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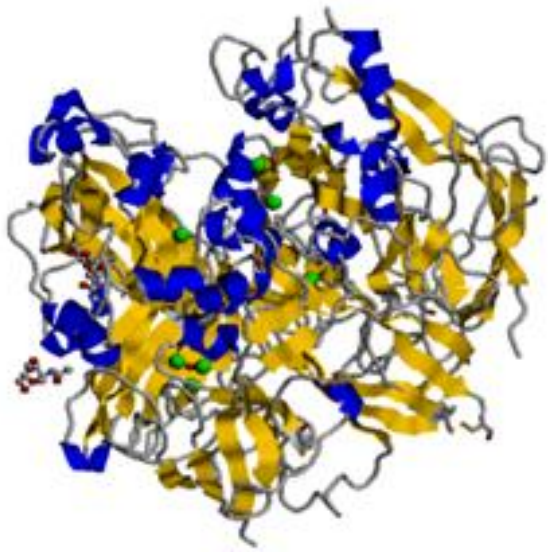
VALIDATION REQUIREMENTS FOR A BIOANALYTICAL LC-MS/MS ASSAY OF TRASTUZUMAB

Outline



- **Introduction**
  - **Trastuzumab as a model compound**
  - **Protein assay by LC-MS/MS**
- **Method development**
  - **Aiming at a generic approach**
  - **Some example results**
- **Issues for method validation**
  - **Selectivity and specificity**
  - **Internal standardization**
  - **Stability**

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Introduction – trastuzumab as a model

- **Trastuzumab has typical treats of biologicals**
  - **~150 kDa, monoclonal antibody**
  - **known sequence**
  - **‘humanized’ sequence, to avoid immunogenic action**
    - » **>90% of amino acid sequence is identical to that of other human antibodies**
  - **main activity: receptor blocking**
  - **is often combined with other drugs**
    - » **as a ‘baseline control’ or**
    - » **in combination therapy**

This makes trastuzumab a good model for development of a generically applicable LC-MS/MS method

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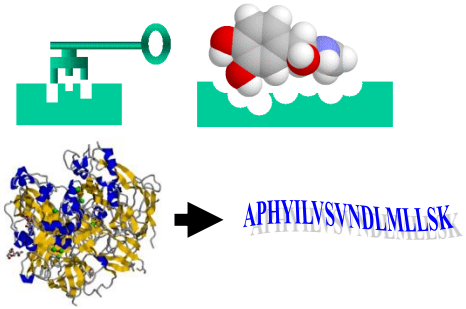
Introduction - Protein assay by LC-MS/MS

- **Advantages over immunochemistry**
  - no development of target specific reagents
  - uniform, ‘generic’, design and control of experiments
    - » possibility for tighter definitions with regard to regulatory issues
    - » less development cost and time
  - larger linear range
    - » no sigmoidal curve fitting or multiple dilutions
  - higher selectivity by sequence than by shape
    - » easier distinction from matrix, e.g. plasma proteins
    - » validation becomes more representative for bioanalysis
  - quality control by internal standard is a feasible option
    - » … if not an obligation



- **LC-MS/MS well accepted for small molecules**
  - widely used for GLP and non-GLP bioanalysis studies
  - some analogy with peptide LC-MS/MS

- **Follows recent technology development**
  - broad application of MS in ‘proteomics’
  - highly sensitive new LC-MS/MS equipment

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Introduction – Protein assay by LC-MS/MS

Many assay options available from 'proteomics'

- **However**

  ***much** of proteomics LC-MS/MS* **compares to** *bioanalytical protein LC-MS/MS*
  almost as
  metabolite LC-MS/MS identification **to** *LC-MS/MS bioanalysis* **for small molecules**

» max. information (full scan)	*vs*	max. sensitivity (MRM)
» few samples	*vs*	thousands of samples
» incidental quantitation	*vs*	precise and accurate quantitation
» any expense on reagents	*vs*	inexpensive reagents
» any sample treatment	vs	quick & reproducible
» exploration	*vs*	production

- **Also**
  - **avoid LBA characteristics from immunoaffinity clean-up**
    - » **IAC → competitive binding, lot-to-lot reagent differences, …**

