TAKING INSULIN ANALYSIS TO THE NEXT LEVEL

APPLICATION OF NEW SCIENCE IN ADVANCED LC-MS BASED WORKFLOWS

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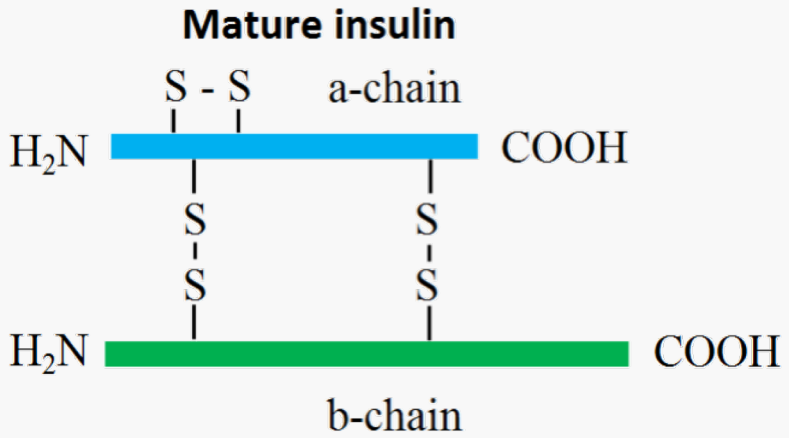
OUTLOOK

1. Charles River Insulin project
2. Top-down insulin analysis
3. Bottom-up insulin analysis
4. Conclusions

Charles River Insulin project

INSULIN

- Diabetes
- Insulin hormone
  - Regulates blood sugar levels
  - Small protein (biopharmaceutical)
- Quantification of Insulin
  - Pharmacokinetics
  - Therapeutic drug monitoring
  - Diagnostics
  - Doping control
  - Forensics



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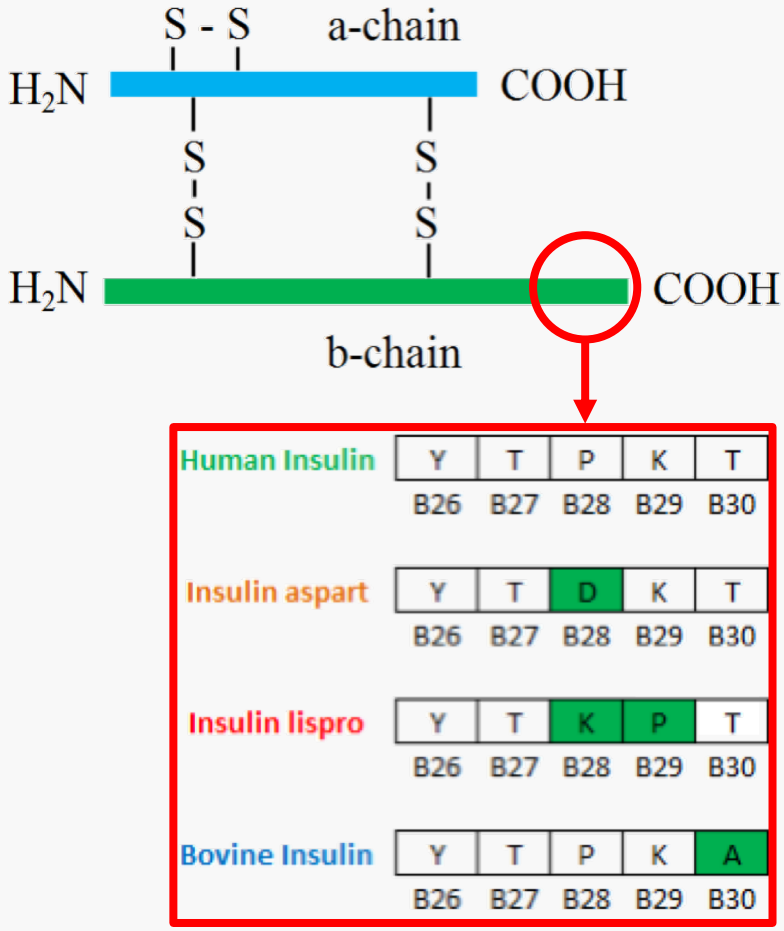
INSULIN ANALOGUES

- Analogues of human or animal origin
  - Human insulin
  - Bovine insulin
- Drug analogues by recombinant biosynthetic technologies
  - Insulin aspart
  - Insulin lispro
  - Insulin glargine
- Analogue specific region on b-chain
  - Insulin aspart: aspartic acid (D) on B28
  - Insulin lispro: interchange lysine (K) and proline (P)
  - Insulin glargine:
    - Two additional arginine (R) at end of b-chain
    - Glycine (G) on A21
  - Bovine insulin: alanine (A) on B30



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TOP-DOWN OR BOTTOM-UP

- Top-down
  - Eluent conditions highly critical
  - Multiple charge states
    - Sensitivity lower
  - CID fragmentation less effective
  - Optimization by infusion

- Bottom-up
  - Proteolysis: enzymatic breakdown of protein
  - More complex sample preparation
  - Few charge states
    - Higher sensitivity
  - Optimization after LC separation

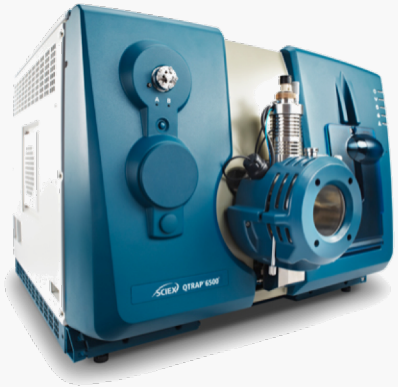

Figure sources:
Waters cooperation , 2011, 720003397EN rev B
Figure of QTRAP 6500+ provided by courtesy of AB Sciex Pte. Ltd

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Top-down insulin analysis

PROJECT OBJECTIVE TOP-DOWN

- To set up analogue specific method for:
  - Insulin aspart
  - Insulin lispro
  - Insulin glargine

