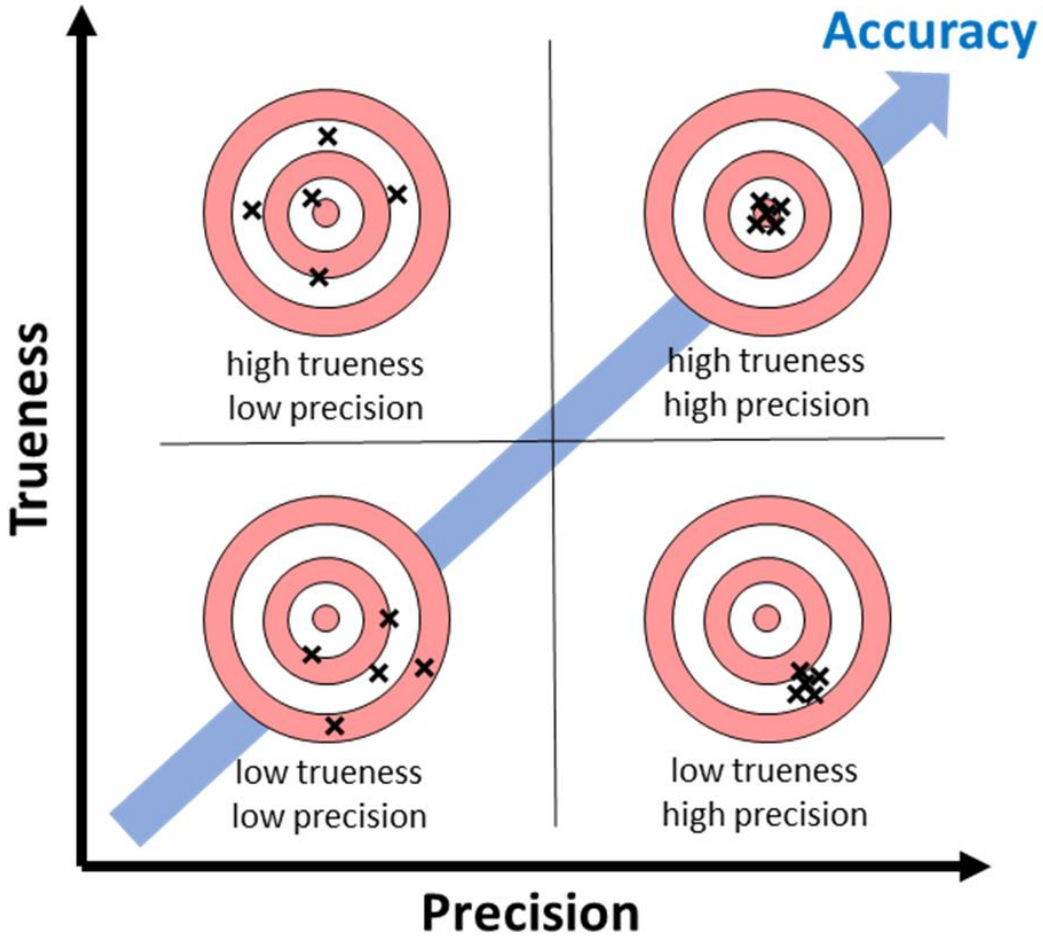
Precision

+

Trueness

=

Accuracy



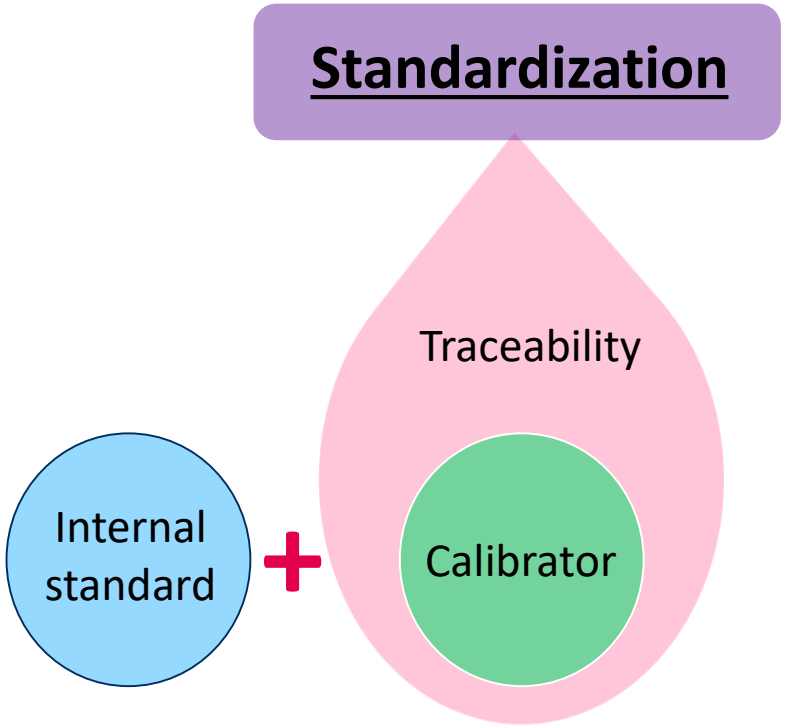
Today is about Trueness and how to achieve this through calibration for protein based MS-tests

Calibration and traceability for MS based methods

Calibrators should ideally be traceable to SI units through standardization.

Measurand should be well-defined

MS is not inherently quantitative → Internal standards are needed for normalization



Internal standards

"A compound added to a sample in known concentration to facilitate the qualitative identification and/or quantitative determination of the sample components."

IUPAC

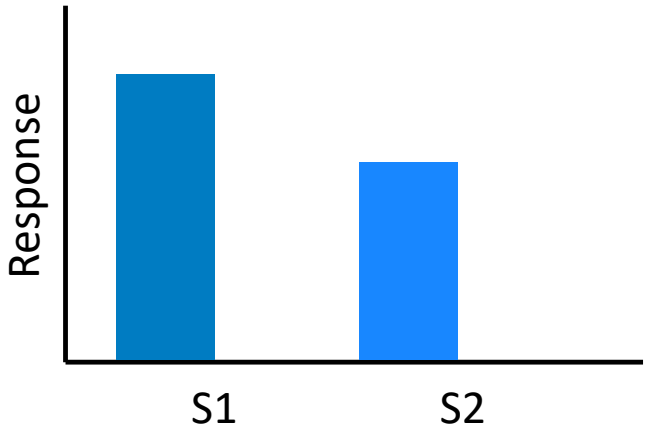
"A chemical substance that is added in a constant amount to calibration standards and unknown samples to correct for loss of analyte during preexamination preparation (e.g. solid phase extraction) or for matrix effects during analysis."

CLSI C62A

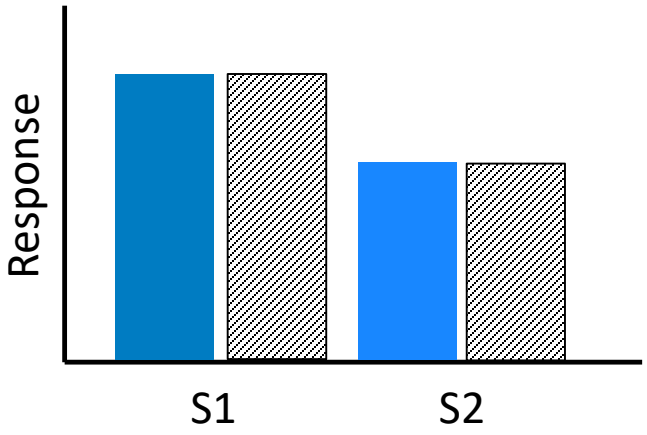
Goals of internal standardization

- Normalization of differences in recovery of sample preparation
- Normalization of differences due to injection variation
- Normalization of differences due to ionization effects
- Aid in identification of target analytes