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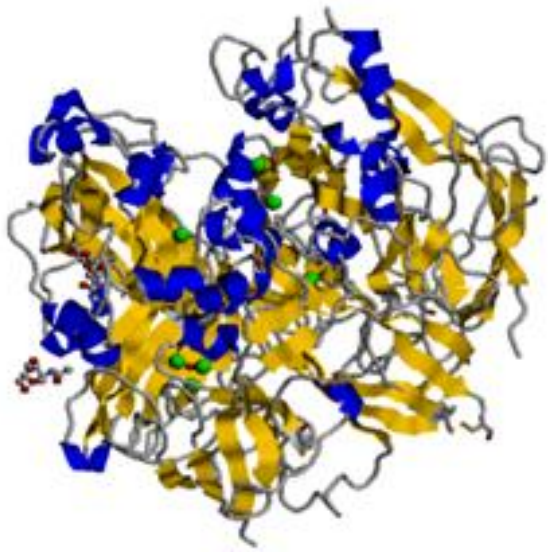

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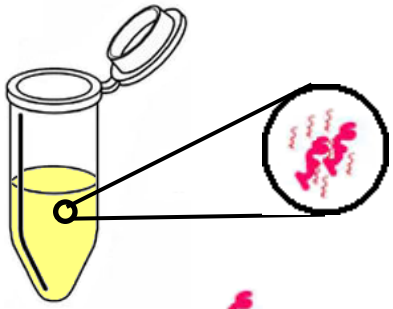
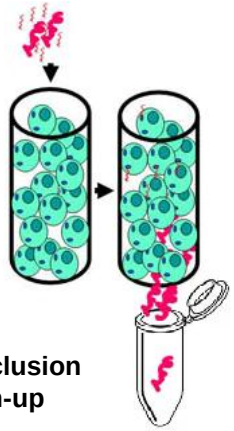
Method development – aiming at a generic approach

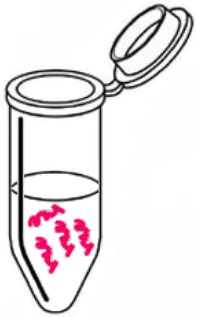


Plasma sample

Large proteins incl. target protein drug

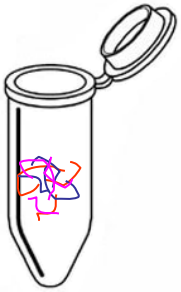


Size exclusion clean-up



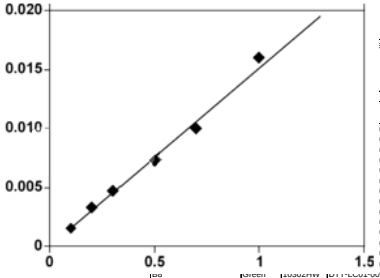
Large proteins only

Reduction, Carboxymethylation, Enzymatic digest



Peptide mixture

Target peptide LC-MS/MS analysis API Q-Trap 5500



eous phosphate buffer solution

	Concentration	Concentration Units	Note
2	122	µg/mL	
1	52.4	µg/mL	
1	16.9	µg/mL	
1	4.84	µg/mL	
1	<3.00	µg/mL	
1	<3.00	µg/mL	
1	<3.00	µg/mL	
2	123	µg/mL	
2	114	µg/mL	
1	26.9	µg/mL	
1	17.6	µg/mL	
1	9.35	µg/mL	
1	4.29	µg/mL	
1	<3.00	µg/mL	
	11.1	µg/mL	
C1	Red	10302HW	DTT-LC01-001	97.5	µg/mL	
C2	Red	10302HW	DTT-LC01-001	36.2	µg/mL	
C3	Red	10302HW	DTT-LC01-001	14.3	µg/mL	
C4	Red	10302HW	DTT-LC01-001	5.45	µg/mL	
C5	Red	10302HW	DTT-LC01-001	<3.00	µg/mL	
C6	Red	10302HW	DTT-LC01-001	<3.00	µg/mL	
C7	Red	10302HW	DTT-LC01-001	9.85	µg/mL	