- Wide analytical range:
  - LLOQ below normal fasted endogenous insulin levels: 350 pg/mL
  - ULOQ at maximum endogenous insulin level: 10000 pg/mL

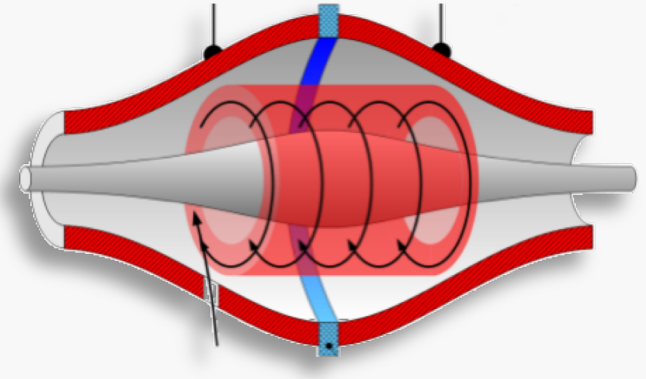


Figure sources:
Waters cooperation , 2011, 720003397EN rev B
http://planetorbitrap.com/data/fe/image/QExactive.jpg

LIGAND BINDING PURIFICATION

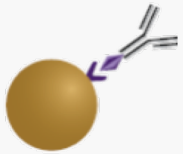
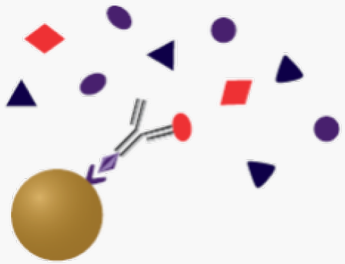
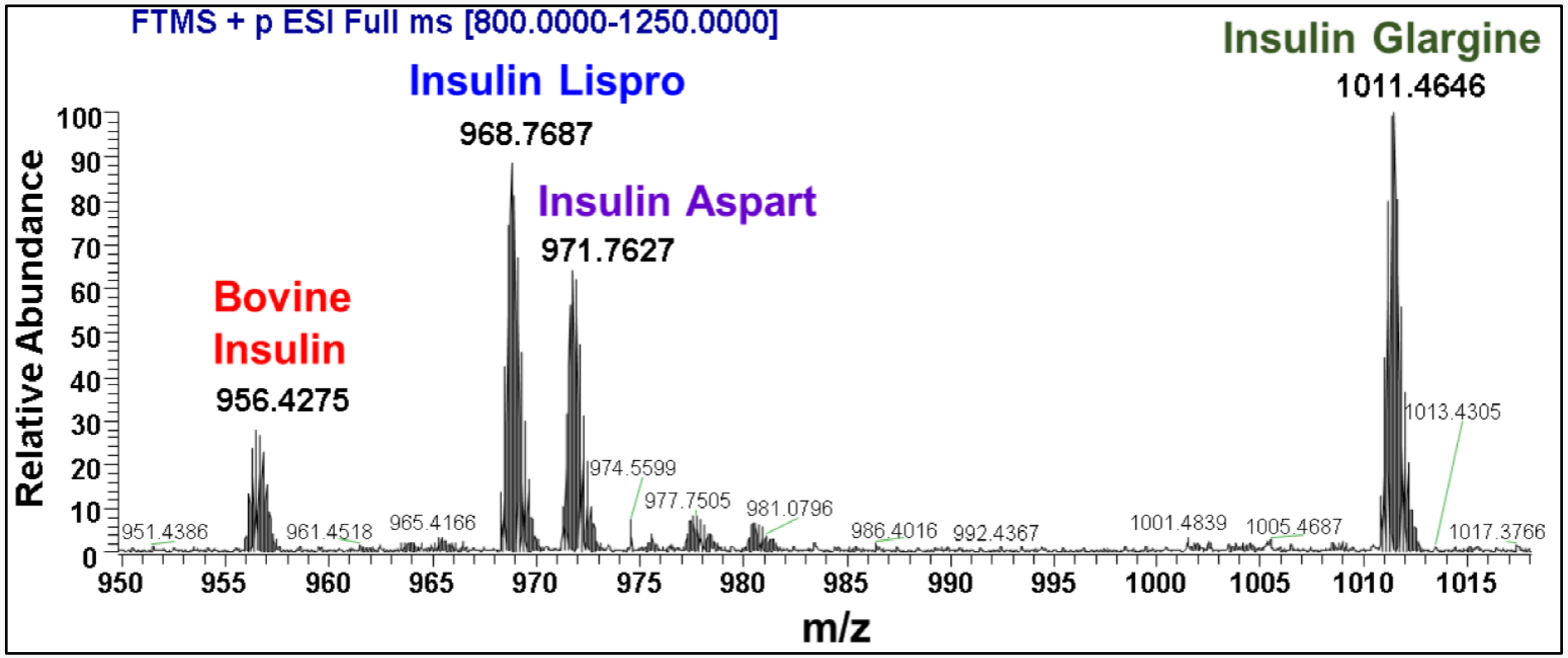
- Biotinylated antibodies 
- Antibodies immobilized on streptavidin magnetic beads 
  - Strongest non-covalent bond in nature
- Insulin capture 
- Unspecific binding removed by bead wash 
- Antigen elution
  - Acidify to remove antigen from antibody

Figure sources:
Dynabeads Streptavidin manual, ThermoFisher, 2016, CO020153 0316
http://www.magnamedics.com/index.php/mm-separator-m96
DOI: 10.1016/j.optlaseng.2017.09.016

HIGH RESOLUTION MASS SPECTROMETRY, OPTIMIZATION

- HR-MS Thermo Q-Exactive
- Easy optimization of $(M+6H)^{6+}$ ions
- Analytes:
  - Insulin lispro
  - Insulin aspart
  - Insulin glargine
- Internal standard:
  - Bovine insulin



Data and figure sources:
Poster AAPS PharmSci360, Nov 2018, T0930-03-017
A Hybrid LBA/LC-MS Assay for Multiplex Quantitation of Insulin Analogs in Plasma, H. Wang,et al., CRL Ashland