Without internal standardization



With internal standardization



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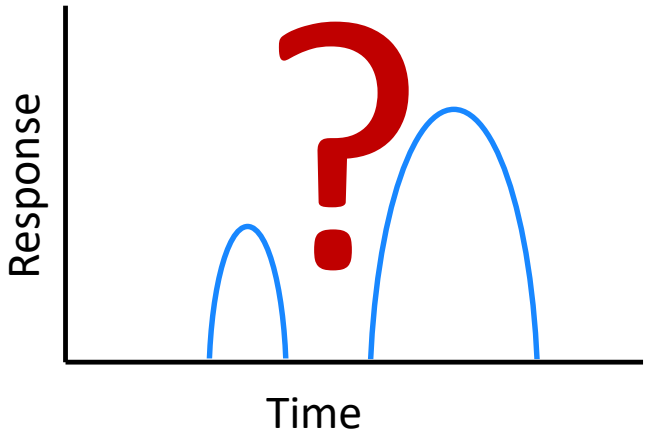
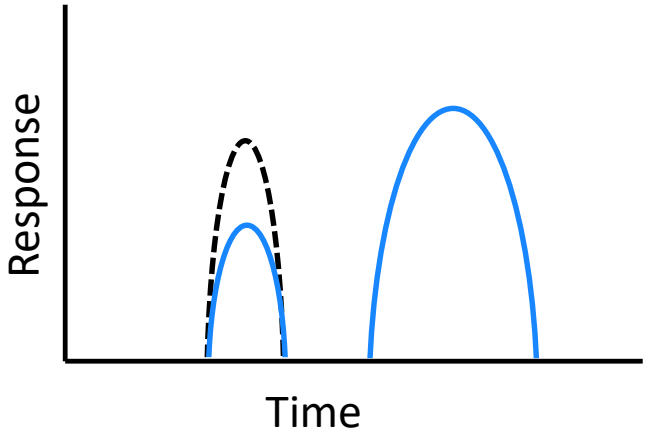
Without internal standardization



With internal standardization



Ideal properties of internal standards

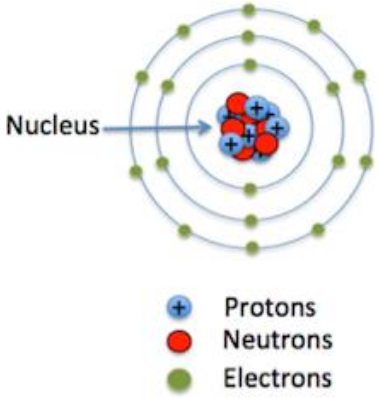
- Same physical and chemical behavior as target analyte
  - → Same LC retention as target analyte
  - → Same ionization efficiency as target
  - → Same fragmentation properties as target

- Different detection properties than target analyte

Stable isotope labelled analogs!

Internal standardization: stable isotope labelling

Isotopes are nuclides having the same atomic number but different mass numbers.



Atomic Number = # of Protons

Atomic Mass = (# of Protons) + (# of Neutrons)

Isotopes may occur naturally

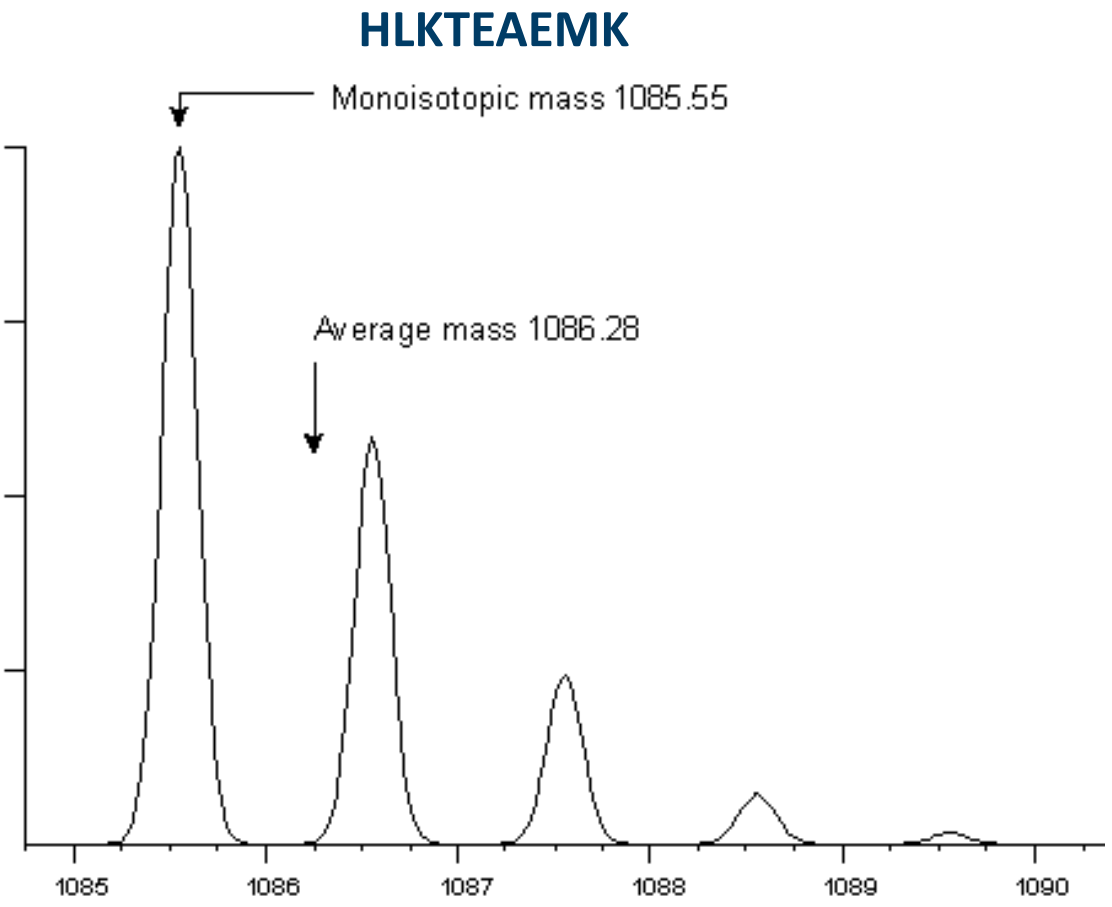
Number of Neutrons = Atomic Mass – Atomic Number

Number of Neutrons = 12 – 6 = 6	Number of Neutrons = 13 – 6 = 7	Number of Neutrons = 14 – 6 = 8
$^{12}_{6}C$	$^{13}_{6}C$	$^{14}_{6}C$
Carbon-12	Carbon-13	Carbon-14
98.9%	1.1%	<0.0001%

Isotope distribution in MS

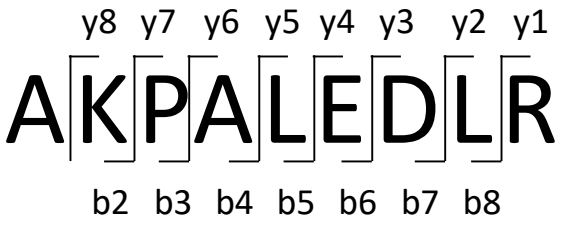
Naturally occurring isotopes, e.g. ^{13}C, result in isotopic envelope in MS.

→ Isotopes may also be used for internal standardization

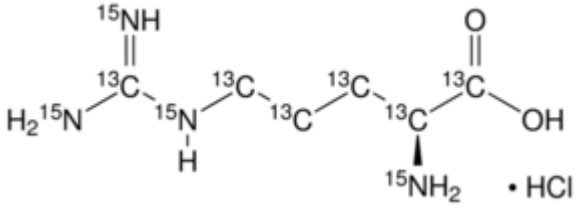


What label to use and where to label?

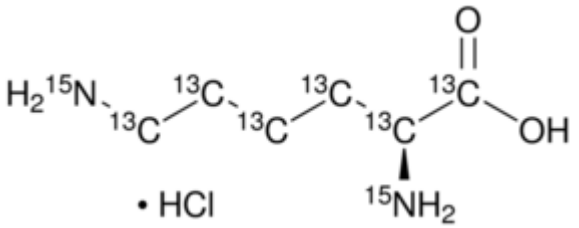
Peptide fragmentation (CID) often results in y-type fragments



b			y
---	1	**A** 9	---
200.1394	2	**K** 8	941.5415
297.1921	3	**P** 7	813.4465
368.2292	4	**A** 6	716.3937
481.3133	5	**L** 5	645.3566
610.3559	6	**E** 4	532.2726
725.3828	7	**D** 3	403.2300
838.4669	8	**L** 2	288.2030
---	9	**R** 1	175.1190

