SCIEX

Answers for Science.
Knowledge for Life.™



Targeted Proteomics – from clinical research to therapeutic drug monitoring

Volker Kruft, Business Development

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Protein/peptide quant in medical research

1) why care about peptide/protein quantitation?

2) how does it work?

2) Mass spectrometry method

3) Application Examples

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Peptide applications in bioanalytics

- Proteomics in academic research

  - Bottom-up Proteomics, i.e. analysis of proteins after endoproteinase digestion

- Clinical metabolism studies

  - Nutrition and glucose metabolism (e.g. Insulin, Ghrelin)

  - Cardiovascular system (e.g. Angiotensin)

  - Neurology (e.g. neuropeptides)

- Food QC and food safety

  - Quality control (e.g. authentification of buffalo mozzarela cheese)

  - melamin scandal

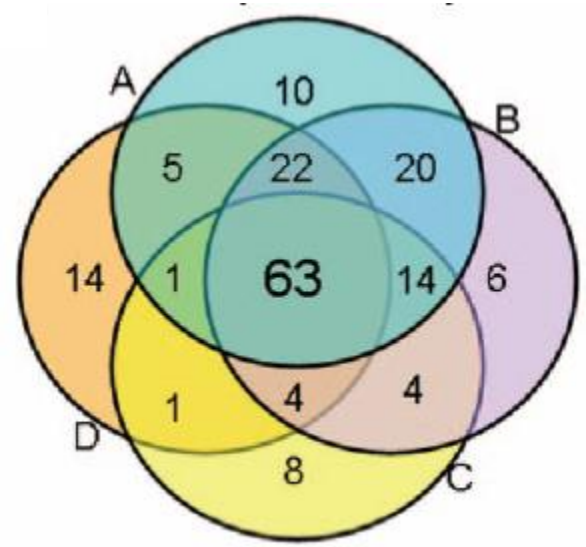
  - food allergens (e.g. nuts, milk, glutens)

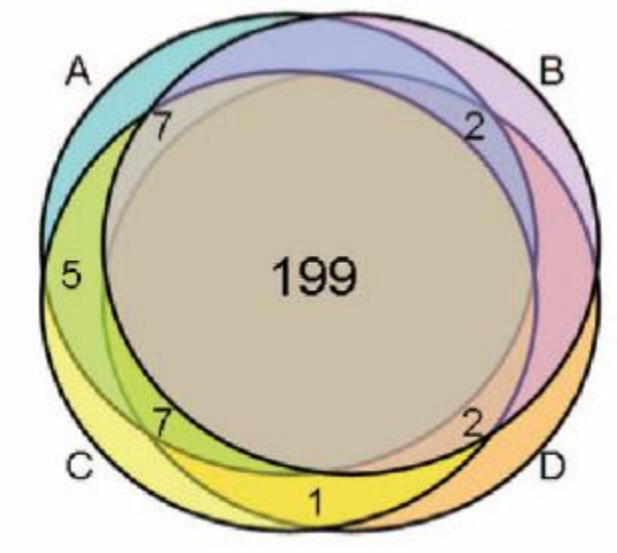
- ….

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Repeated Experiments: Non-Targeted vs. Targeted Protein ID





Little reproducibility in non-targeted experiments (LC-MS/MS)

Excellent reproducibility in targeted experiments (LC-MS/MS)

Taken from: "Human mammary epithelial cells (HMCA) stimulated with EGF"

Wolf-Yadlin et al., PNAS 104, 5860-5865 (2007)

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The challenge of data interpretation