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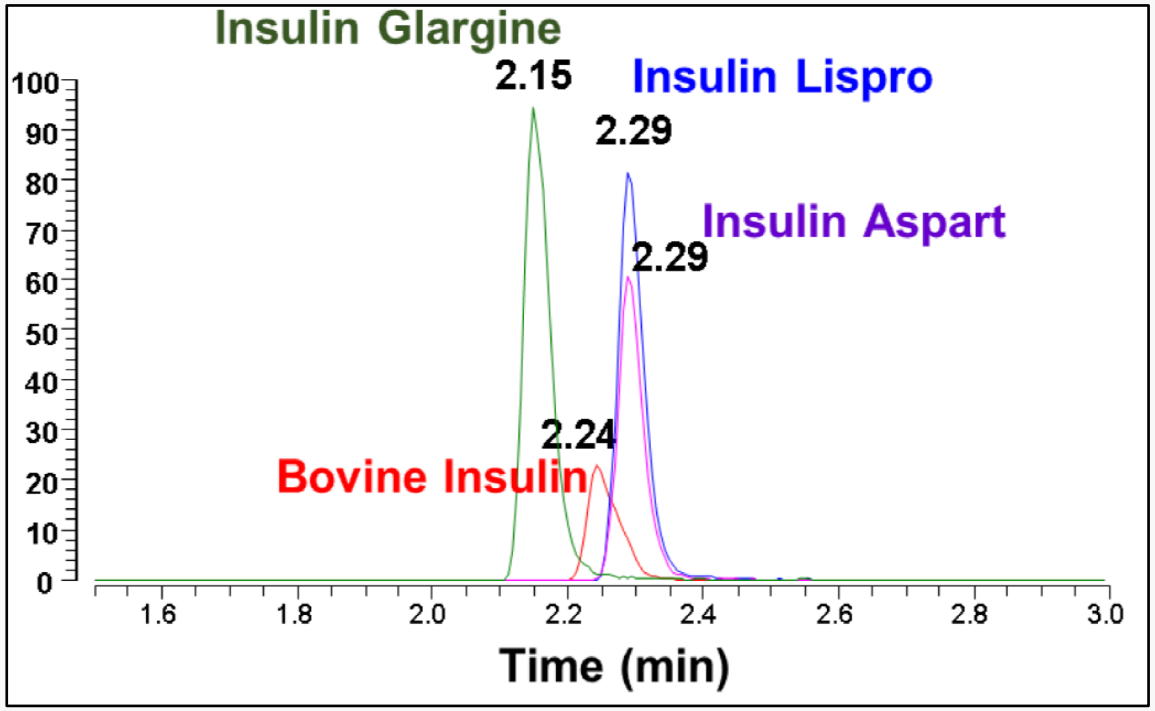
SAMPLE PREPARATION & CHROMATOGRAPHIC SEPARATION

- Matrix: Minipig plasma
- 250 µL of sample processed by Ligand binding purification
- UPLC
- Reversed phase column
- 0.4 mL/min



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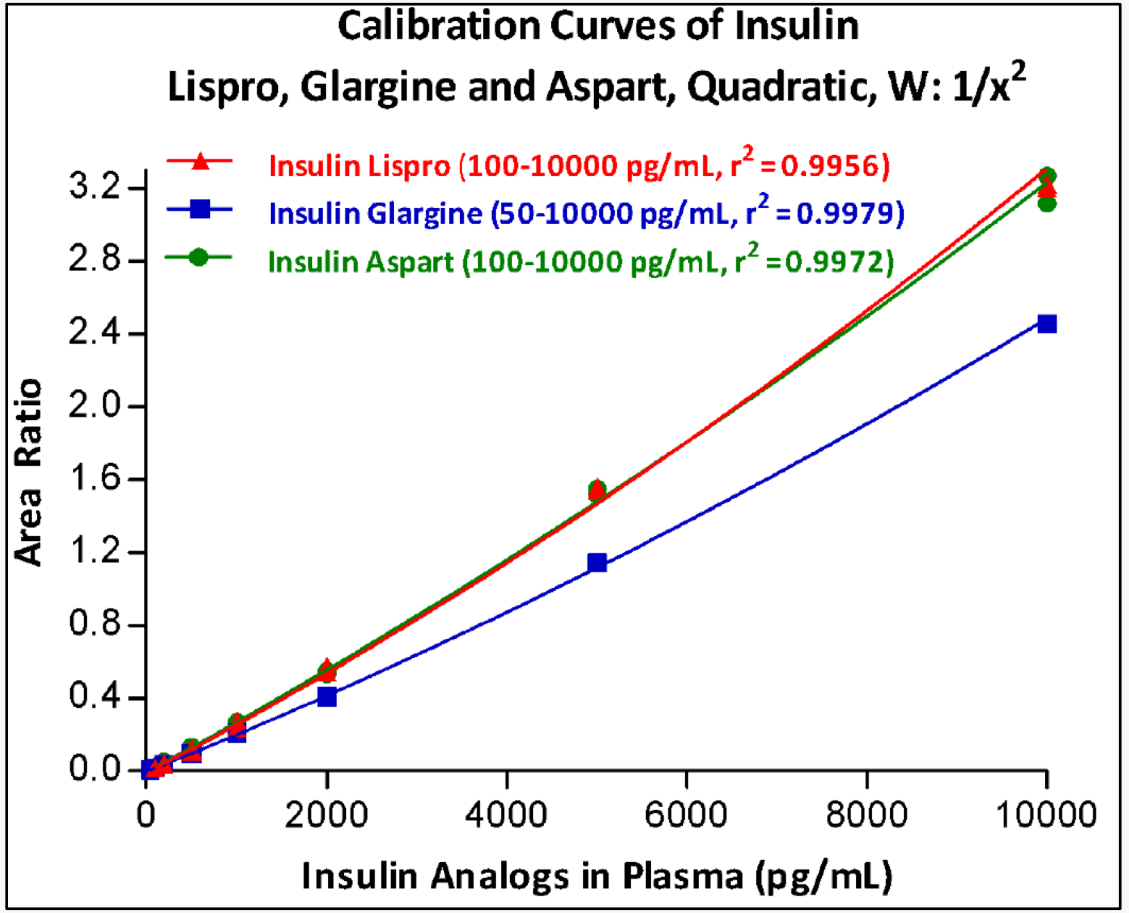
RESULTS LBA-LC-HRMS