Protein drug quantity

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Results for trastuzumab

1. **Evaluated trastuzumab trypsin digest *in silico***
   - **several trastuzumab digest peptides do not appear in other human proteins**

2. **Targeted these peptides in experiments**
   - **full scan product ion MS/MS**
   - **first in neat solution, then in human plasma**
   - **one selective peptide with best performance: DTYIHWVR**

3. **Minimized R_t and collected MS/MS spectra over CE ramp**
   - **assessed best MRM transitions**
     - » $[M+H]^{2+} \rightarrow y''_4$ = 545.6 → 597.3
     - » $[M+H]^{2+} \rightarrow y''_3$ = 545.6 → 460.3

4. **Ran experiments with plasma samples**

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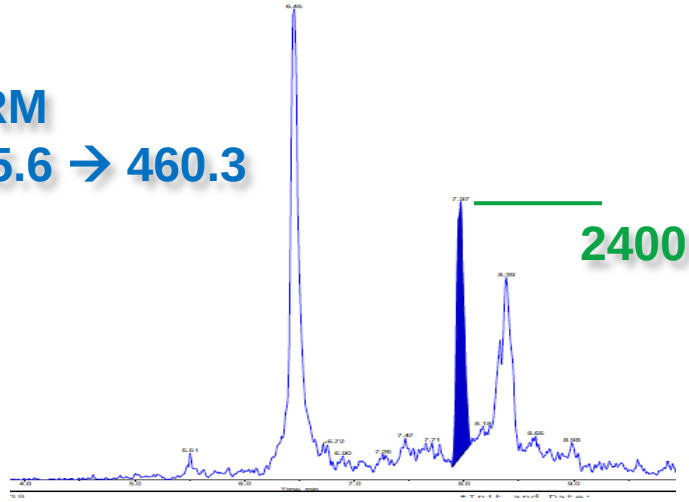
Results for trastuzumab (2)

MRM
545.6 → 460.3



MRM
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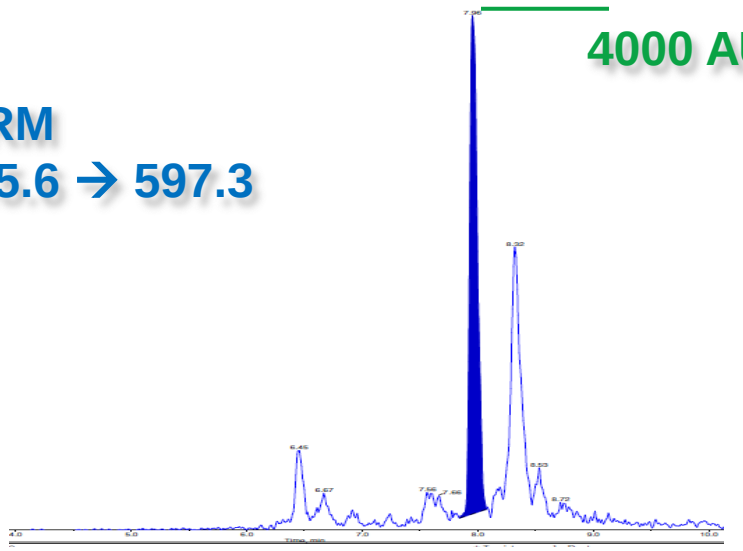

Typical result from

10 µg/mL plasma
- **LLOQ ELISA: 2.0 µg/mL serum**

@ 25 µL sample applied to SEC

Common peptide LC
- **BEC 300 C18 column (2.1 x 50 mm, 3.5 µm particles)**
- **H_2O:ACN (0.1 % FA) gradient**