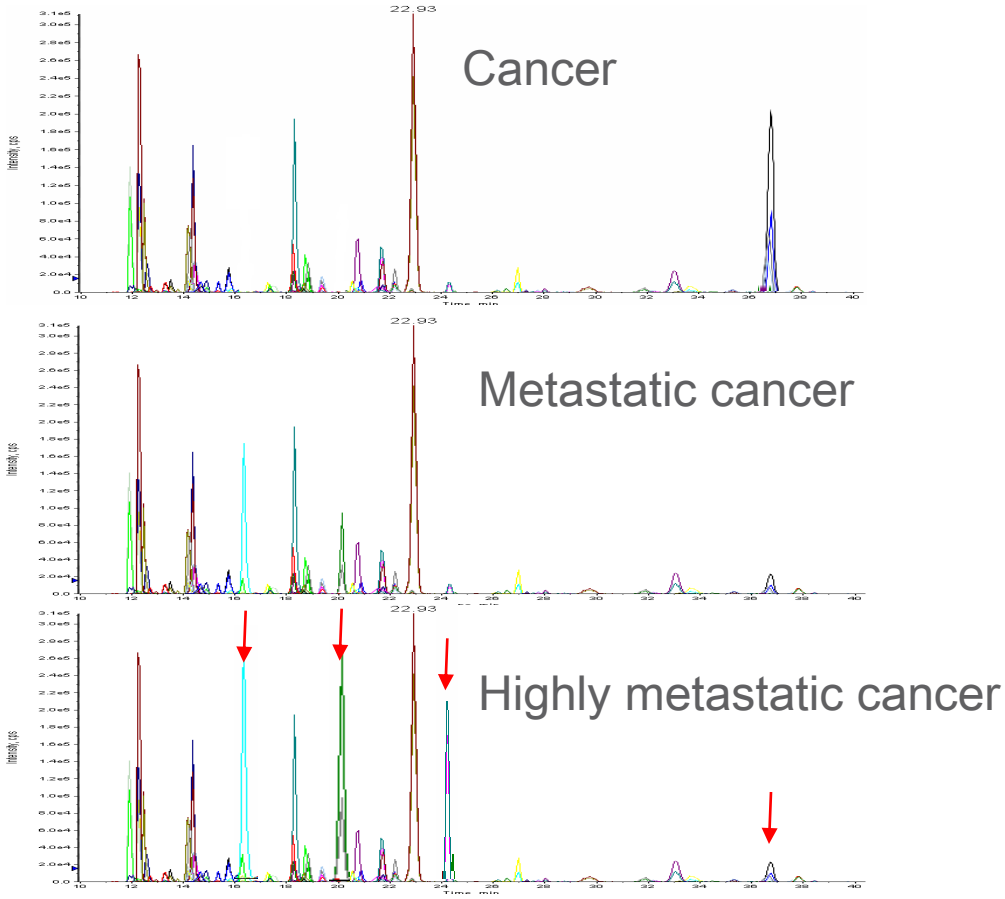


From: www.genomicglossaries.com/images

© 2011 AB Sciex

Targeted MRM Profiling Assay
For Verification of Candidate Biomarkers

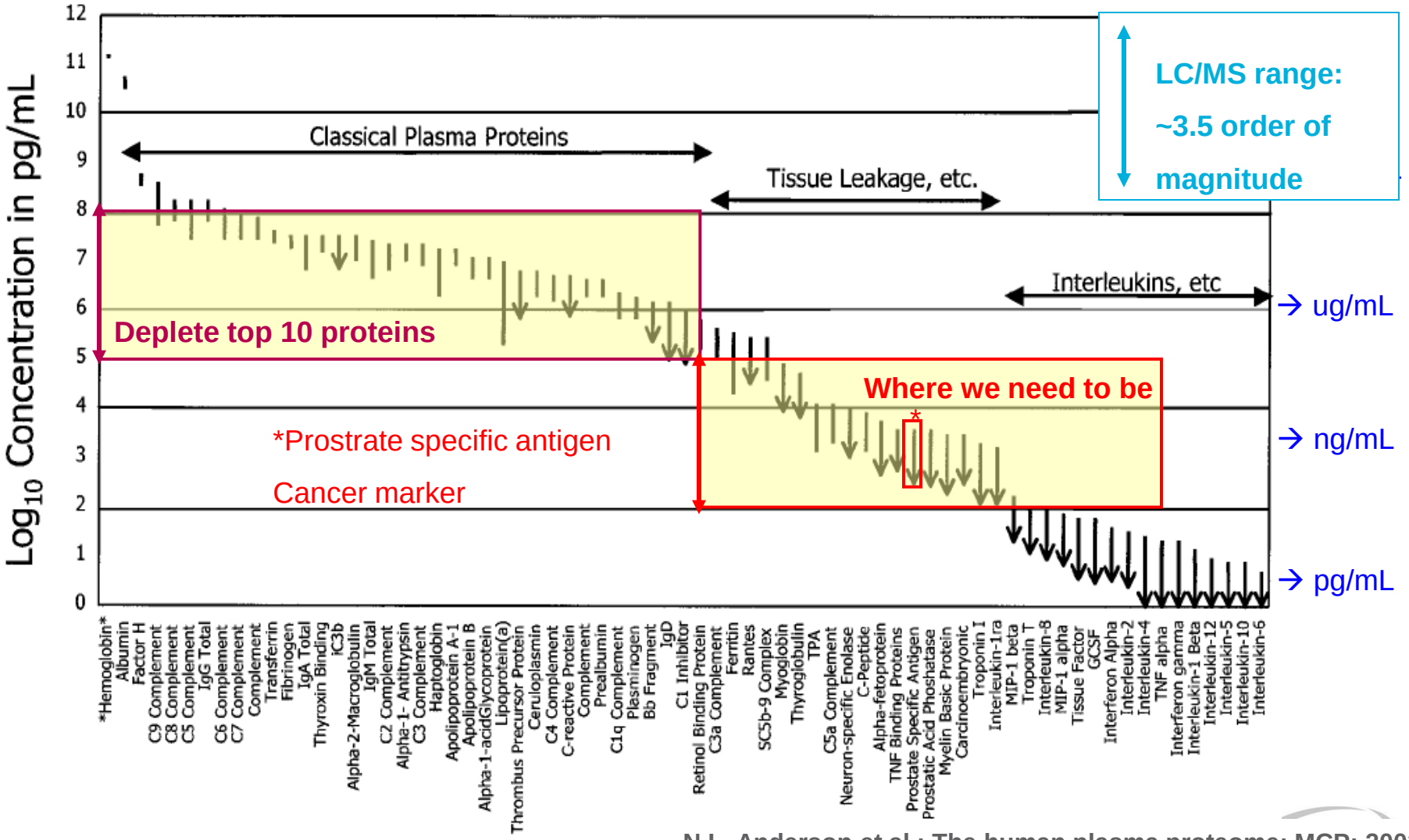




- MRM is a targeted MS scan
- MRMs can be multiplexed: >100 in a single run
- Industry standard MS quantitation technique
- Quantitively monitor peptides to **all** of our candidate biomarkers

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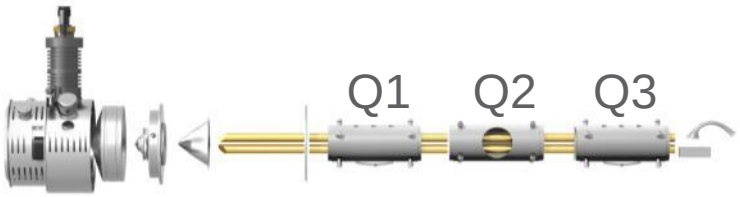
The Dynamic Range Challenge: Plasma Proteomics

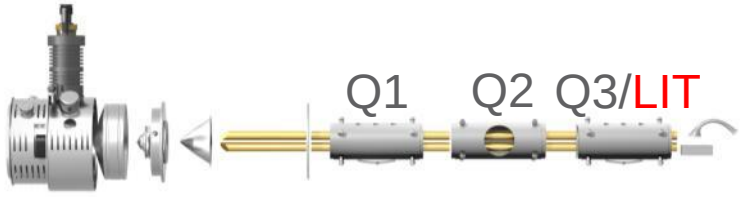


N.L. Anderson et al.; The human plasma proteome; MCP; 2002

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Advantage of QTRAP® Technology for Unknown Screening and Identification