- Quantification by adding up analogue isotope responses
- Extracted ion chromatogram (EIC)
- Quadratic regression
- Good correlation $r^2 > 0.99$ (n = 2)
- Background signal in matrix blank was less than 20% of LLOQ



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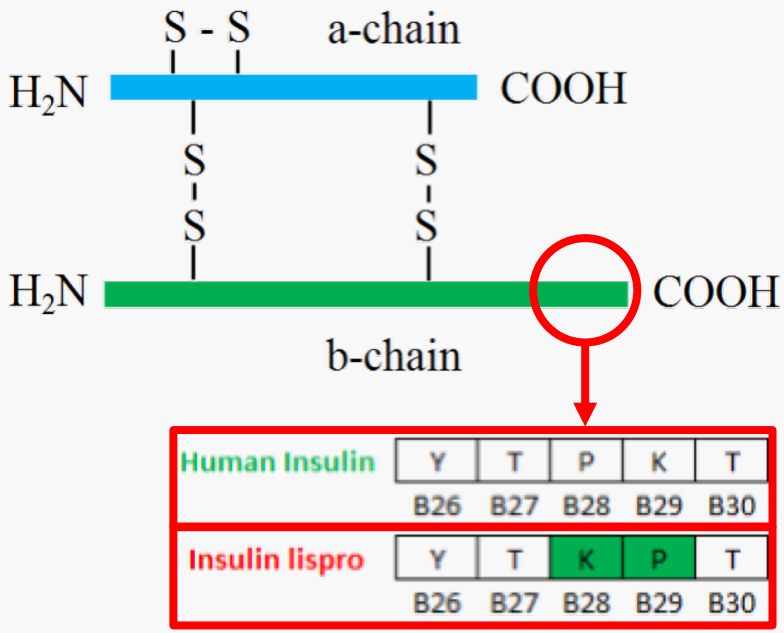
HUMAN INSULIN / INSULIN LISPRO SEPARATION CHALLENGE

- Interchange lysine (K) and proline (P)
- Intact insulin LC-MS
  - Separation by LC very hard
  - MW identical
  - Comparable fragments
  - Interference according to S. Taylor et al. (2016) [1]



- Intact insulin 2D-LC-MS published by E. Chambers et al. (2014) [2]
  - Limited retention time difference (4.28 / 4.30 minutes)

- What about only analyzing analogue specific region?
  - Bottom-up approach

[1] DOI: 10.1016/j.cca.2016.01.019
[2] DOI: 10.1021/ac403055d

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Bottom-up insulin analysis

PROJECT OBJECTIVE BOTTOM-UP

- To set up an LC-MS (QqQ) method for Insulin lispro
    - Analogue specific for insulin lispro
    - Larger Dynamic range compared to ELISA (2 decades)
    - Range: 0.07 to 20 ng/mL

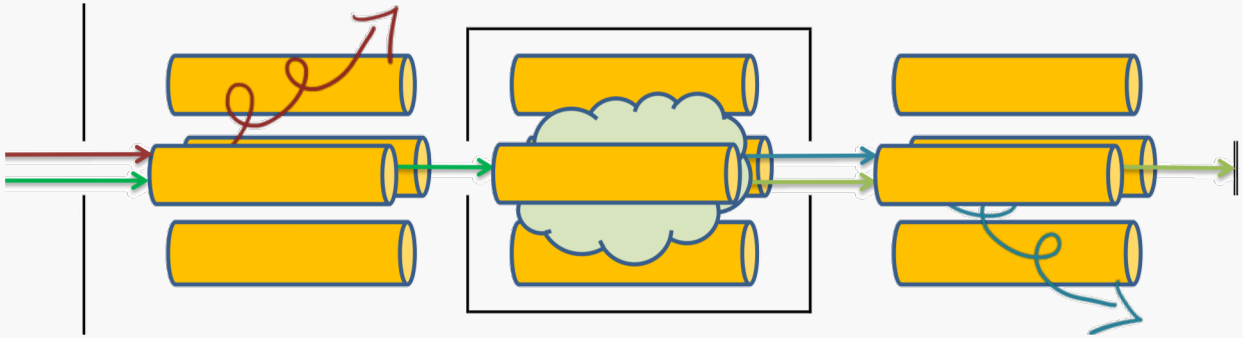

Figure sources:
Waters cooperation , 2011, 720003397EN rev B
Figure of QTRAP 6500+ provided by courtesy of AB Sciex Pte. Ltd

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BOTTOM-UP: PROTEOLYSIS

- Enzymatic breakdown of protein to peptides

- Trypsin protease
  - Arginine residue (R)
  - Lysine residue (K)
  - No cleavage when proline at C-terminus
- Glu-C protease
  - Glutamic acid residue (E)
  - Aspartic acid residue (D) in presence of PO_4