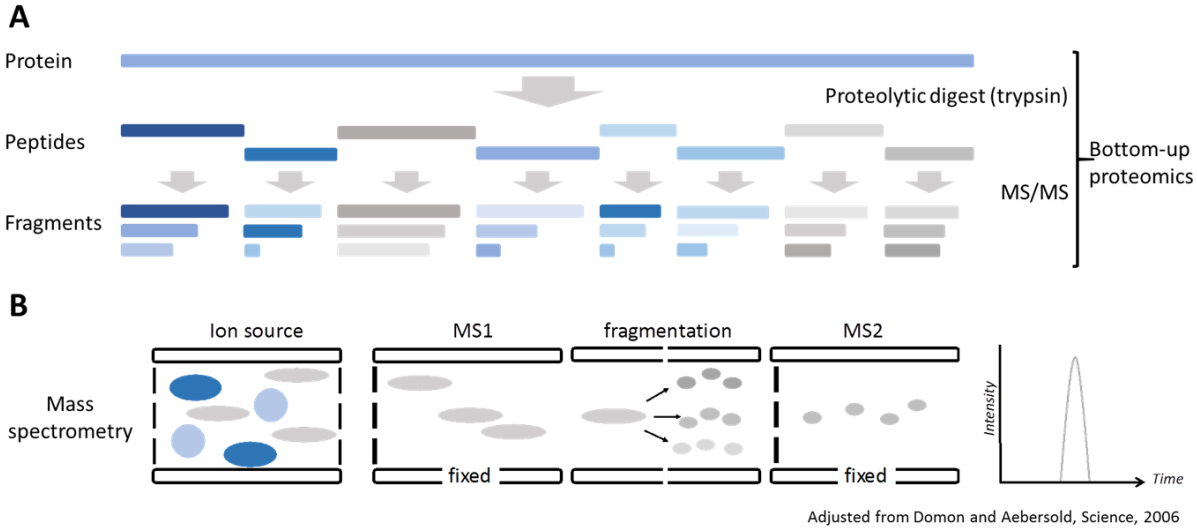


Arginine (+10)



Lysine (+8)

Quantitative bottom-up proteomics



Adjusted from Domon and Aebersold, Science, 2006

Advantages of bottom-up proteomics:

- Antibody independent
- Enables multiplexed tests
- 'lower' production costs
- Allows for molecular characterization of the measurand

Assumptions of bottom-up proteomics:

- Intact protein present in matrix
- No modifications in quantifying peptides (unless these are targeted)
- Equimolar or at least stable digestion of proteins independent of matrix

Internal standard should also correct for sample preparation

Types of IS for quantitative bottom-up proteomics

Several types of internal standards have been developed:

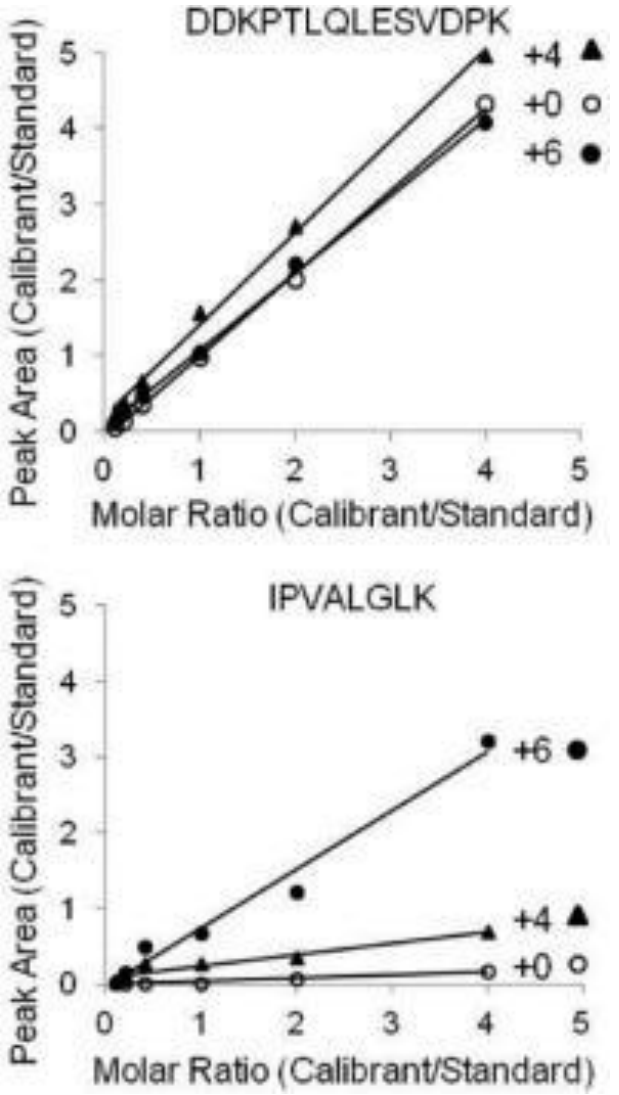
- SIL protein
  - ^{13}C, ^{15}N labelled R or K in protein
  - Expensive
  - Often not commercially available
- SIL peptides
  - ^{13}C, ^{15}N labelled R or K in peptide
  - Relatively easily obtained
- Winged SIL peptides
  - ^{13}C, ^{15}N labelled R or K in peptide
  - Peptide extended with several AA on both ends
- QPrEST
  - 50-150 AA section of target protein, ^{13}C, ^{15}N labelled R or K
  - Only commercially available
- QConCat
  - Artificial sequence created by ‘pasting’ several peptide sequences together
  - ^{13}C, ^{15}N labelled R or K

Properties of SIL peptides

Stable isotope-labeled peptides

1. co-elute with the targeted analytes
2. fragment to yield the corresponding, mass-shifted peptide backbone fragment ions
3. have (in the absence of interference) identical relative abundances of the fragment ions as the endogenous peptide
4. compensate for ion suppression and poor spray stability

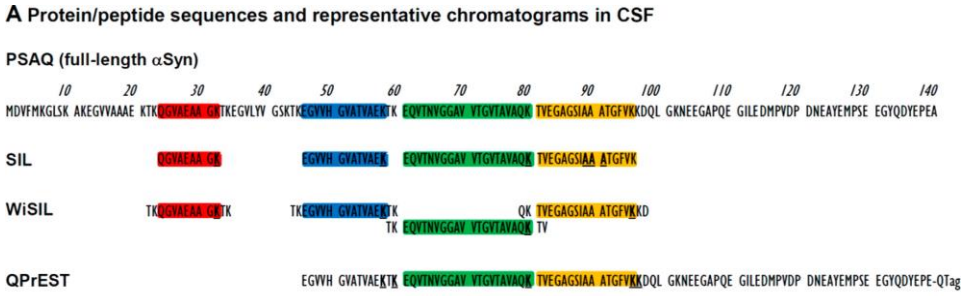
BUT: SIL-peptides do not correct for digestion

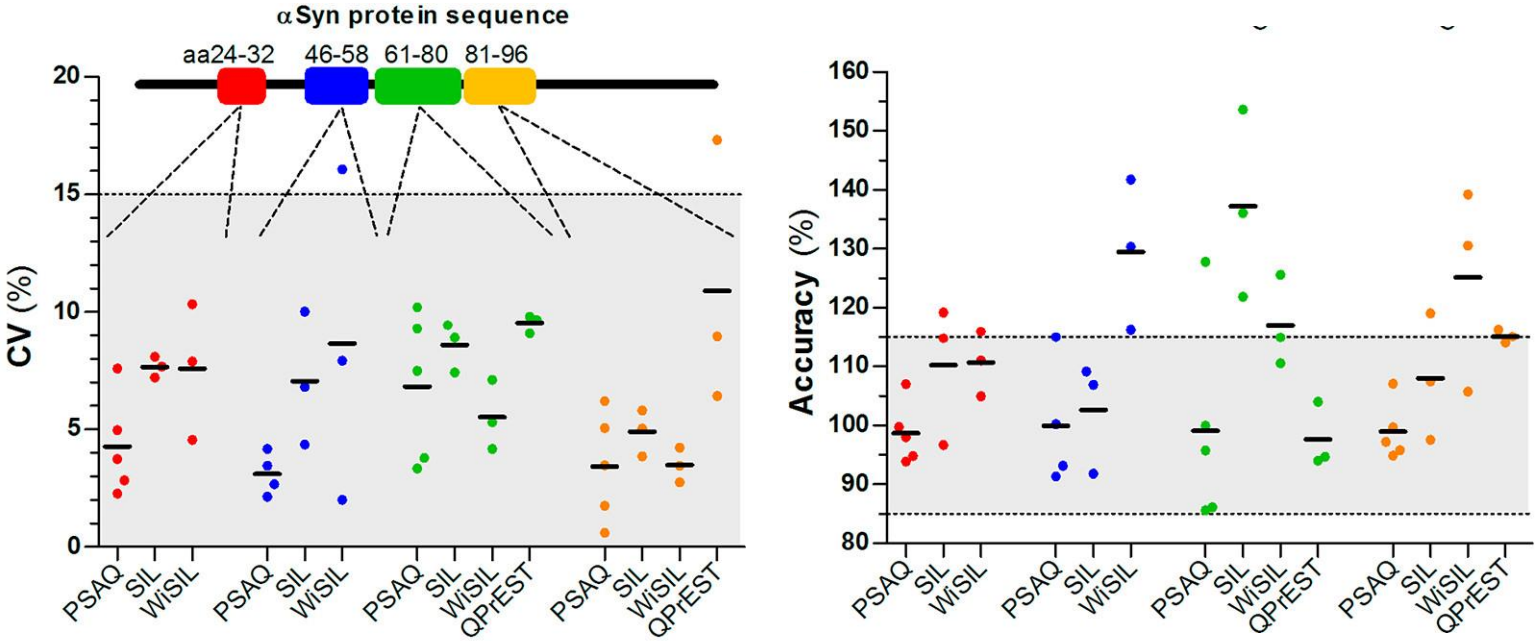


Scott et al., Anal Chem, 2015

Example of the use of SIL peptide vs SIL protein

- A-synuclein quantitation from CSF
- Comparison of SIL protein (PSAQ), SIL peptide (SIL), winged SIL peptide (WiSIL) and QPrEST
- Evaluation of both precision (%CV) and bias (%).