Triple Quad

- MRM Quantitation
- ID with MRM ratio
- Low sensitivity and slow MS and MS/MS

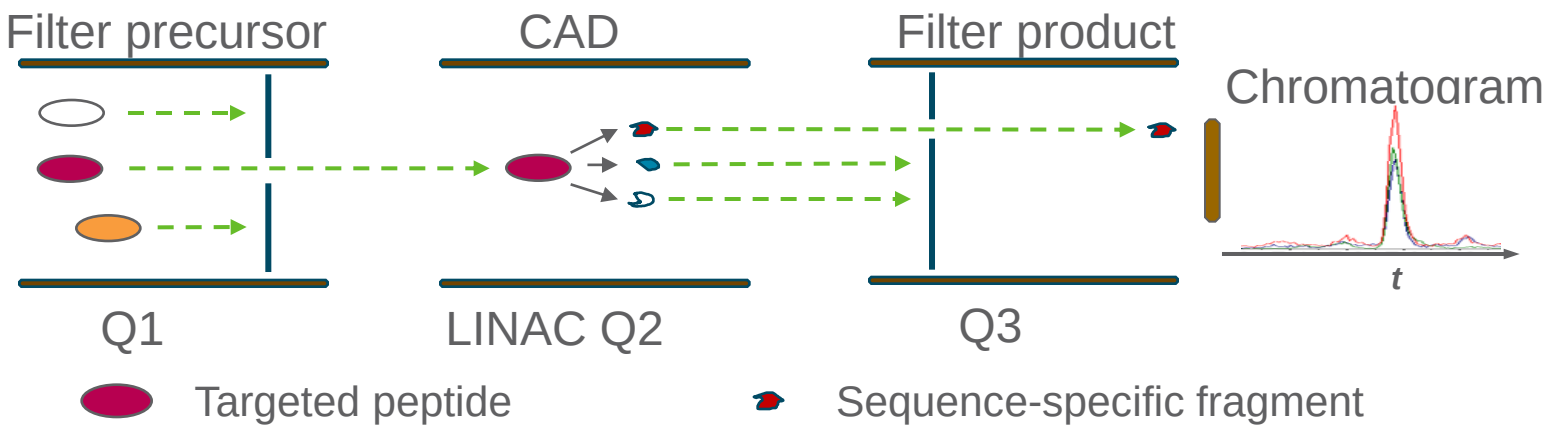
QTRAP® system

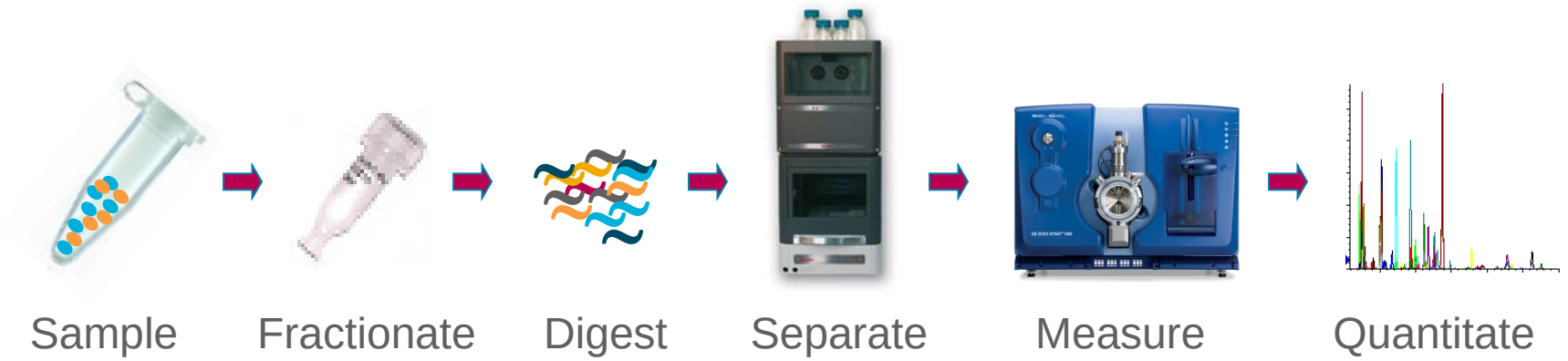
- MRM Quantitation
- ID with MRM ratio
- High sensitivity and fast MS, MS/MS, MS/MS/MS, and ER

information to detect and identify compounds

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Protein quantitation by Multiple Reaction Monitoring

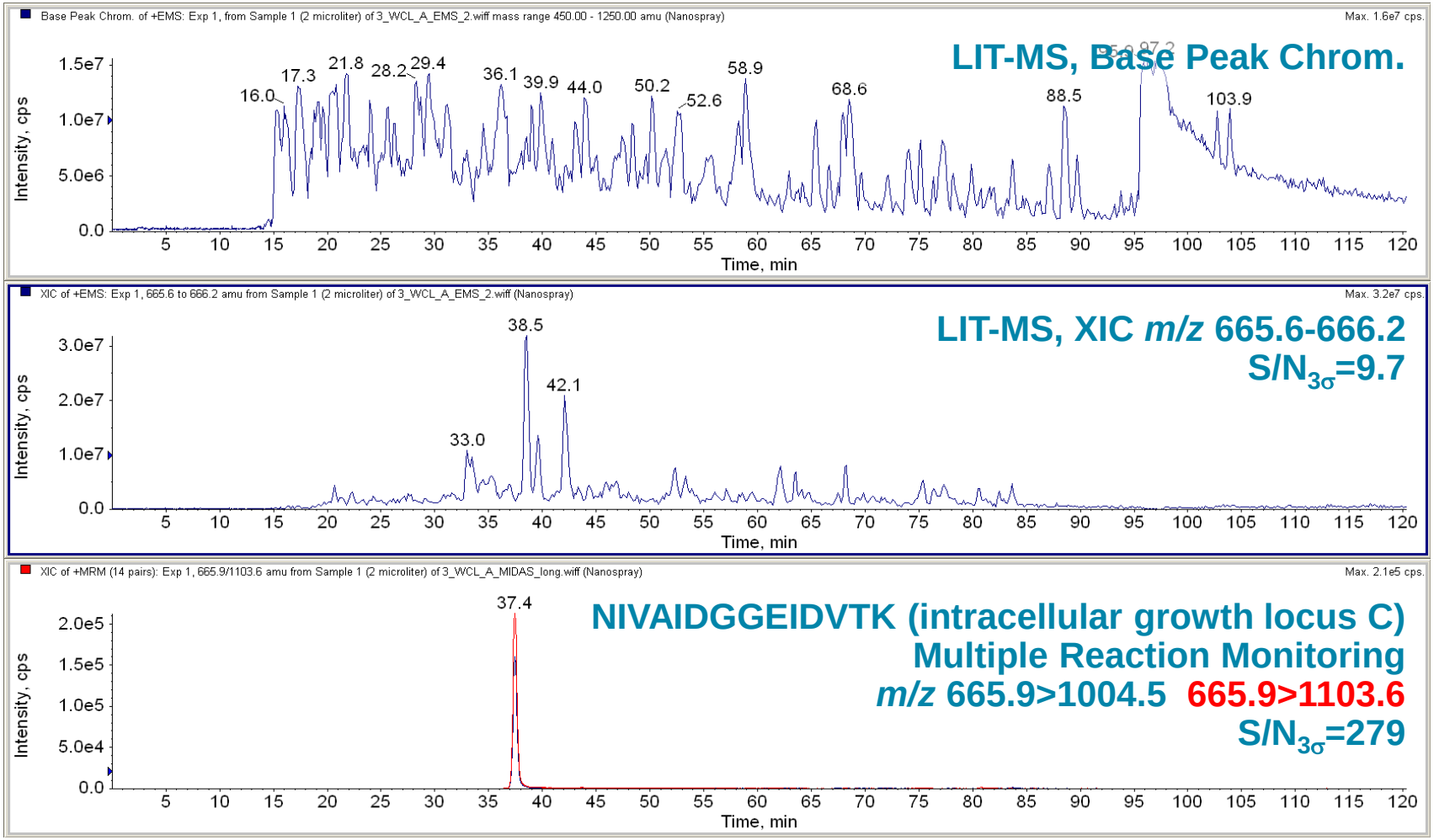




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Peptide MRMs: selectivity

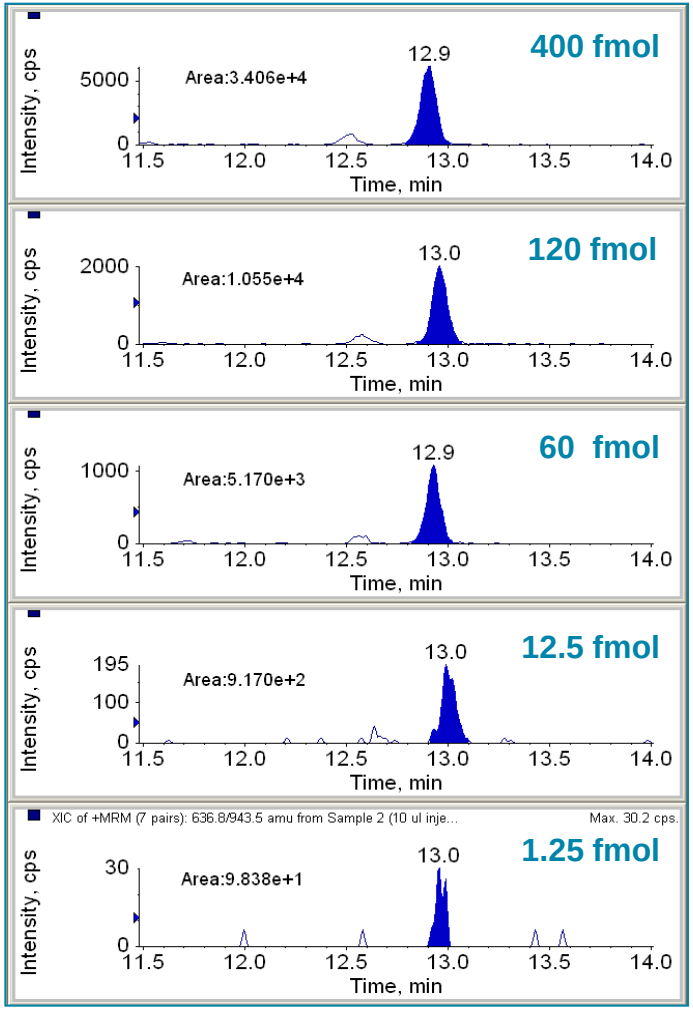
Francisella tularensis s. SCHU S4, tryptic digest of cell lysate