- Next: *In-silico* proteolysis

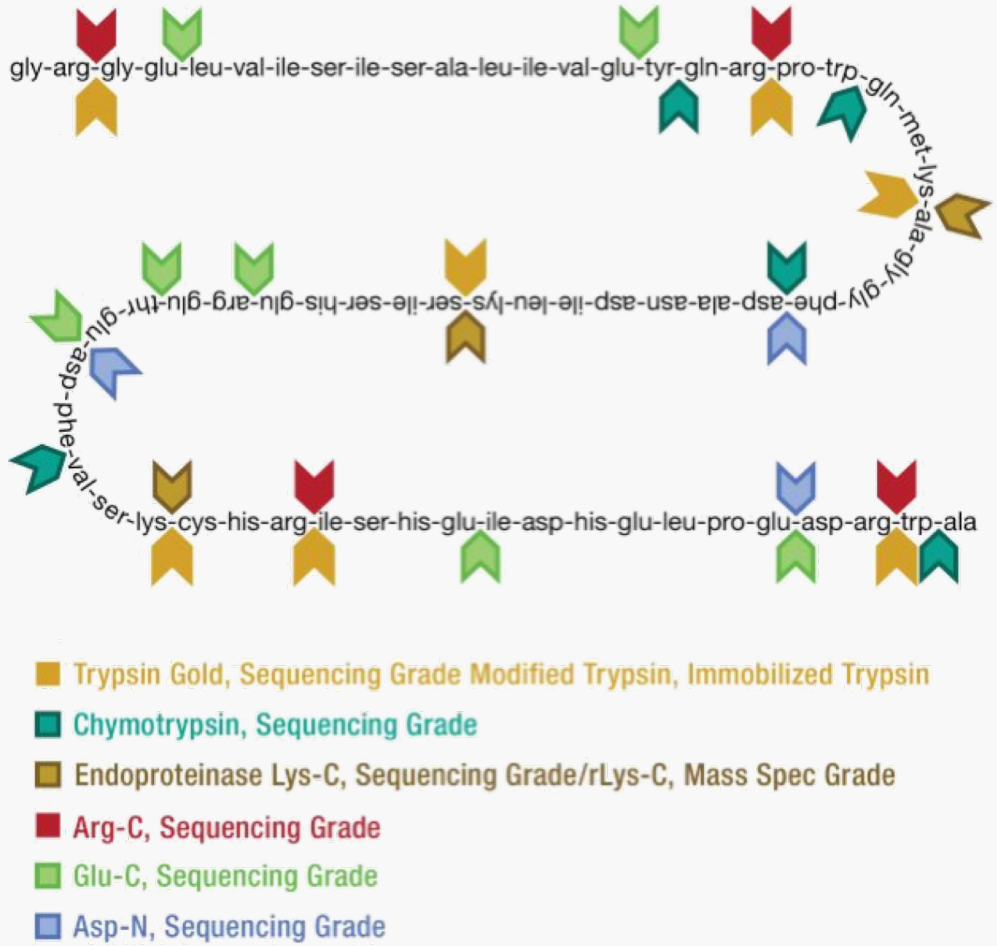


Figure source:
https://embed.widencdn.net/img/promega/etotmupu8q/640px/8634ME.jpeg?keep=c&crop=yes&u=7fvzhm

PROTEOLYSIS

- Proteolysis
  - Enzymatic breakdown of protein to surrogate peptides
  - Trypsin protease
  - Glu-C protease
    - Glutamic acid residue (E)
  - First: *In-silico* proteolysis

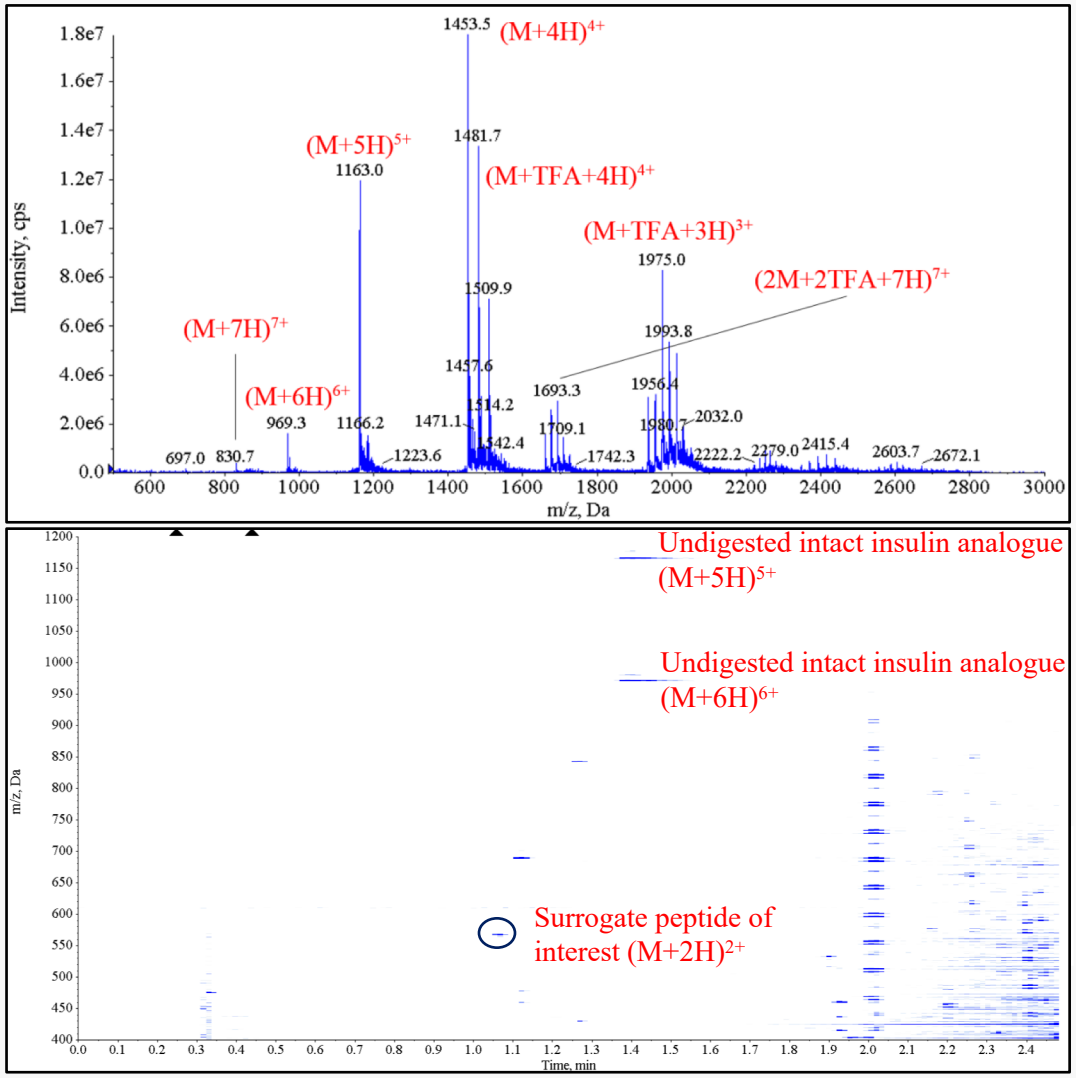
- BLAST
  - Unique surrogate peptides
  - Generic surrogate peptides

Insulin analogue	Trypsin proteolysis	Glu-C proteolysis
Human insulin	GFFYTPK	RGFFYTPKT
Bovine insulin		RGFFYTPKA
Rat insulin-1		RGFFYTPKS
Rat insulin-2	GFFYTPMS	RGFFYTPMS
Insulin lispro	GFFYTKPT	RGFFYTKPT
Insulin aspart	GFFYTDK	RGFFYTDKT

MASS SPECTROMETRY, PRECURSOR ION OPTIMIZATION

- Intact (top-down)
  - Like a small molecule: direct infusion

- Analogue specific region (bottom-up)
  - Infusion optimization not feasible
    - Sample too complex
  - LC separation before optimization needed.



