Calibrated Quantification

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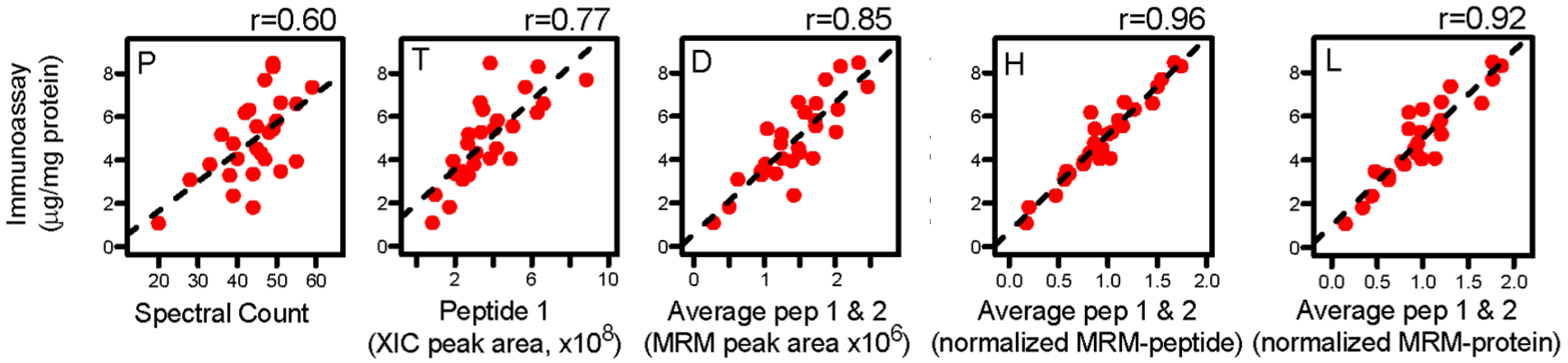
Why Calibrate?

reduce magnitude of analytical variance
(and increase accuracy of relative quantification)