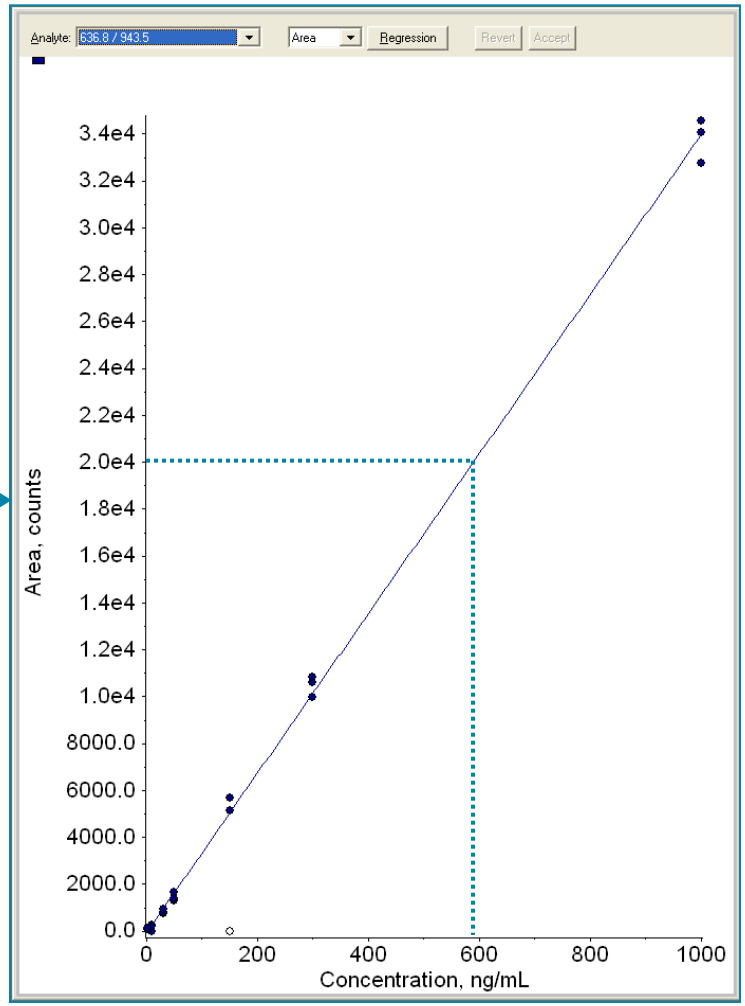
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Peptide MRMs: linearity of quantitation

LSPAELTDAVK (Prostate Specifica Antigen), LC/MRM, 250 µl/min



Peak area is linearly related to amount injected

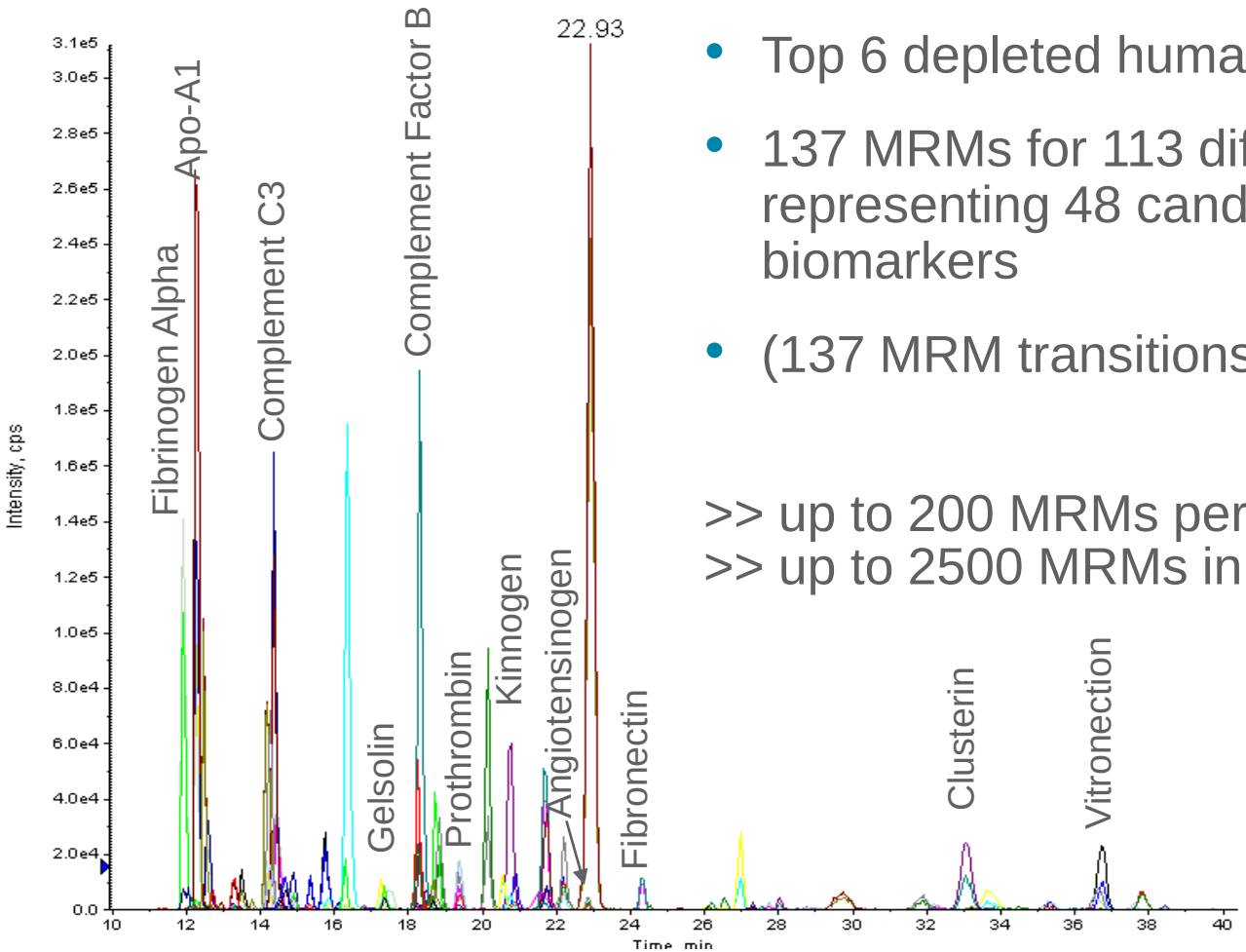


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Peptide MRMs: Multi-compound methods

Protein biomarkers for cardiovascular disease (Anderson et al., MCP 2005)