Oeckl et al. JPR, 2018

Calibrators

Properties of calibrators:

- Should behave similar to native materials
  - → Digestion
  - → Matrix effects
  - → commutability
- Should provide accurate results over the full measurement range
- Should be value assigned in a traceable manner to ensure comparability of results

Internal calibrators vs external calibrators

Internal calibration

Addition of a well-characterized and value assigned internal standard that also functions as a calibrator

SIL proteins, winged peptides etc, who correct for the full sample preparation procedure. Typically these materials do not behave the same during digestion and therefore this approach is currently not favoured in clinical chemistry.

External calibration

Native samples of a known concentration that behave the same as samples of interest. The external calibrators undergo the exact same sample processing as samples

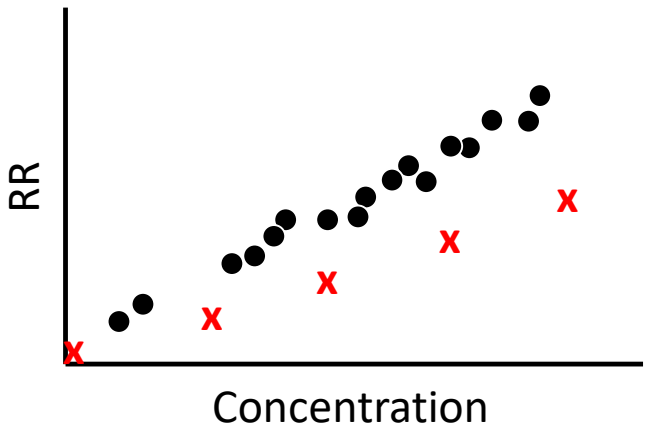
LUMC prefers External calibration

Commutability of calibrators is required!

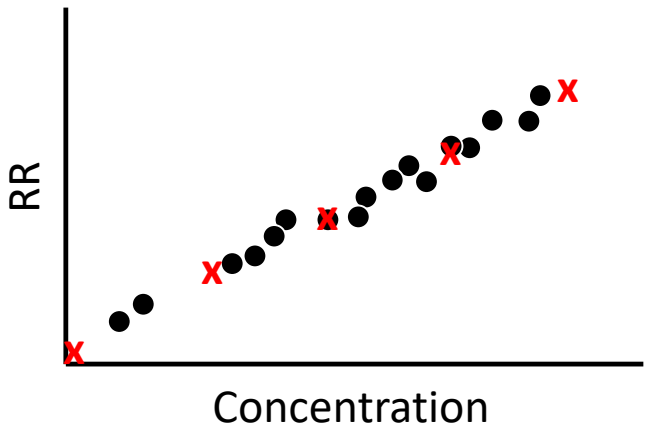
Commutability is *"the property of a material, demonstrated by the closeness of agreement between the relation among the measurement results for a stated quantity in this material obtained according to two give measurement procedures, and the relation obtained among the measurement results for other specified materials."*

The calibrators should behave the same as the native materials!