Apolipoprotein E



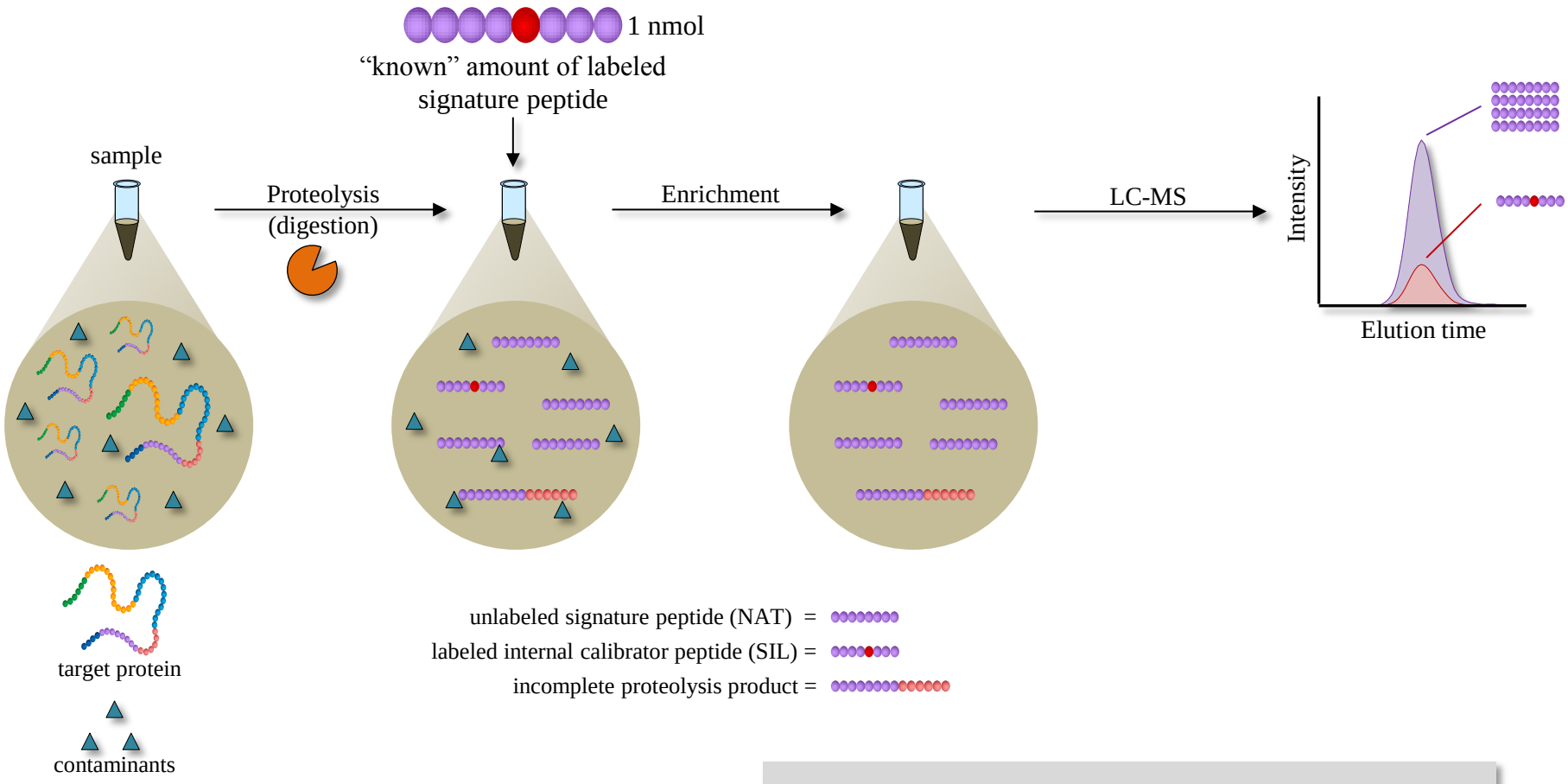
Un-calibrated

"Calibrated" to Internal Standard