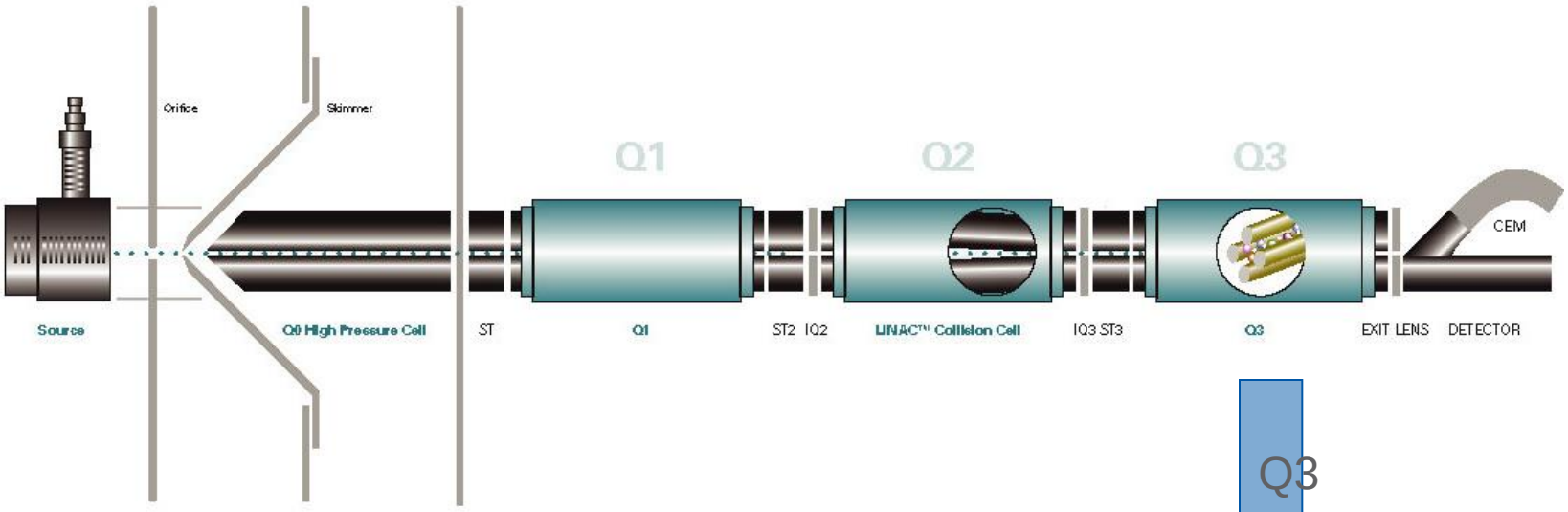


- Top 6 depleted human plasma
- 137 MRMs for 113 different tryptic peptides representing 48 candidate protein biomarkers
- (137 MRM transitions) * (20 ms/MRM) = 2.74 s cycle time

>> up to 200 MRMs per LC/MS/MS routine
>> up to 2500 MRMs in testing

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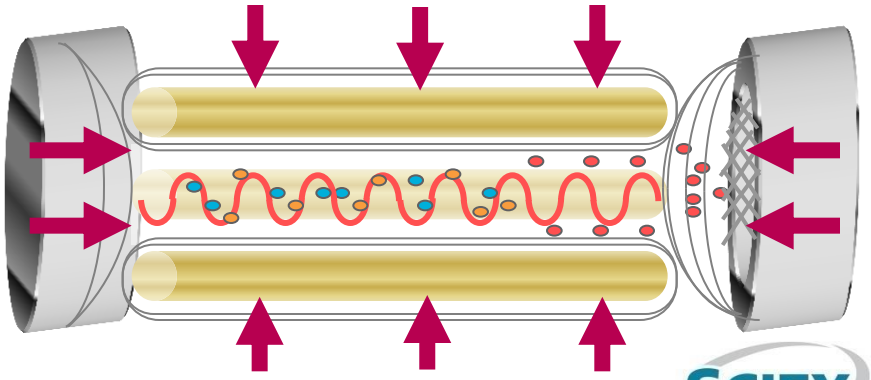
Triple Quadrupole Linear Ion Trap Technology



Q3

- < 1ms to switch between QQQ and QqLIT modes

- full scan MS/MS sensitivity ↑ >500x over QQQ

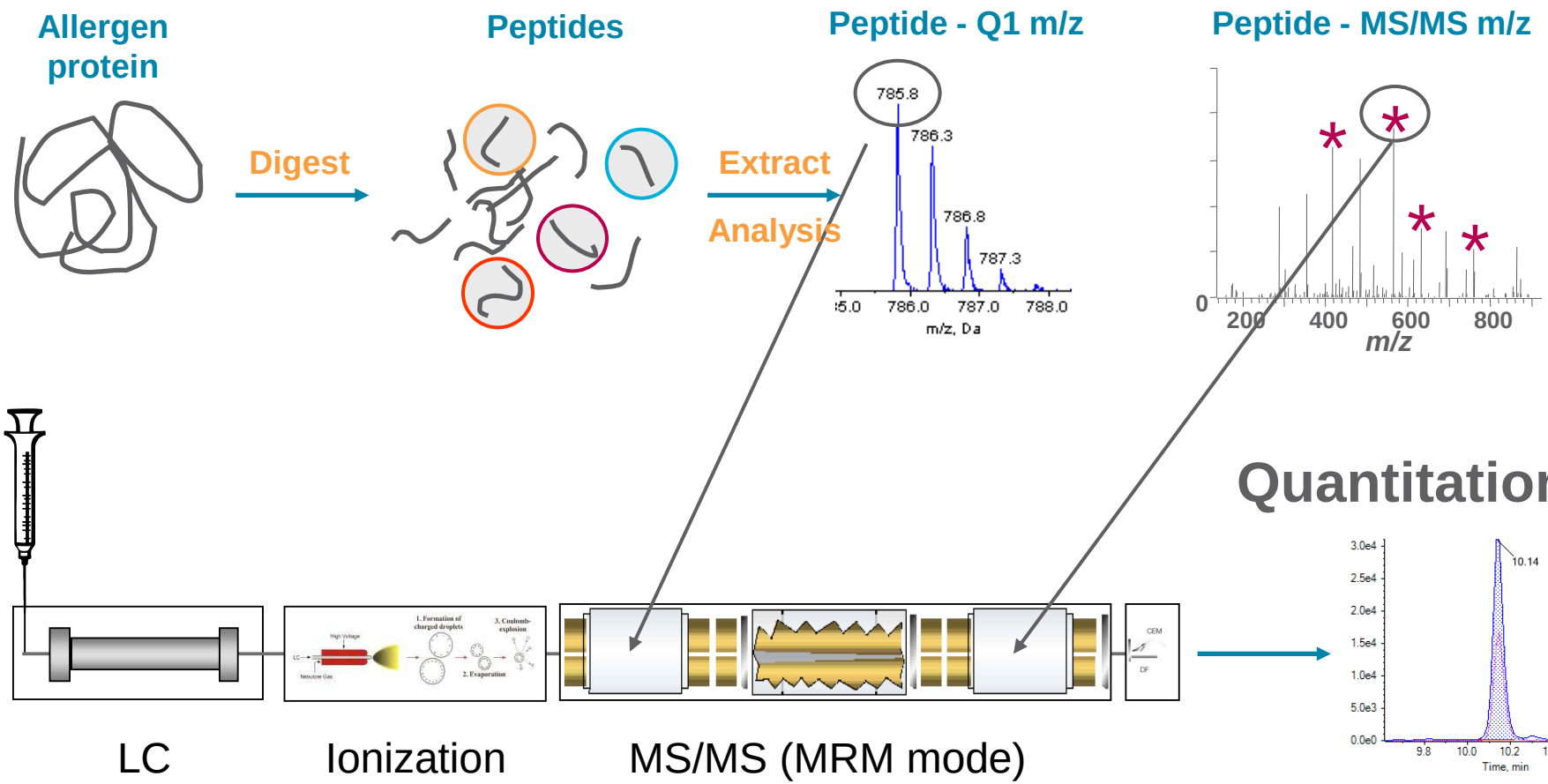


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Peptide quantitation by LC-MS/MS