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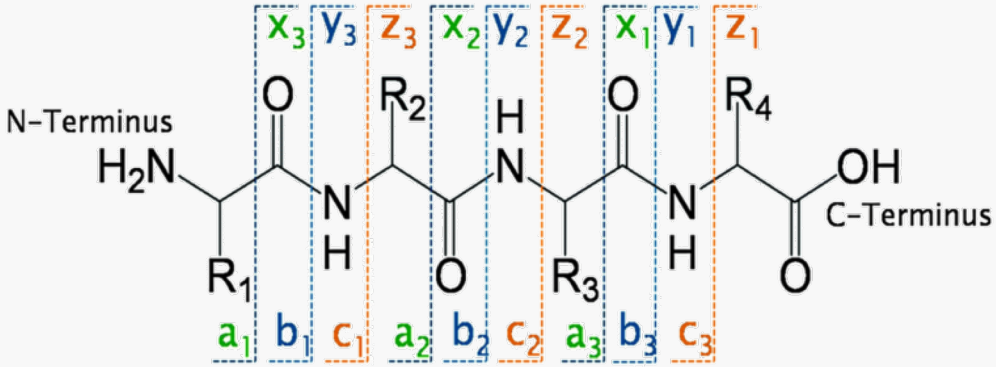
MASS SPECTROMETRY, PRODUCT ION OPTIMIZATION

- Nomenclature



- Proline (P) effect
  - Strong peptide bond (because of secondary amine)
  - Predominance b_i and y_i ions formed at N-terminus proline