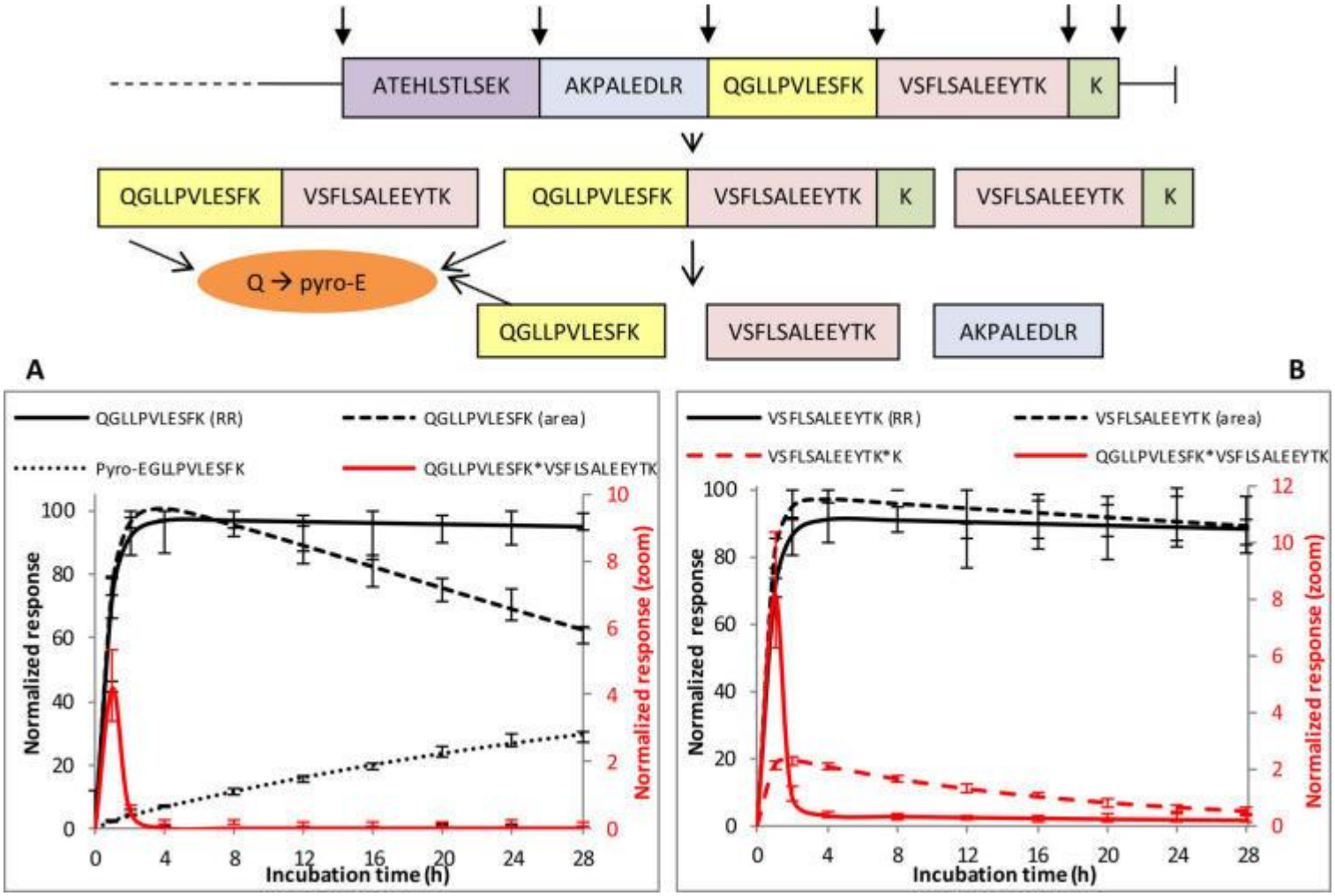
Non-commutable calibrators



Commutable calibrators



Digestion efficiency is important



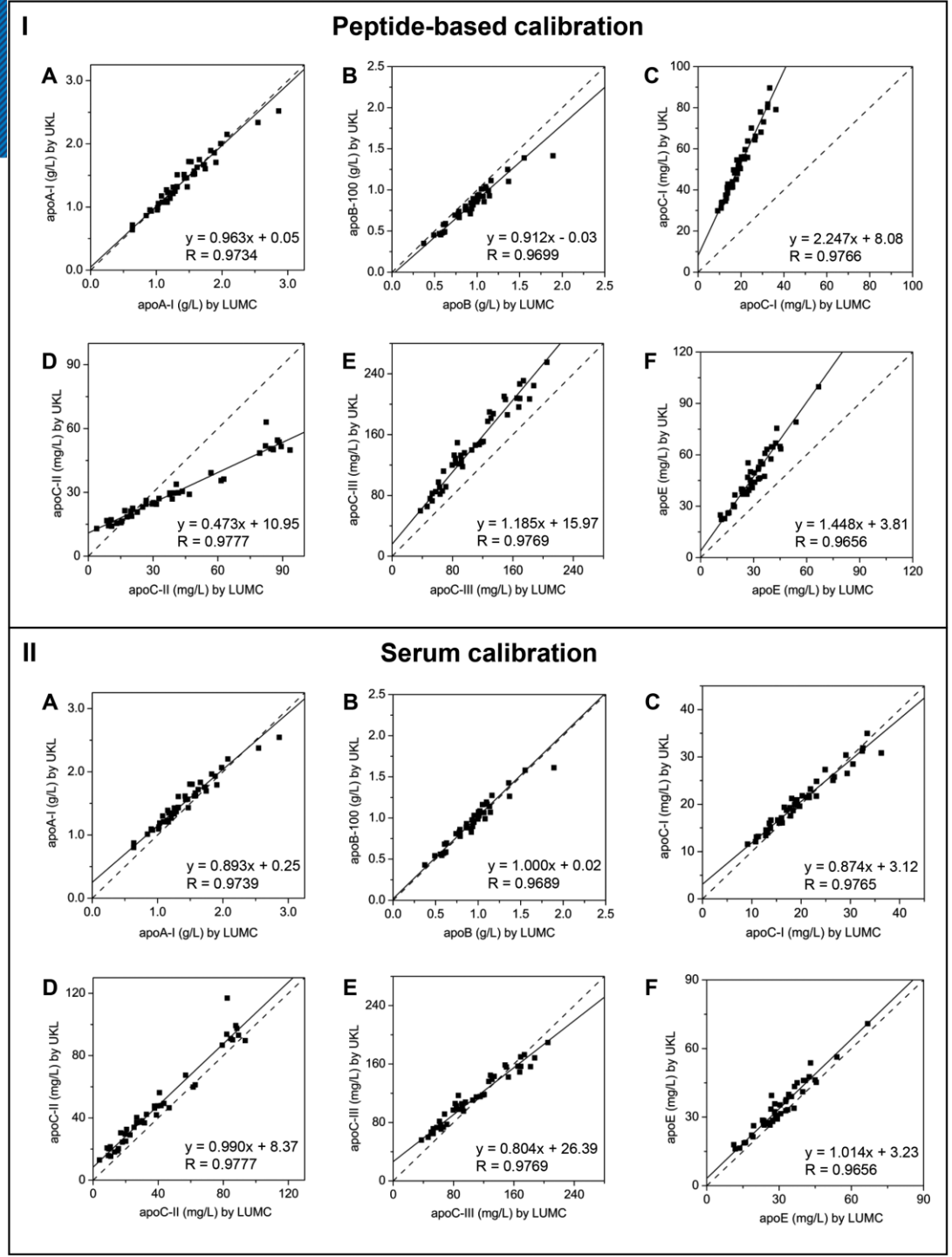
vd Broek et al., JPR, 2013

Peptide based calibration often results in bias

Quantitation of six apolipoproteins

Calibrators:

1. peptides in HSA
2. Value assigned native serum samples

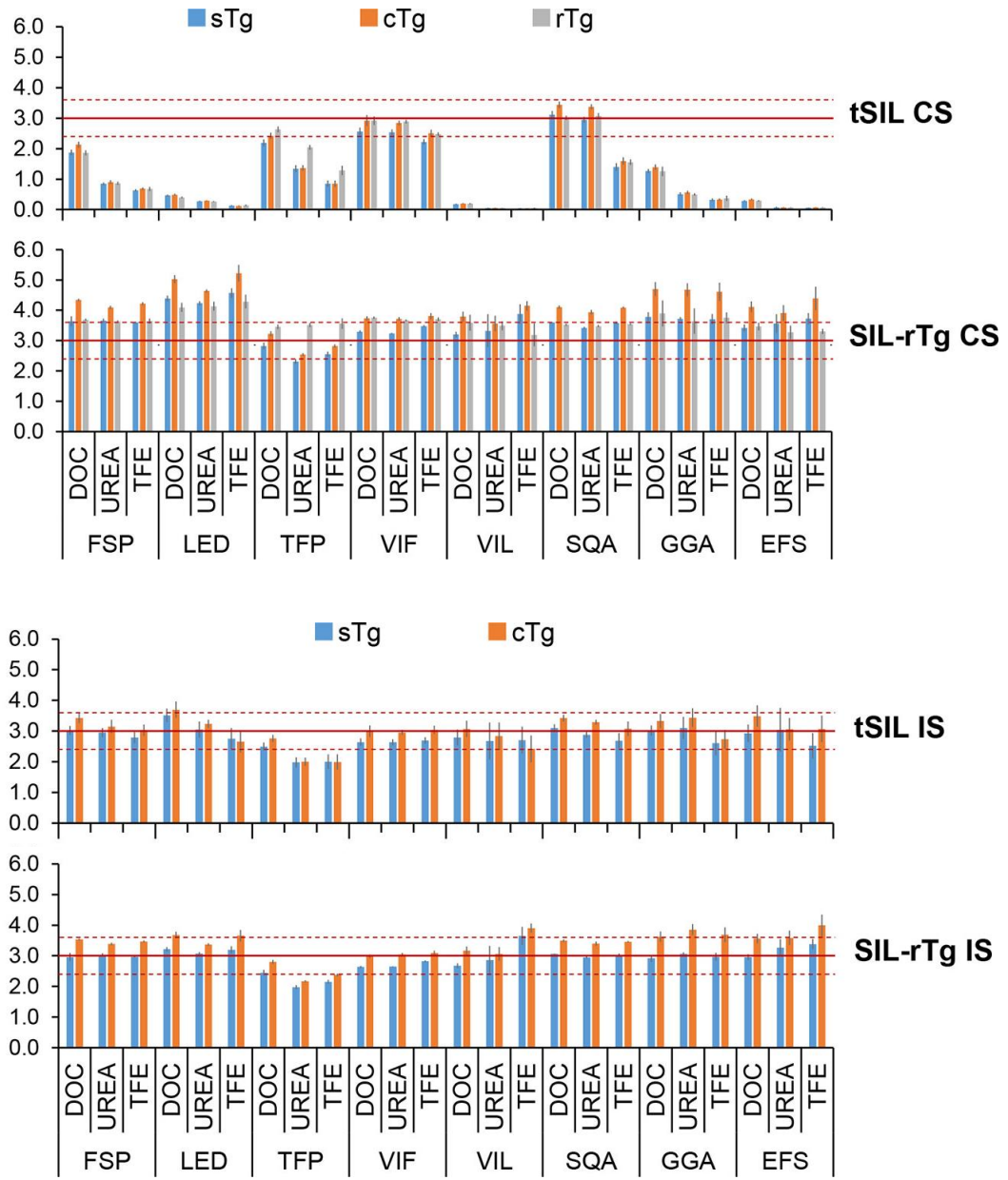


Dittrich et al., Proteomics, 2018

Comparison of calibration strategies

Comparison of accuracy (precision and bias) of quantitation of thyroglobulin

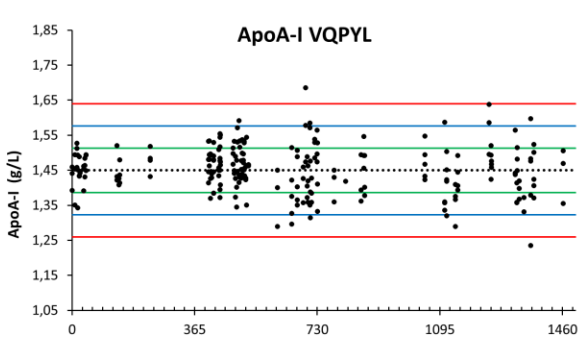
- Internal calibration using SIL peptides or SIL protein (top)
- External calibration using using SIL peptides or SIL protein (bottom)