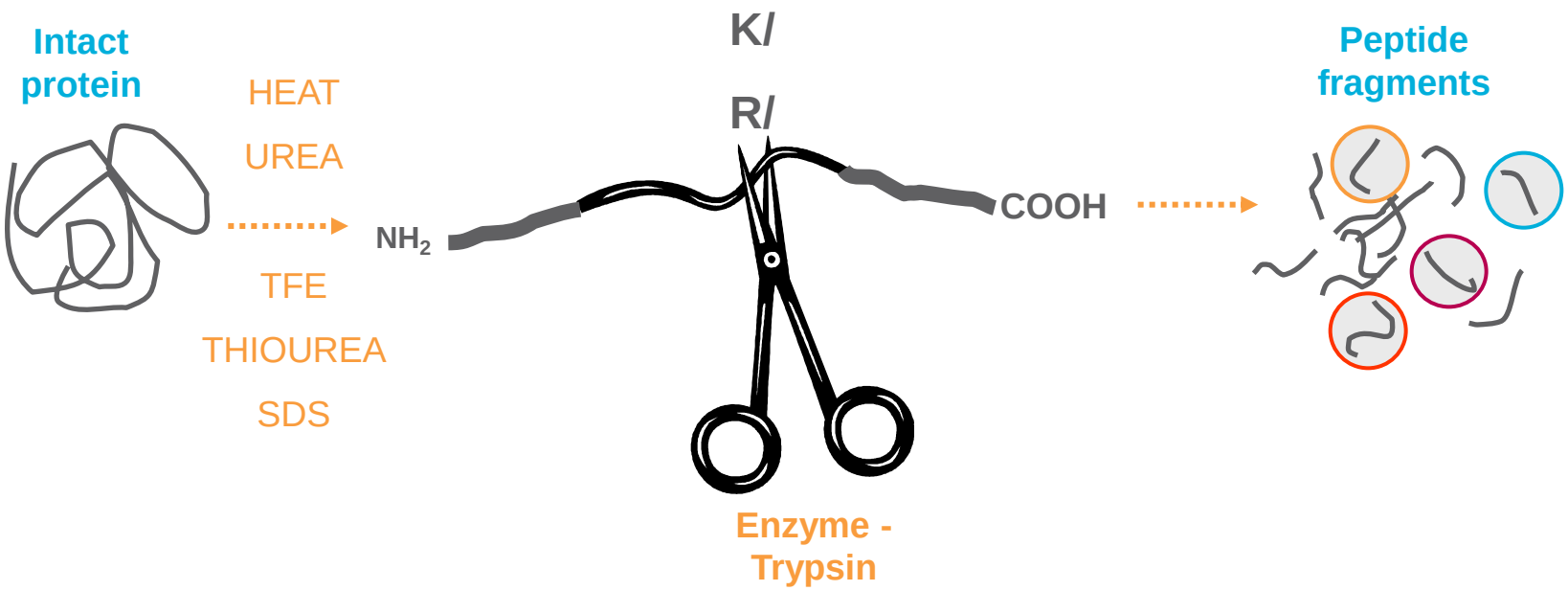
Sample Preparation

Identification



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Proteins have first to be denatured



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Choosing the right peptides: *proteotypic peptides*

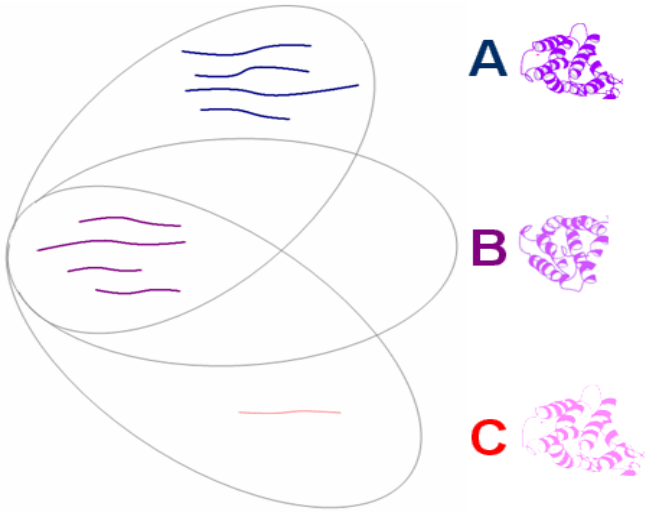
Describing a peptide sequence that is found in only a single known protein and therefore serves to identify that protein or an isoform of that protein.

- Problem solved when using data imported from ProteinPilot. (**Progroup Algorithm™**)
- Other tools for finding these peptides:
  - NCBI blast search: *most straight forward approach.*
  - Use of proteotypic peptide libraries for DB search:

Nature Biotechnology **25**, 125 - 131 (2006)
Published online: 31 December 2006 | doi:10.1038/nbt1275

Computational prediction of proteotypic peptides for quantitative proteomics

Parag Mallick[1,2,3], Markus Schirle[4], Sharon S Chen[3], Mark R Flory[1], Hookeun Lee[1,5], Daniel Martin[1], Jeffrey Ranish[1], Brian Raught[1], Robert Schmitt[4], Thilo Werner[4], Bernhard Kuster[4] & Ruedi Aebersold[1]