Courtesy of Andrew Hoofnagle, UW

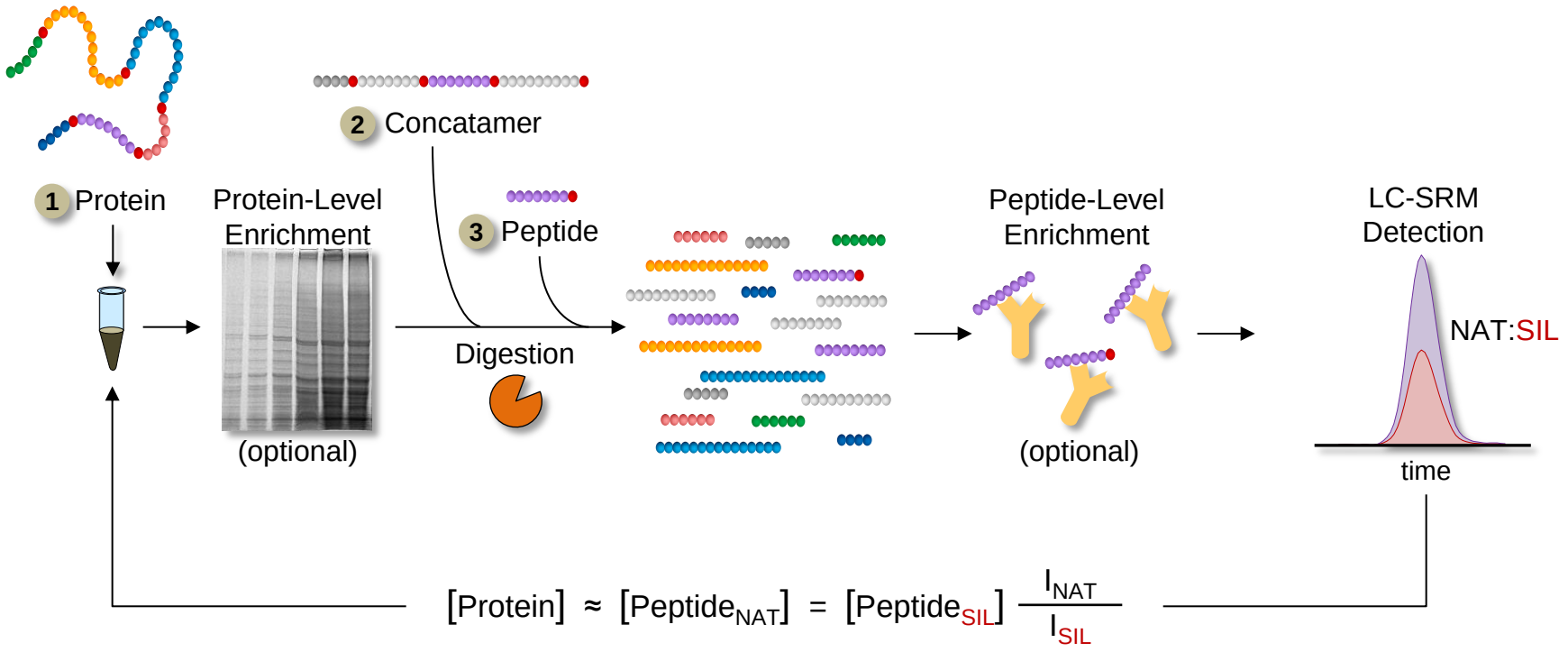
A.N. Hoofnagle *et al.*, *Clin. Chem.*, **2012**, 58(4), 777-781.