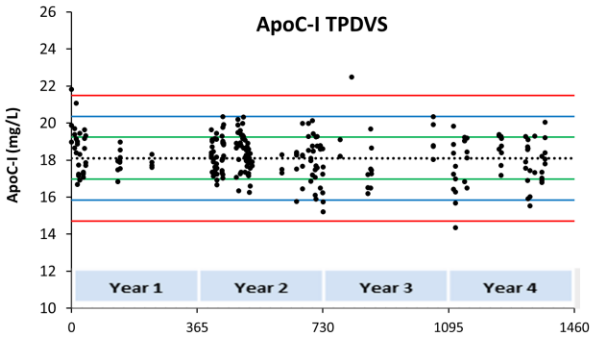


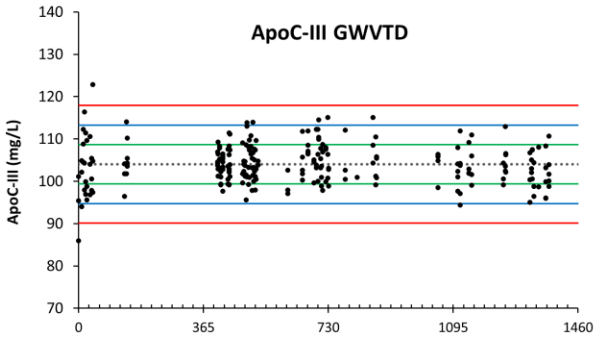
Shuford et al., Anal Chem, 2017

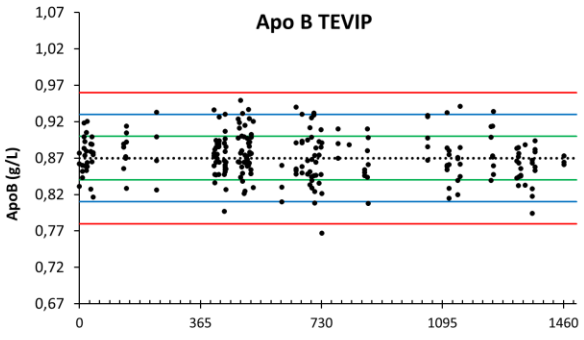
Long-term stability of results

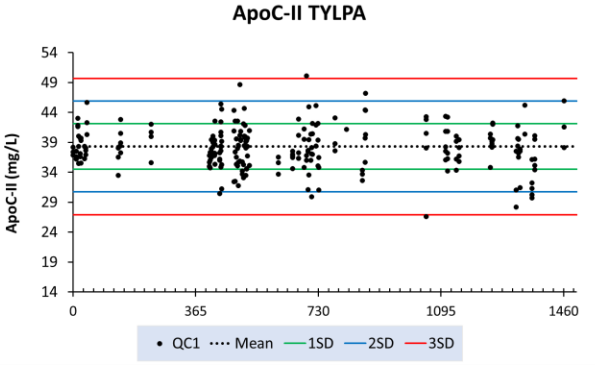
Proper selection of both internal standard and external calibrators, enable long-term stability of measurement results, even over multiple lot numbers of trypsin, several operators and two LC-MS instruments!

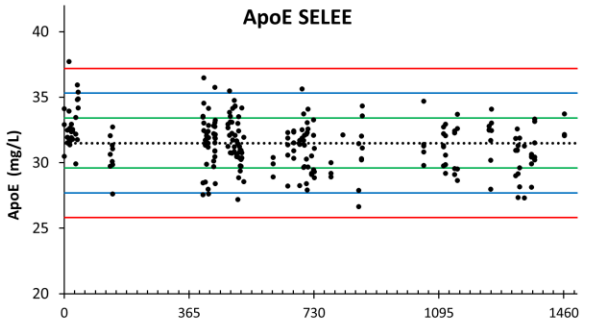












Standardization through metrological traceability

Metrological traceability

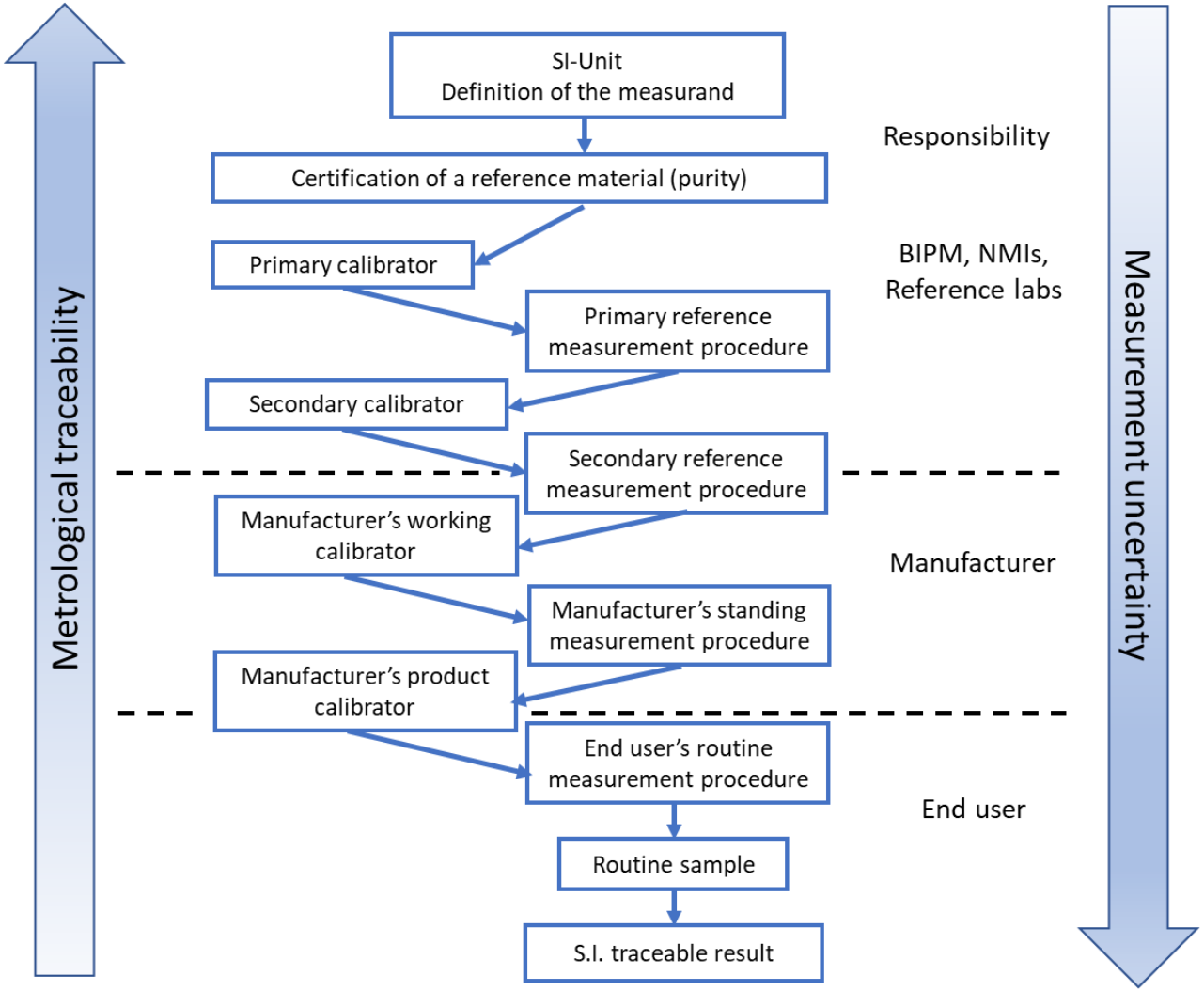
"Property of a measurement result whereby the result can be related to a reference through a documented unbroken chain of calibrations, each contributing to the measurement uncertainty"

Standardization and metrological traceability is described in several ISO guides

Information on standardization of measurands can be found in the JCTLM database: https://www.bipm.org/jctlm/

Currently, a complete traceability chain is in place for only ~10% of clinical chemistry analytes!