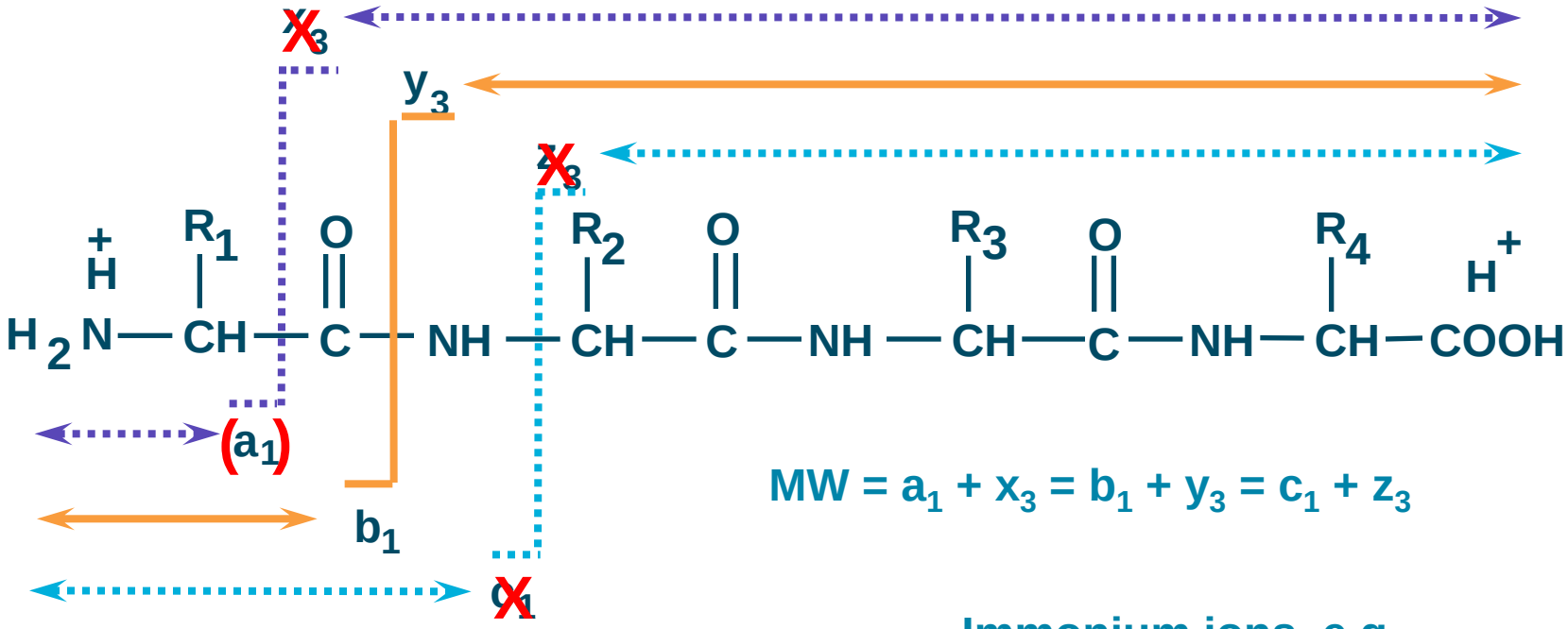


  - STEPP: SVM Technique for Evaluating Proteotypic Peptides

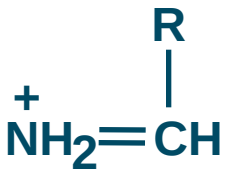
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Peptides: CID-MS/MS fragmentation

Papayannopoulos et al., Mass Spectrom. Rev. 14, 49-73 (1995).



$MW = a_1 + x_3 = b_1 + y_3 = c_1 + z_3$



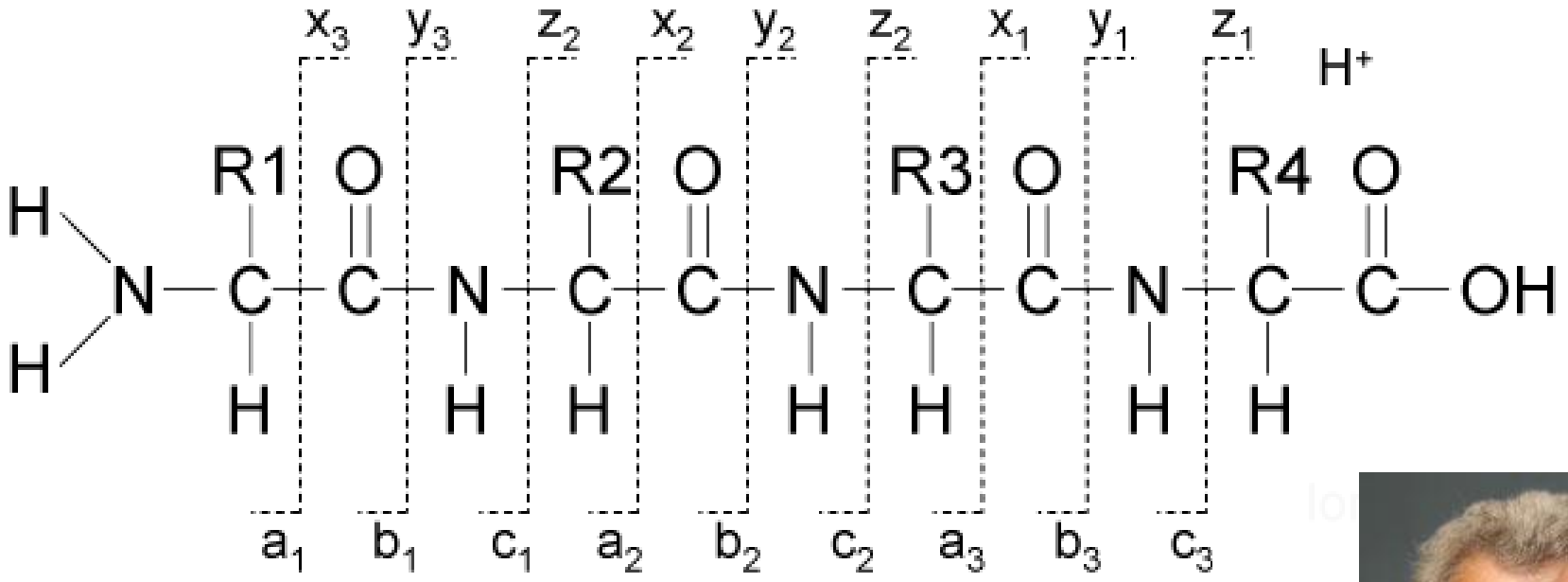
Immonium ions, e.g.

m/z 110 Histidine (H)

m/z 120 Phenylalanine (F)

m/z 136 Tyrosine (Y)

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www.ionsource.com



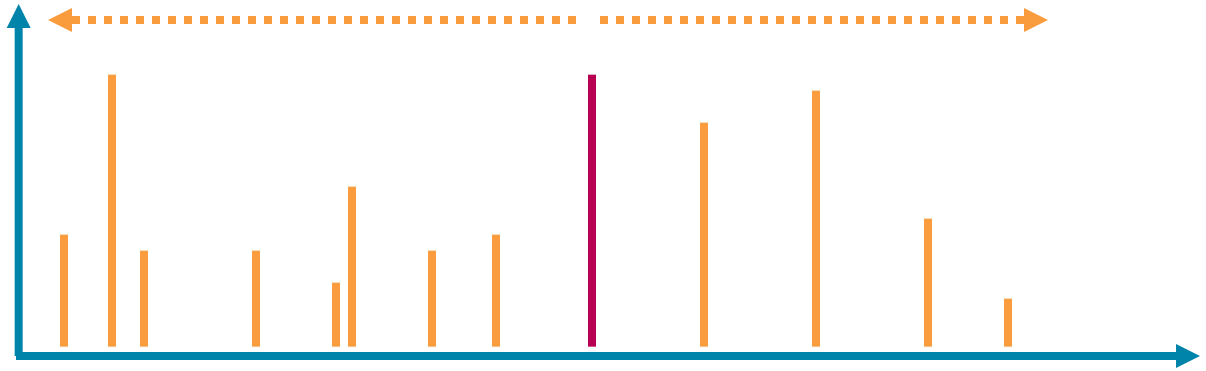
Roepstorff P, Fohlman J. Proposal for a common nomenclature for sequence ions in mass spectra of peptides. Biomed Mass Spectrom. 1984 Nov;11(11):601.