API 5500 Triple quad MS/MS @ Q1, Q3 unit resolution

Conclusion
LLOQ of <1.0 µg/mL plasma is no problem

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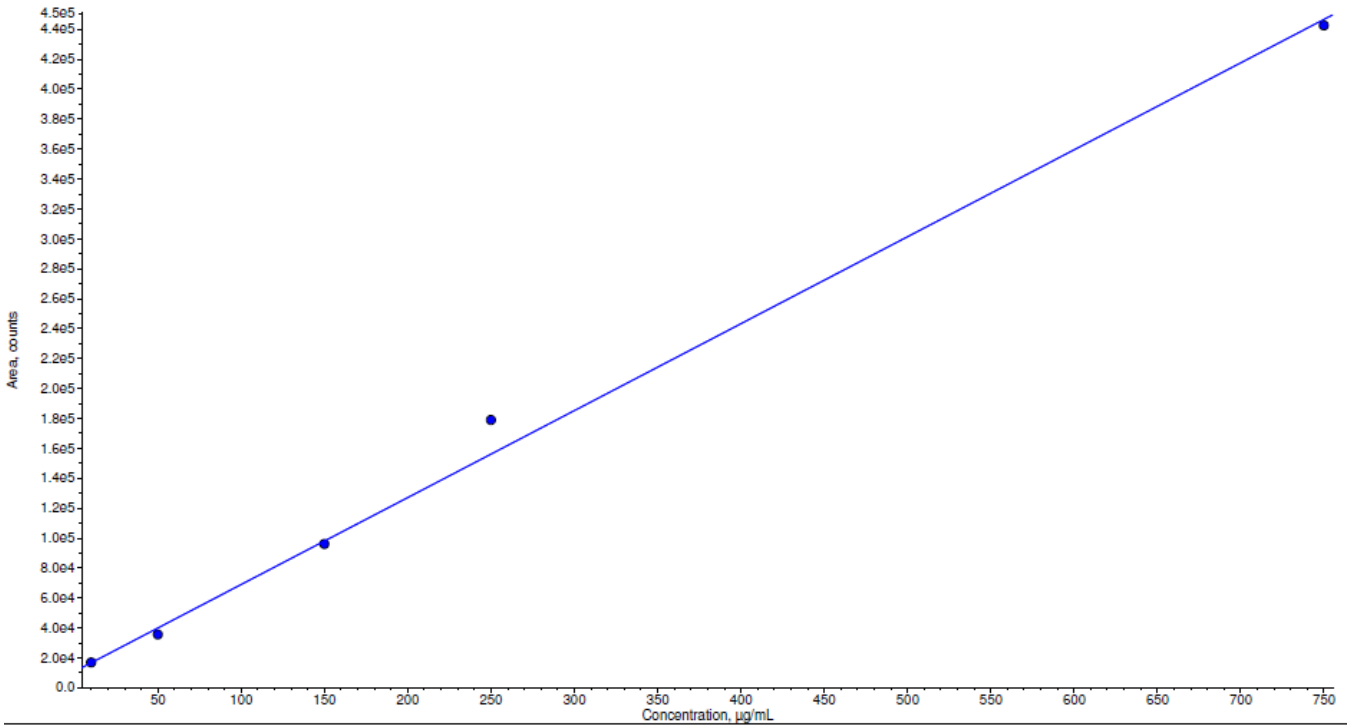
Results for trastuzumab (3)



First test of calibration from human plasma

- **using MRM 545.6 → 597.3**

Linear over 10 – 750 µg/mL, 10 µg/mL >> LLOQ

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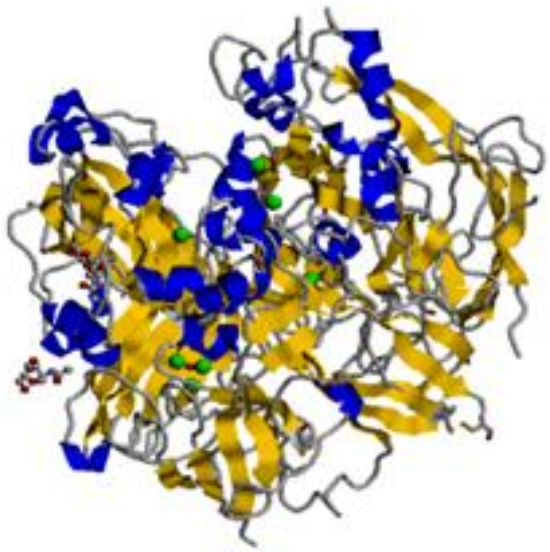