Internal Calibration



$$\frac{[NAT]}{[SIL]} = \frac{I_{NAT}}{I_{SIL}} \Rightarrow [NAT] = \frac{I_{NAT}}{I_{SIL}} \times [SIL]$$

Internal Calibration Hierarchy

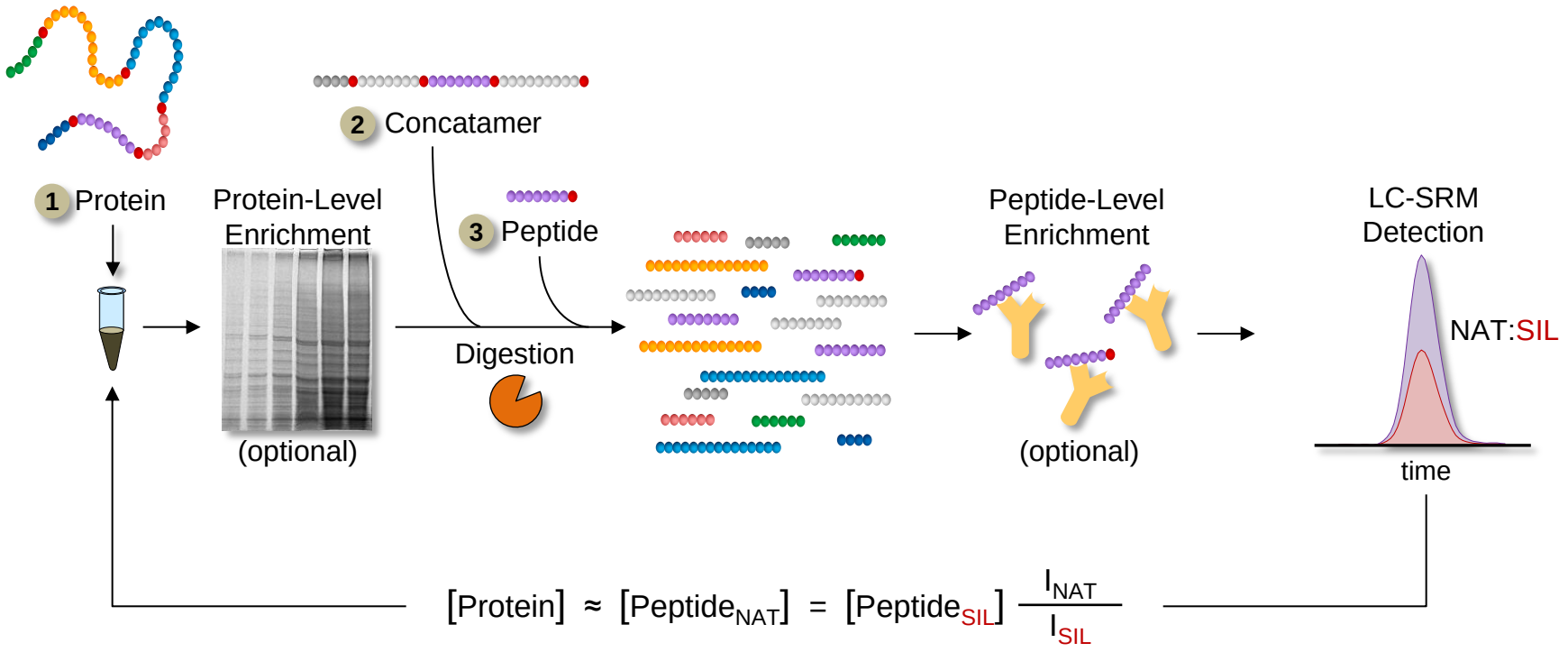


1 **Full-length Protein:** Desiderio, D. M. and co-workers, *Biol. Mass Spectrom.* **1991**, 2(2), 149-156
Brun, V. and co-workers, *Mol. Cell. Proteomics* **2007**, 6(12), 2139-2149

2 **Peptide Concatemer:** Beynon, R. J. and co-workers, *Nat. Methods* **2005**, 2(8), 587-589
Beynon, R. J. and co-workers, *Nat. Protocols* **2006**, 1(2), 1029-1043

3 **(Partial) Peptide:** Barr, J. R. and co-workers, *Clin. Chem.* **1996**, 42(10), 1676-1682
Gygi, S. P. and co-workers, *P. Natl. Acad. Sci. USA* **2003**, 100(12), 6940-6945.