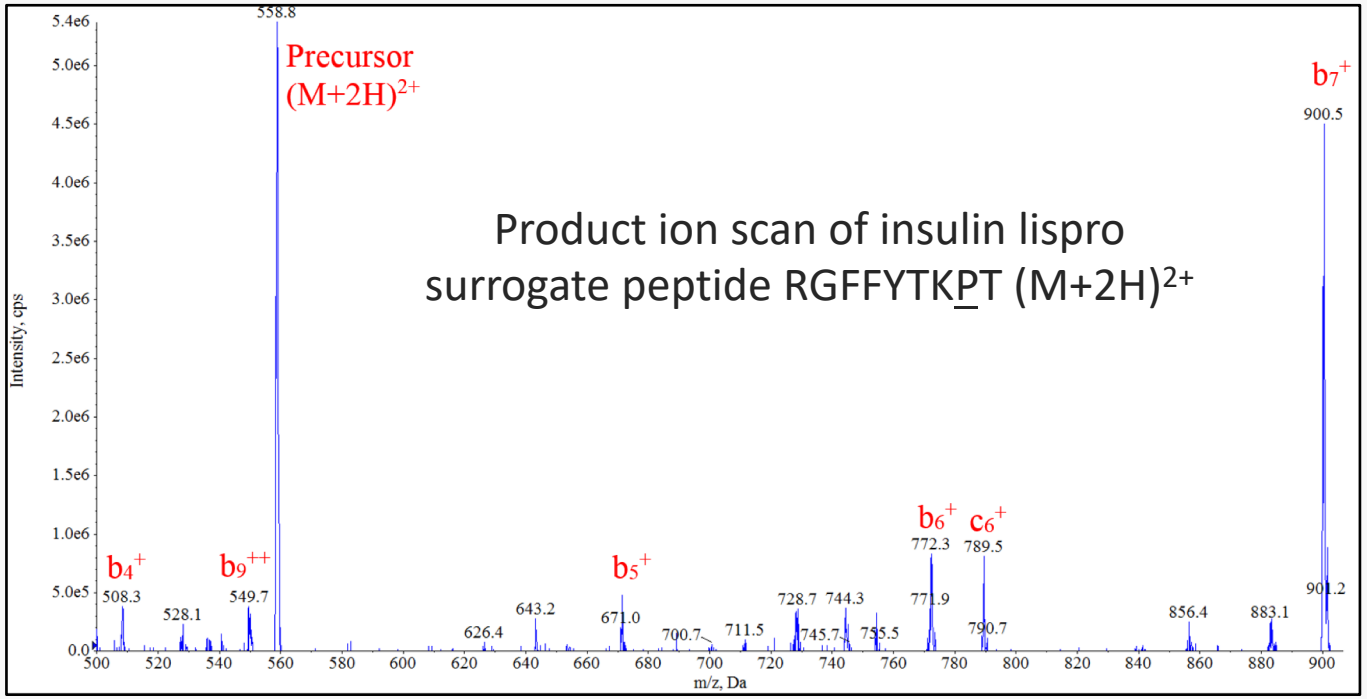


Figure source:
DOI: 10.1039/C3CS60444F

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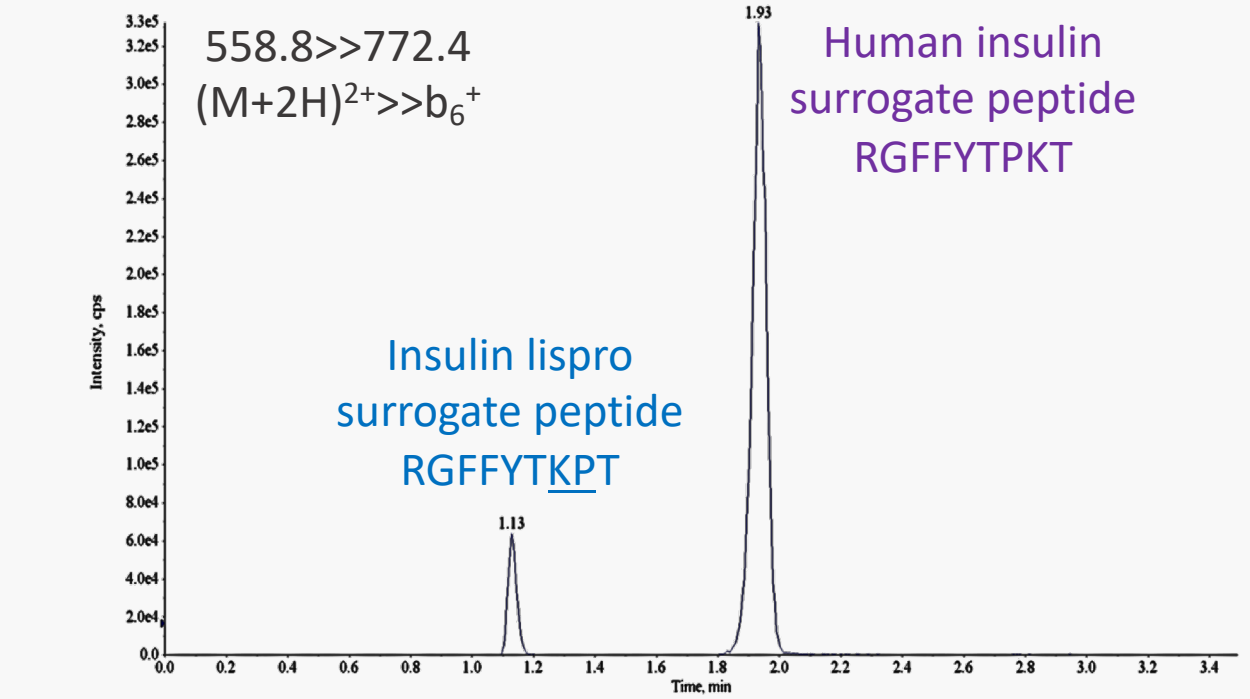
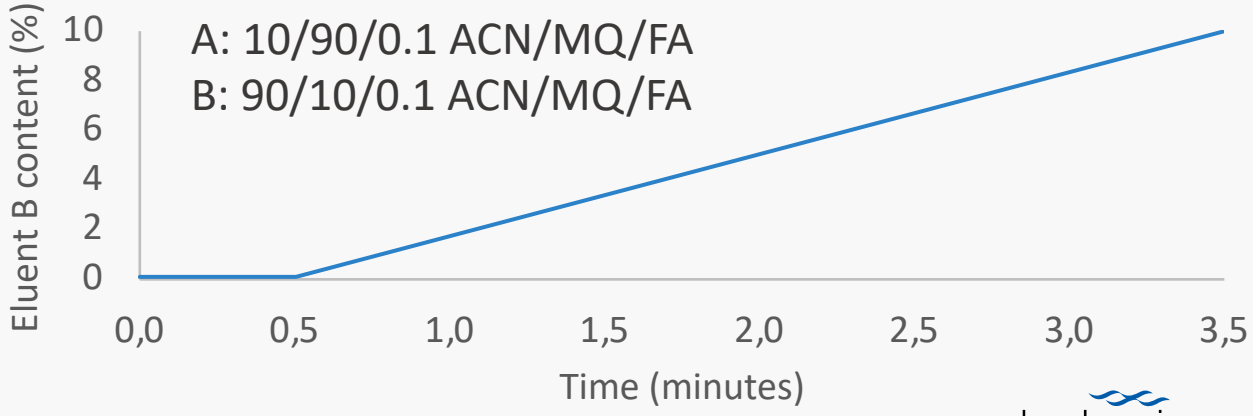
CHROMATOGRAPHIC OPTIMIZATION

- Reminder: Intact insulin quantification cannot separate insulin lispro from human insulin

- <u>We can!</u> Now we have the first method to perform insulin lispro and human insulin selective LC-MS quantification in one assay