Metrological traceability chain to achieve standardization



Adapted from ISO 17511:2003

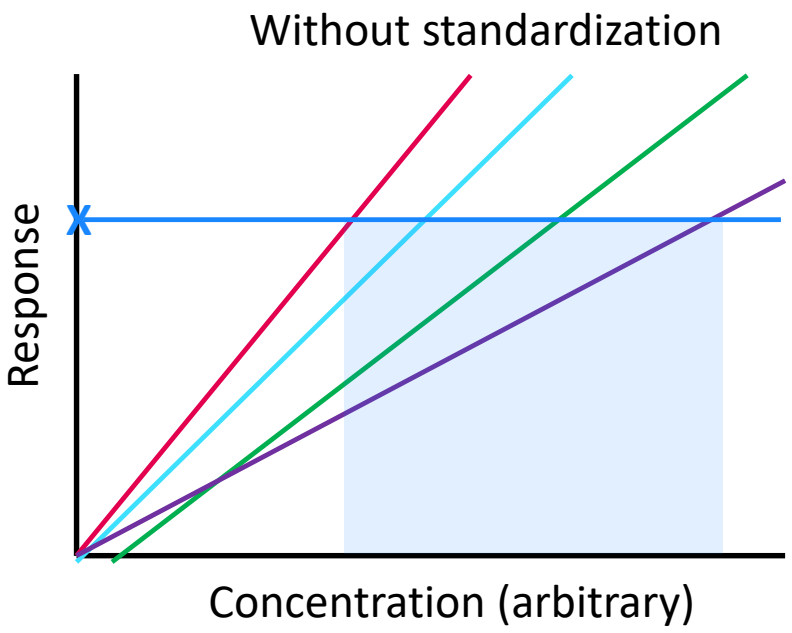
For LDTs, the end users have the manufacturers responsibility

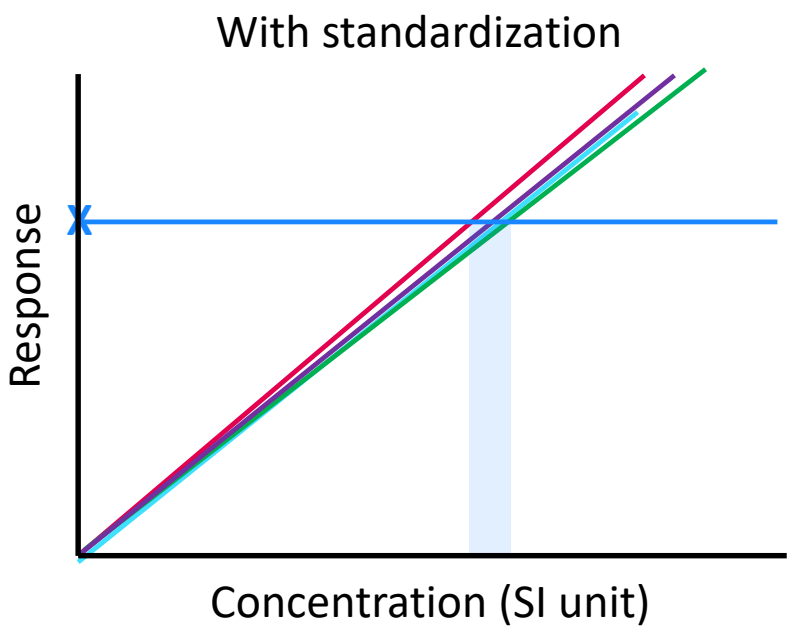
Goals of standardization

Standardization enables comparability of results between laboratories through anchoring to SI units.

Should ideally be aimed at early in method development phase to avoid patient harm and duplication efforts.

If standardization is not feasible, harmonization towards a consensus reference material may be considered.





A well-defined measurand is important!

Units

The **International System of Units (SI)** is the modern form of the metric system and is the most widely used system of measurement. It comprises a coherent system of units of measurement built on seven base units, which are the second, metre, kilogram, ampere, kelvin, mole, candela.

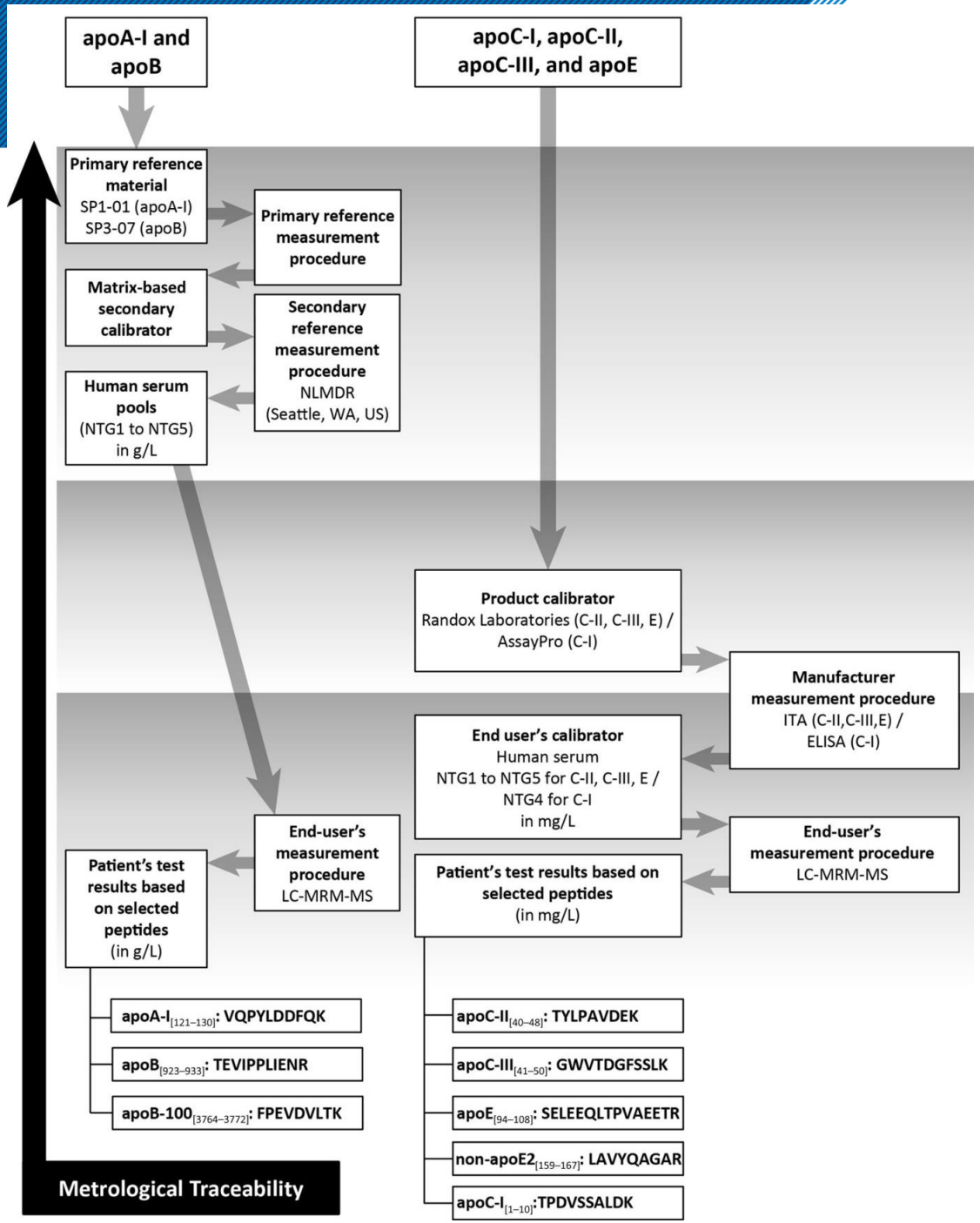
amount of substance is the number of discrete atomic-scale particles in a sample. It is expressed in moles (mol).

1 mole is defined as exactly $6.02214076 \times 10^{23}$ particles (Avogadro number).

SI units

Base quantity Grandeur de base	Base unit Unité de base	
Name Nom	Name Nom	Symbol Symbole
length longueur	metre mètre	m
mass masse	kilogram kilogramme	kg
time temps	second seconde	s
electric current courant électrique	ampere ampère	A
thermodynamic temperature température thermodynamique	kelvin kelvin	K
amount of substance quantité de matière	mole mole	mol
luminous intensity intensité lumineuse	candela candela	cd

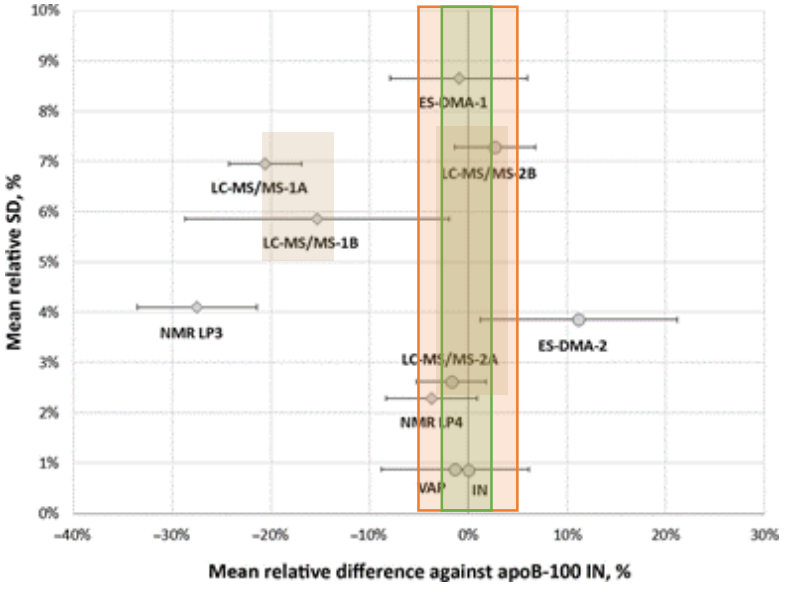
Metrological traceability chain for apolipoproteins