Internal Calibration

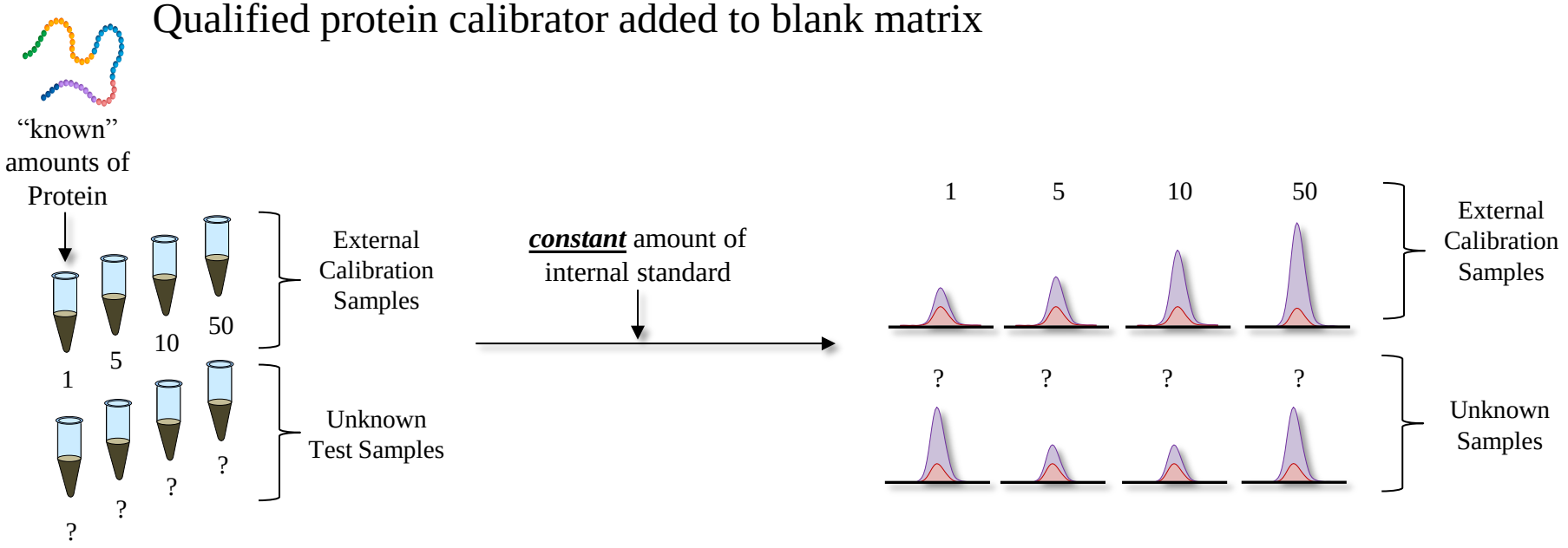


Pitfalls:

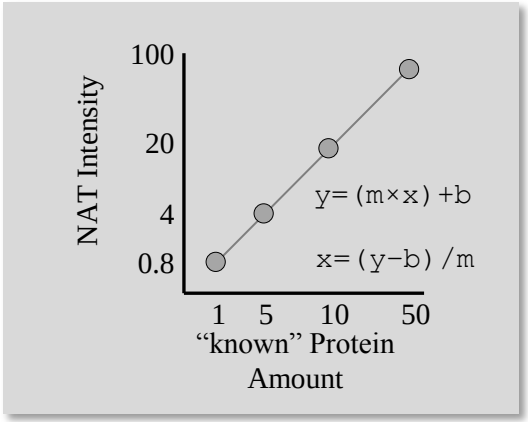
- Assume digestion is stoichiometrically complete (peptide and concatamers)
  - Results vary by digestion/denaturation conditions
- Reproducibility between digestions/days is critical
  - Internal Calibrator must be stable
- Response assumed to be linear

External Calibration: Multipoint

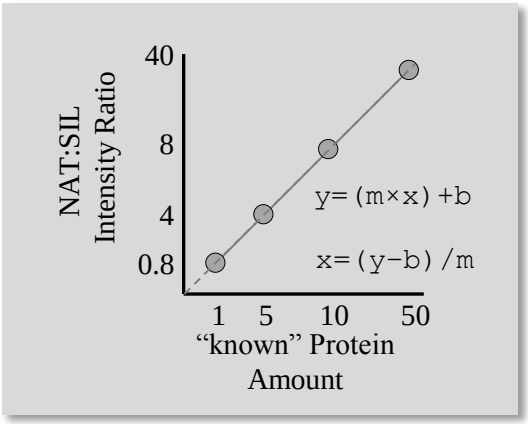
Qualified protein calibrator added to blank matrix