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Issues for method validation (1)

Drug entities will strongly resemble endogenous molecules

- **inherent to avoiding adverse immune system reaction**
- **use is more host-specific than for 'small molecules'**

- **Selectivity and specificity issues different from 'small molecule'**
  - **is a target peptide exclusively representative for the target protein?**
    - » **can be evaluated *in silico* in early method development**
      - – **calculated proteolytic cleavage**
      - – **protein and digest peptide sequence matching against the host genome**
    - » **post-translational modifications (PTM) may interfere with analysis**
      - – **natural: enzymatic cleavage, oxidation, phosphorylation, glycosylation, …**
      - – **artificial: oxidation, adducts, …**
    - » **requires critical evaluation and further empirical investigation**
      - – **digest and analyze**
        - o **neat protein *vs* neat protein in matrix**
        - o **fresh vs stored**

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Issues for method validation (2)

There is no consensus approach for internal standardization

- **no IS used in LBA assays – multiple analysis**
- **stable isotope labeled IS common for 'small molecules'**
  - **but stable isotope labeling is not trivial for large molecules**

- **IS options differ from those for 'small molecules'**
  - ***current best practice*: add synthesized stable isotope labeled peptide**
    - » **addition after a digestion step**
      - – **not suitable for validation of protein clean-up and digestion performance**

…

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Issues for method validation (3)

…

- options for validation of an entire work flow, sample → data
  - » add an unlabeled protein
    - establish a second target peptide
      - different sequence → different behavior in analysis
      - comparison will always be disputable
  - » create intact protein IS by differential stable isotope labeling

Not suitable for regulatory bioanalysis

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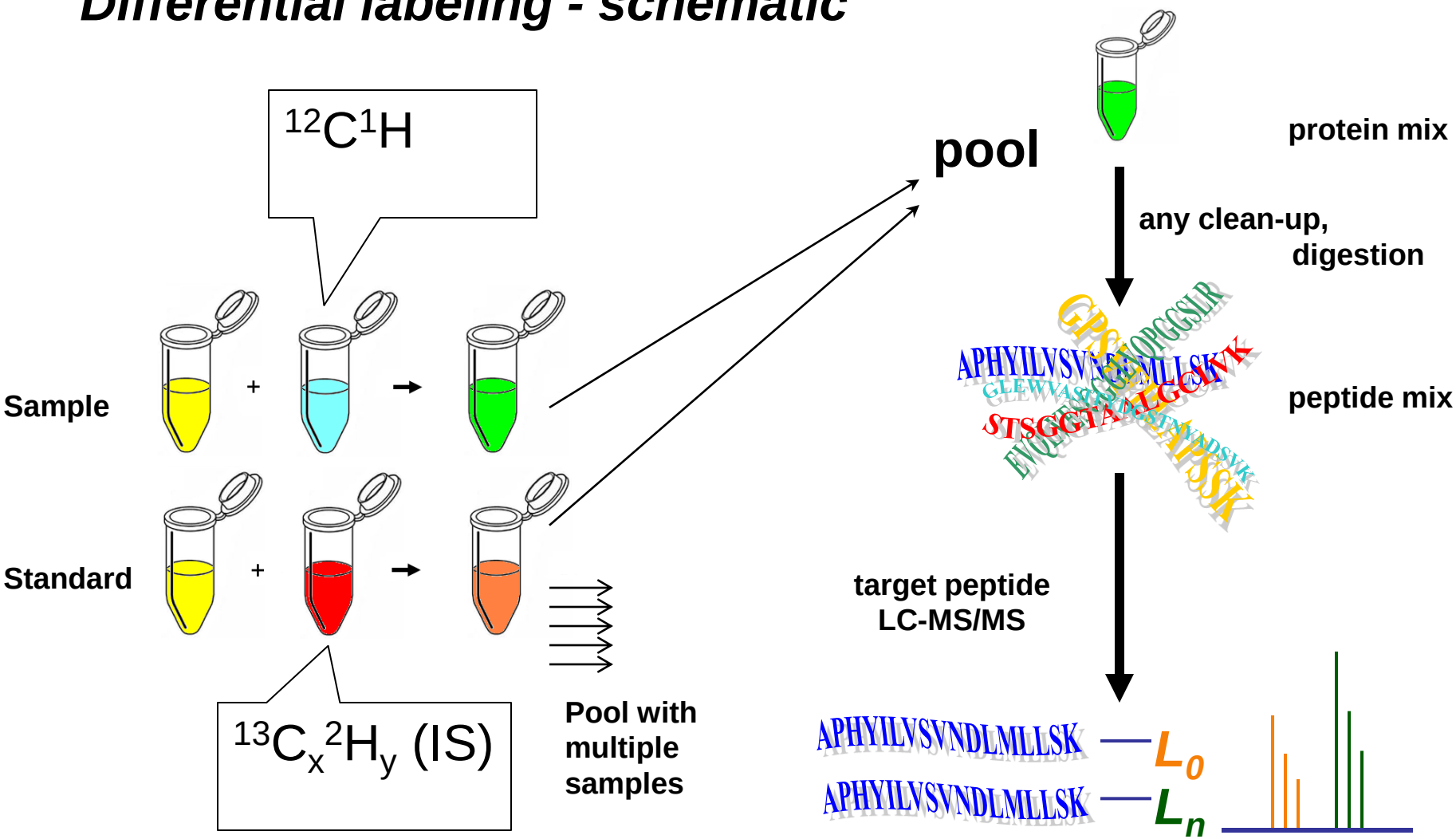
Differential labeling - schematic