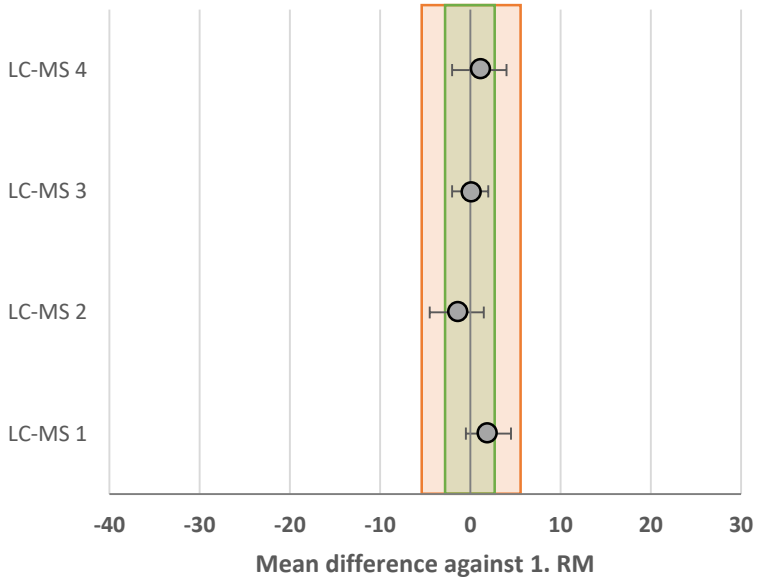
vd Broek et al., Clin Chem, 2016

Traceability and standardization of apoB: BioSITrace

Current situation, ApoB



Ideal situation, ApoB



ApoB Tea = 11.5%
Based on biological variation

Delatour et al. Clin Chem 2018

Different calibrators and traceability

Traceability to WHO-IFCC 1. reference material

International efforts in working groups e.g. by IFCC

The issue of proteoforms and standardization

Proteoforms are all of the different molecular forms in which the protein product of a single gene can be found, including changes due to genetic variations, alternatively spliced RNA transcripts and post-translational modifications.

→ Genetic modifications

Insertions

Deletions

Point mutations

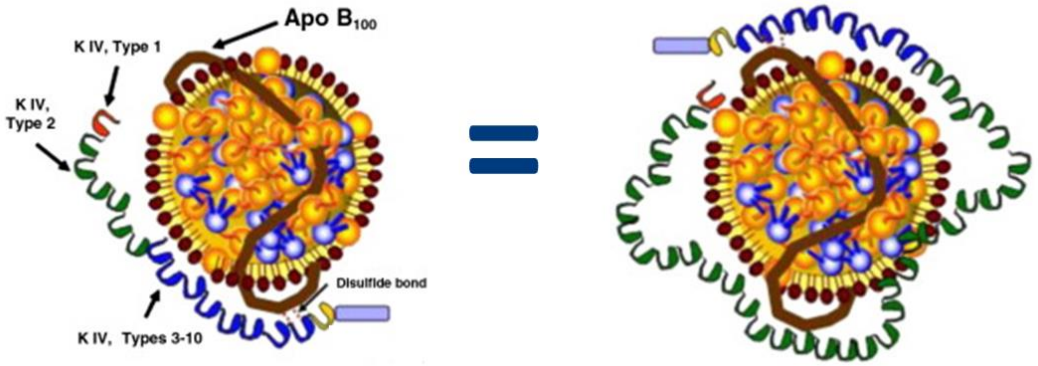
→ Oxidation

→ Phosphorylation

→ Glycosylation

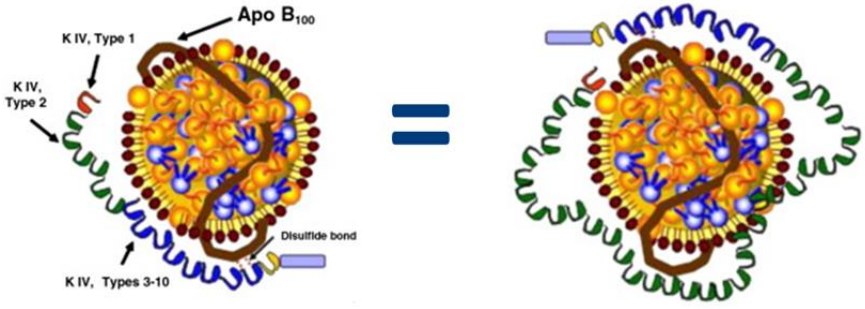
→ Etc..

The example of apo(a)



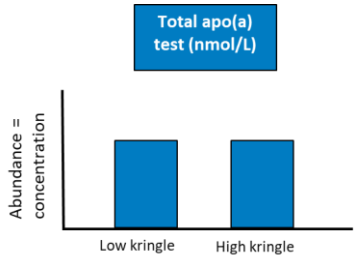
Expression of concentrations in mol/L enables unequivocal results!

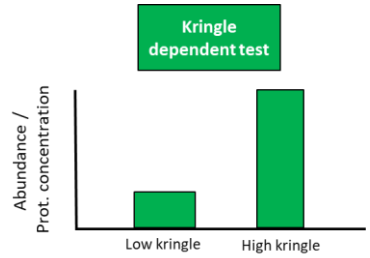
Effects of apo(a) proteoforms on standardization

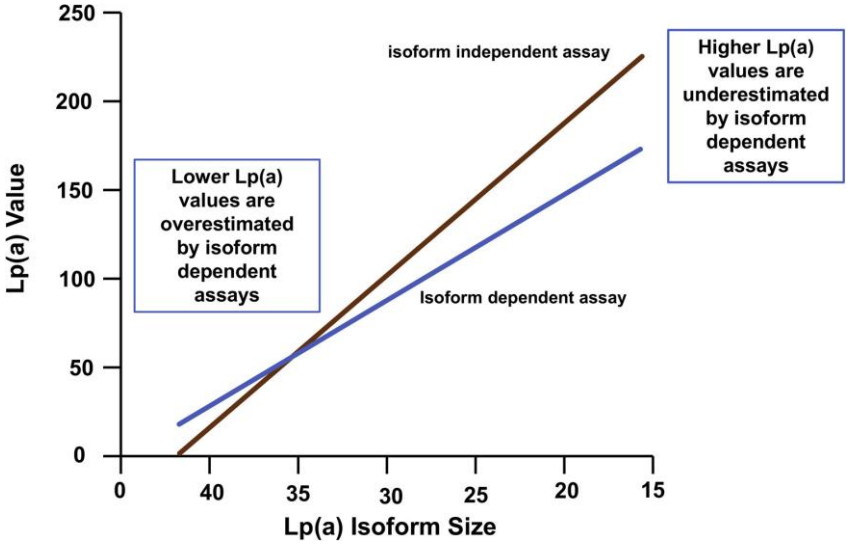


# of kringles IV-2	AA mass (Da)	# of N-glycans	N- & O-glycan mass (Da)	Total apo(a) mass
3	186945	14	77671	264616
10	275250	21	116506	391756
20	406480	31	171985	578465
30	536379	41	227464	763843
40	628098	51	282943	911041
50	798294	61	338422	1136716

Kringle-dependent vs kringle-independent quantitation







Most proteins exist in multiple proteoforms!
PSA, antithrombin, apoCIII, etc.

Tsimikas et al., J Clin Lipidol, 2018

Proteins should be quantified in mol/L!

Take home messages

- Accuracy is the product of precision and trueness
- Trueness can only be achieved through a proper calibration strategy
- The internal standard should be selected carefully, depending on the application
- Calibrators should be commutable and traceable to SI units through a metrological traceability chain
- Proteins should ideally be quantified in molar units

Literature

Internal standards for protein analysis

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Standardization

- **Tate and Panthegini**, Standardization – The theory and practice
- **Van den Broek et al.** Quantifying Protein Measurands by Peptide Measurements: Where Do Errors Arise?, JPR, http://pubs.acs.org/doi/abs/10.1021/pr5011179
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Prime time for Precision Diagnostics driven by unmet Clinical Needs

14 November 2019

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Acknowledgements

Clinical Chemistry

- Christa Cobbaert
- Yuri van der Burgt
- Tirsa van Duijl
- Fred Romijn
- Nico Smit
- Mervin Pieterse
- Maarten van Hoorn
- Caithlin Peelen
- Arnoud van der Laarse

Marie Curie Fellowships

E.C. Noyons stipend

Leiden University
Medical Center

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