Without Internal Standard

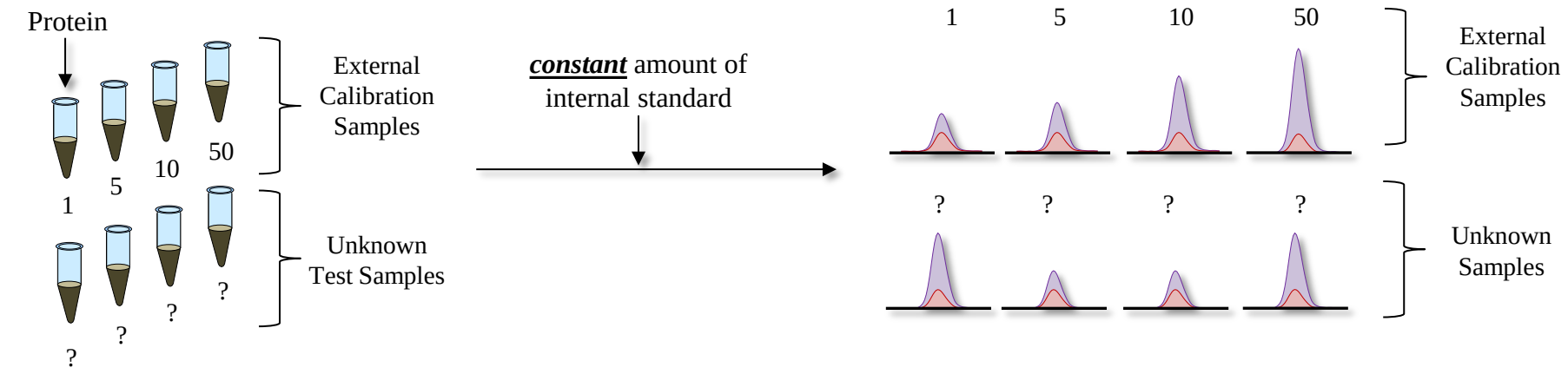


With Internal Standard



External Calibration: Multipoint

Qualified protein calibrator added to blank matrix

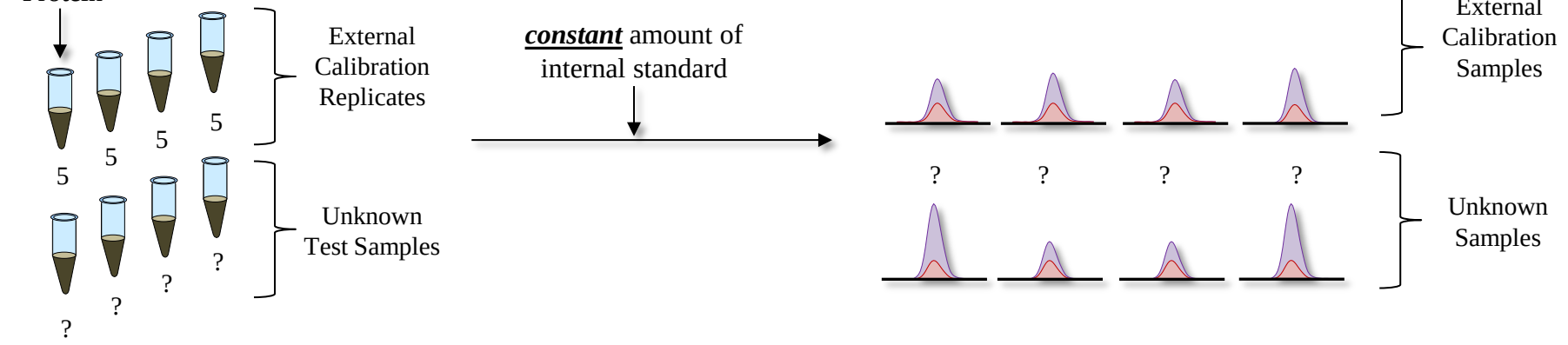


Pitfalls:

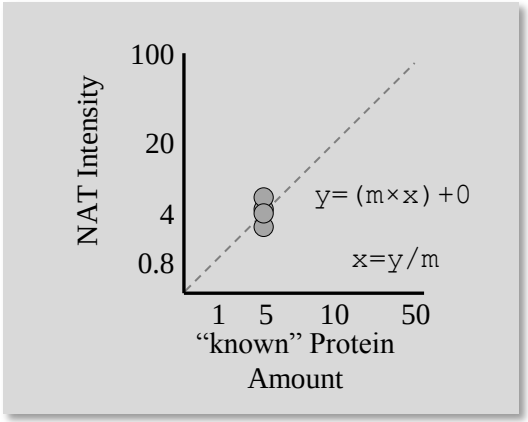
- "Blank Matrix" must be essentially identical to sample matrix (requires verification)
- Qualified protein should have same structure as endogenous protein (size, conformation, PTMs, etc)
- External calibrators should be processed in parallel with unknown samples (added cost/time).
- External calibrators must be stable

External Calibration: Single-point

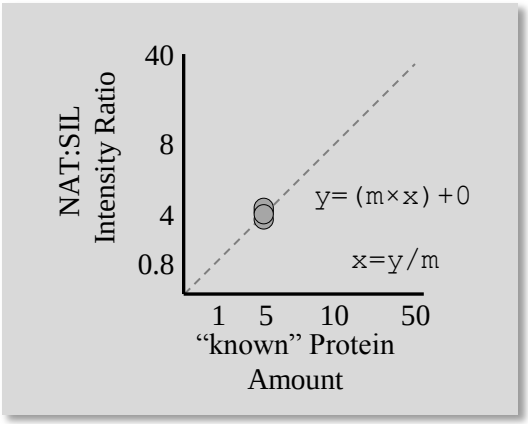
Qualified protein calibrator added to blank matrix