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Not suitable for regulatory bioanalysis

  - » create intact protein IS by differential stable isotope labeling
    - – not suitable for work flow with IAC type clean-up
      - o labeling denatures protein
    - – may interfere with digestion
      - o depending on enzyme, e.g. lysine methylation blocks trypsin cleavage
    - – commercial differential labeling: ICat™, ITraq™, …
      - o aimed at protein identification; methods generally target cysteine residues (-SH)
      - o relatively expensive, IP
    - – organic chemistry for other side chains?
      - o amino groups, carboxylic acids
      - o remains to be tested….

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Issues for method validation (4)

Some stability issues will be different from small molecules

- biological stability ≠ chemical stability
  - » 'intact sequence' need not reflect 'intact activity'
  - » biological stability is implicitly tested in some LBA
    - – ligand may be the drug target

Potential issues

- ease of oxidation of particular amino acid residues
  - » avoid in target peptide selection, *if possible*
  - » may be assessed by 'full scan' or 'parent ion scan' LC-MS
    - – elaborate tests
- less response is not necessarily due to instability
  - » proteins and peptides may precipitate
    - – may cause sample extract inhomogeneity over time
    - – solubilization may require attention during method development
      - o addition of surfactants is generally not compatible with ESI

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Issues for method validation (5)

- **Potential issues…**
  - **degradation in samples due to biological activity**
    - » **endogenous proteases**
      - – **any proteolytic activity may interfere with assay**
        - o **inhibitor additives may even be required *at sampling time***
    - » **bacterial or fungal activity**
      - – **sterilize**
        - o **protein denaturation will not interfere with LC-MS/MS**
  - **'less assay response' is not necessarily due to instability**
    - » **proteins and peptides may precipitate**
      - – **may cause sample extract inhomogeneity over time**
      - – **solubilization may require attention during method development**
        - o **addition of surfactants is generally not compatible with ESI**
  - **sample processing may be lengthy**
    - » **limited options for validation against 'freshly prepared'**

Problems apply equally to all assay methods

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Conclusions

- **The time is right for ‘large molecule LC-MS/MS’ bioanalysis**

- **Method validation for ‘large molecule LC-MS/MS’ has some specific issues when compared to ‘small molecule LC-MS/MS’**
  - **Development of a suitable work-flow**
  - **Selectivity and specificity**
  - **Use and choice of internal standard**
  - **Stability issues**
  - **Other issues that may come up by application of LC-MS/MS and that have not been recognized by LBA**

- **It is due time to establish consensus guidance on regulatory demands for large molecule LC-MS/MS**

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