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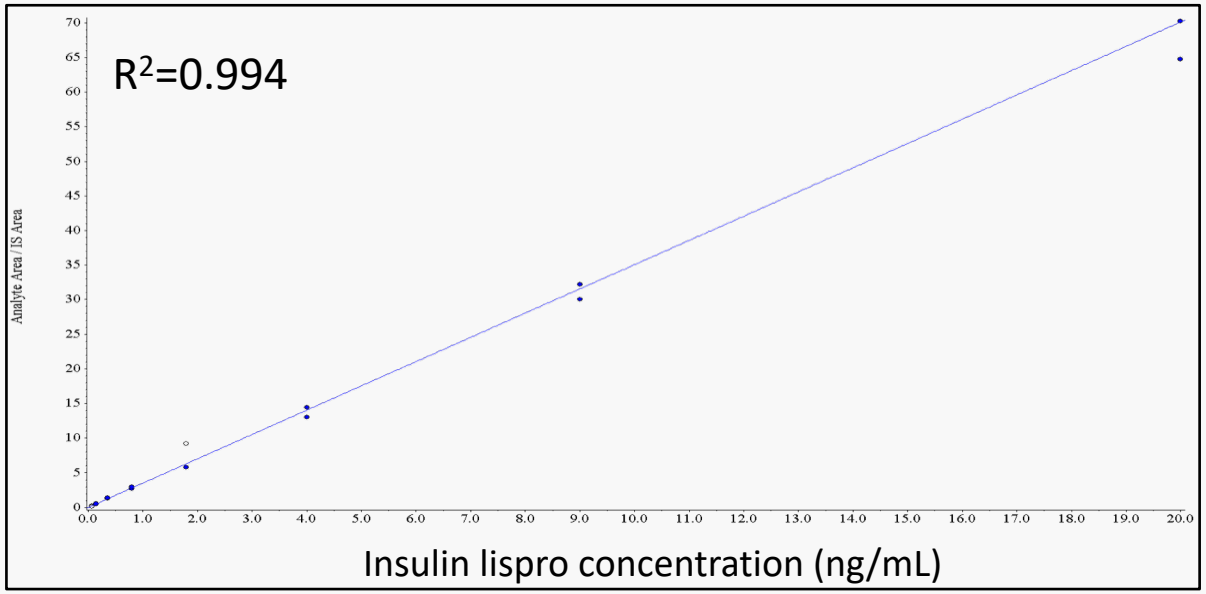
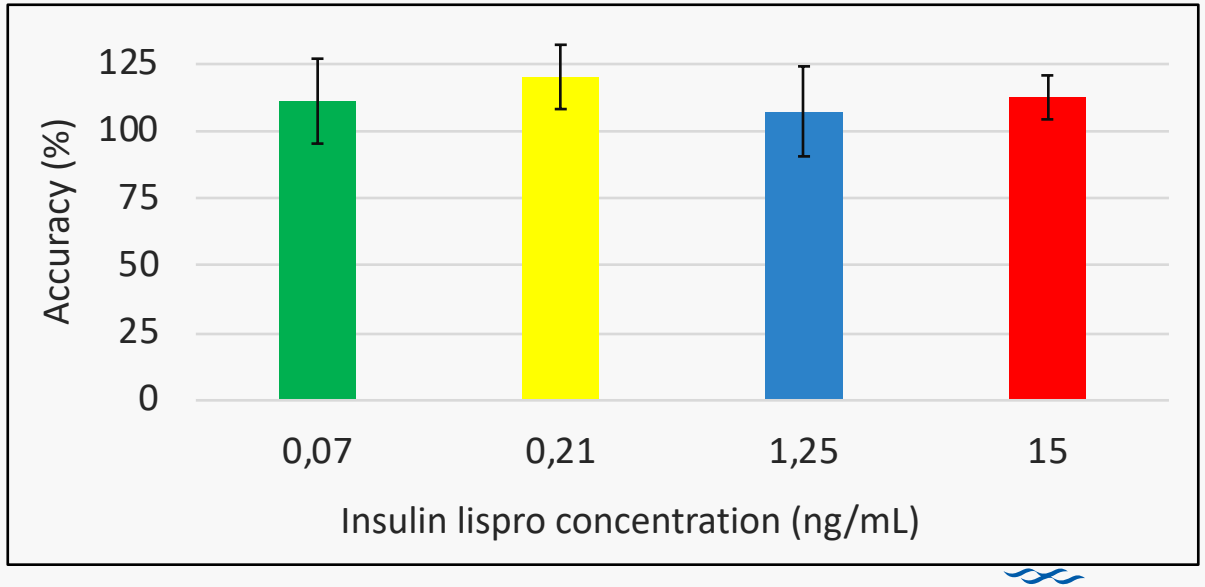
INSULIN LISPRO

- 500 µL human K_2EDTA plasma processed
- Double Blank
  - No internal standard response
- Blank
  - No insulin lispro response
- Calibration samples
  - Duplicate sample preparation
  - Calibration curve according to criteria
  - 87.5 % accepted
- QC samples
  - 5 replicates of
    - QC-LLOQ 0.07 ng/mL
    - QC-L 0.21 ng/mL
    - QC-M 1.25 ng/mL
    - QC-H 15 ng/mL





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Conclusions

CONCLUSIONS

- LC-HRMS multiplex quantification
  - Easy method development
  - Method developed in analytical range 0.05 to 10 ng/mL for insulin glargine
  - 0.1 to 10 ng/mL for insulin lispro and insulin aspart

- Insulin lispro bottom-up LC-MS
  - Separation for insulin lispro and human insulin surrogate peptides
  - Method developed in analytical range 0.07 to 20 ng/mL insulin lispro

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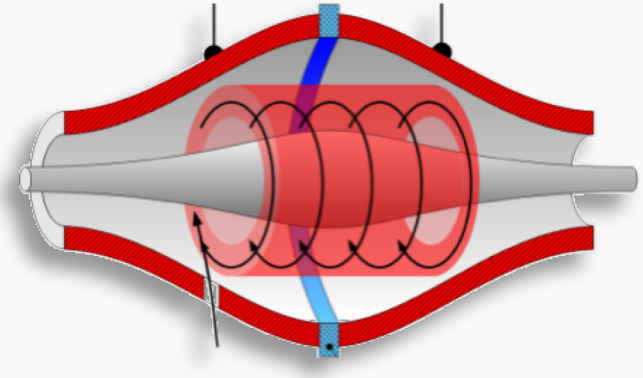

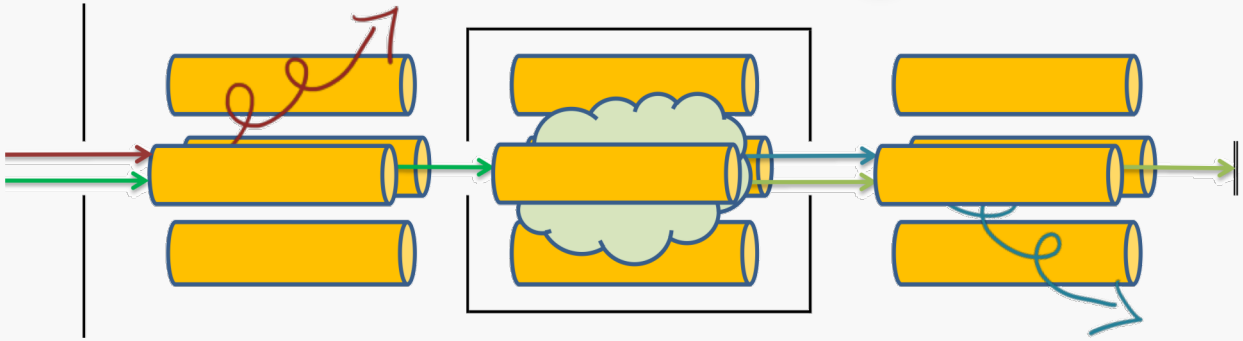

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Thank you

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