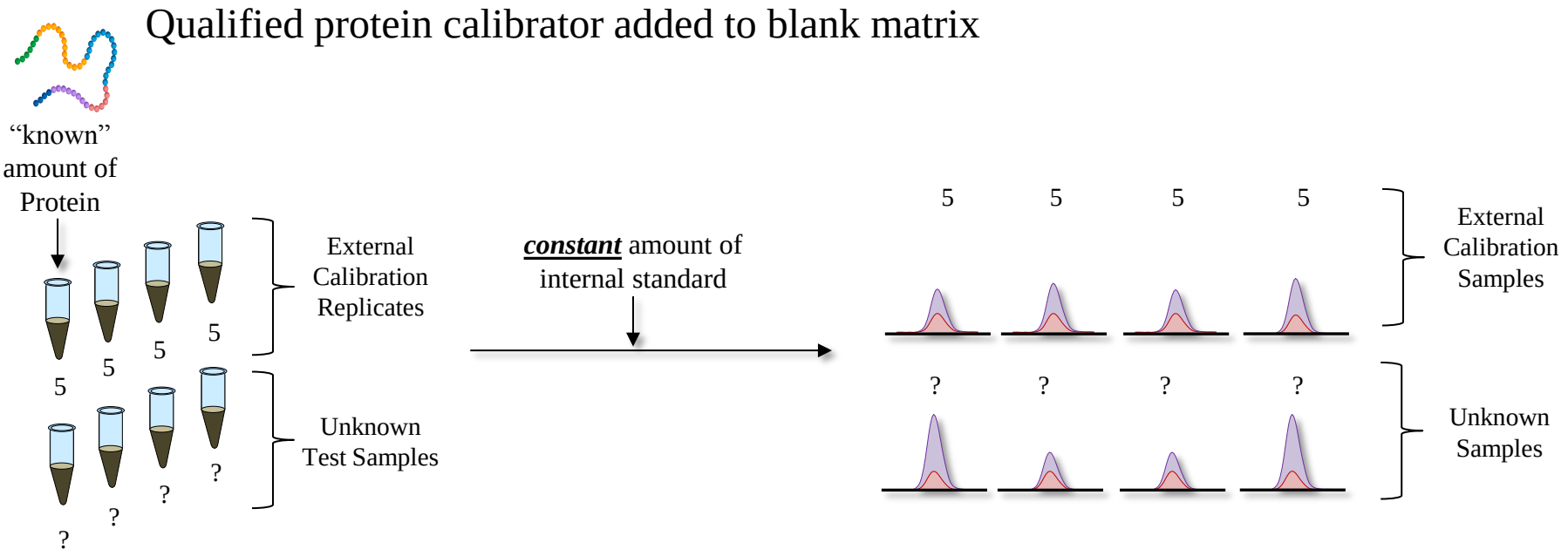
Without Internal Standard



With Internal Standard



External Calibration: Single-point



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Additional Reading

Source of Analytical Variance in Proteomics
H.D. Cox *et al.*, *Clin. Chem.* **2016**, 60 (3), 541-548.

Peptide Degradation during Digestion
C.M. Shuford *et al.*, *Mol. Cel. Proteomics*, **2012**, 11 (9), 814-823.

More Peptides = More Confidence
Q. Fu *et al.*, *Clin. Chem.* **2016**, 62 (1), 198-207.

Single-point Calibration with Sample Pool
S.A. Agger *et al.*, *Clin. Chem.* **2010**, 56 (12), 1804-1813.

Analytical Verification in Translational Research
R.P. Grant and A.N. Hoofnagle, *Clin. Chem.* **2014**, 60 (7), 941-944.