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Choosing Q3 fragment masses for MRM

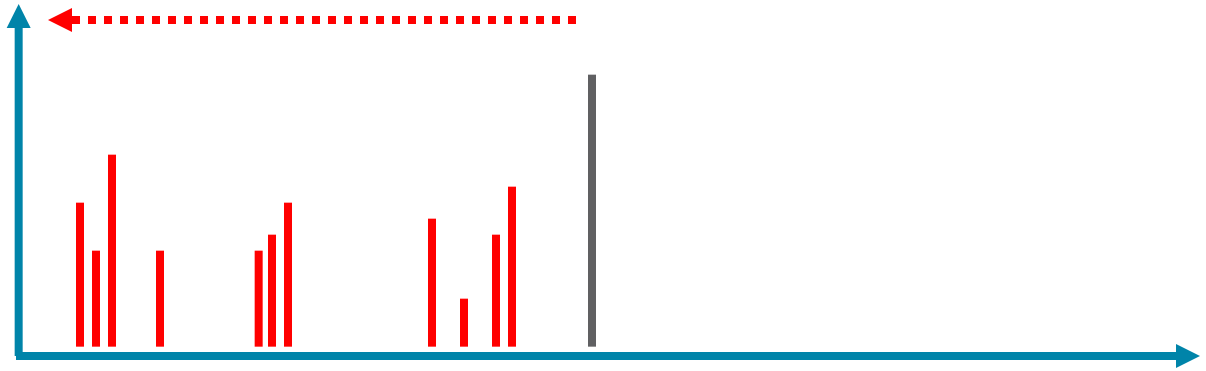
Peptide^{2+}

Fragments above and below precursor *m/z*



Non-peptide^{+}

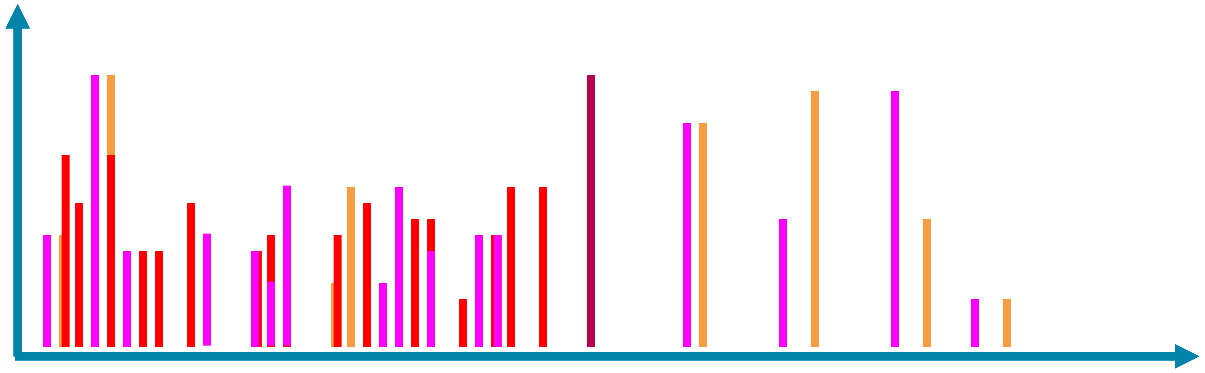
Fragments only below precursor *m/z*



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Choosing Q3 fragment masses for MRM

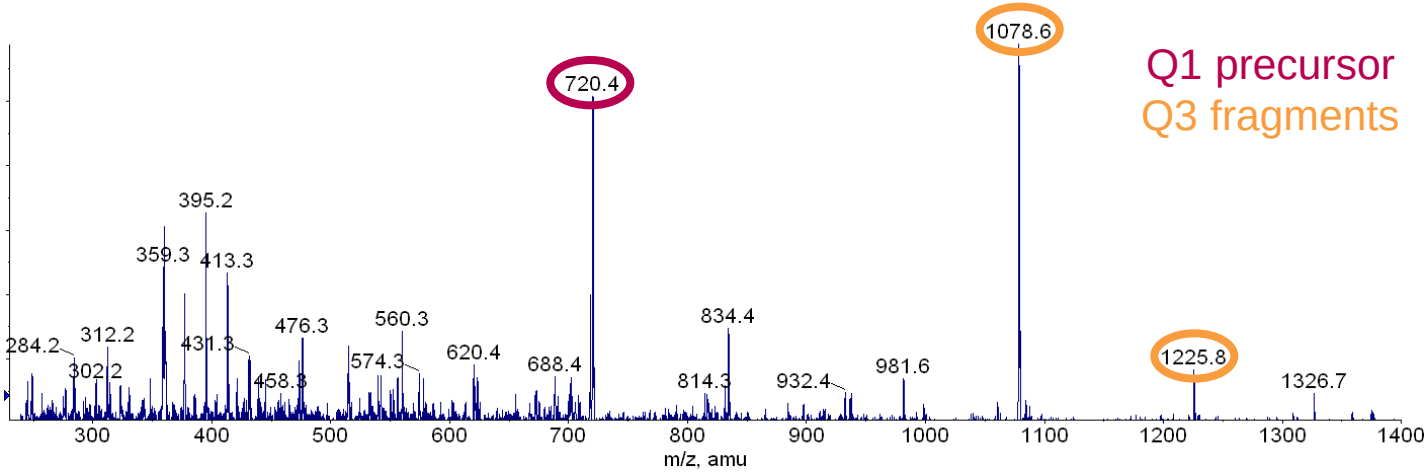
Overlay of multiple peptide and non-peptide fragment patterns....



...as in real life:

m/z 720.4^{2+}

@ 37V CE



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Optimization example EAETFPFNPFDLTK $^{2+}$

Default parameters as taken from the Parameter Settings

	Q1	Q3	Dwell time [ms]	DP [V]	EP [V]	CE [V]	CXP [V]
3+ prec.	554.9	727.4	100	60	10	46.5	12
	554.9	841.5	100	60	10	46.5	12
	554.9	988.5	100	60	10	46.5	12
	554.9	1085.6	100	60	10	46.5	12
	554.9	1232.6	100	60	10	46.5	12
	554.9	1333.7	100	60	10	46.5	12
2+ prec.	831.9	727.4	100	60	10	46.5	12
	831.9	841.5	100	60	10	46.5	12
	831.9	988.5	100	60	10	46.5	12
	831.9	1085.6	100	60	10	46.5	12
	831.9	1232.6	100	60	10	46.5	12
	831.9	1333.7	100	60	10	46.5	12

3-6 transitions to test

Parameter Settings

Parameter Settings

Rolling Coll. Energy

Parameter Settings

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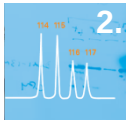
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The MIDAS™ Workflow

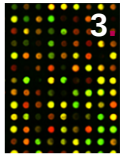
MRM Initiated Detection And Sequencing Using the MIDAS™ Workflow



1. Literature



2. Proteomics



3. Genomics

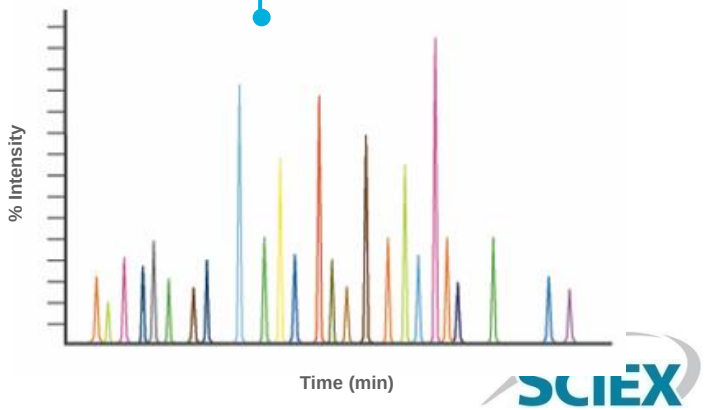
Protein Sequence

MSAIQAAWPSGTECIAKYNFHGTAEQD
LPFCK**GDVLTIVANVTK**DPNWYKAKNK
VGREGIIPANYVQKREGVKAGTKLSLMP
WFHGKITREQAER**LLYPPETGLRLVR**E
STNYPGDYTLCVSCDGKVEHYRIMYHA
SKL**SIDEEVYFENLK**MQLVEHYTSDAD
GLCTRLIKPKVMEGTVAAQDEFYRSGW
ALNMKELKLLQTIGK**GEFGDVMLGDYR**
GNKVAVKCIKNDATA…

In silico MRM transitions

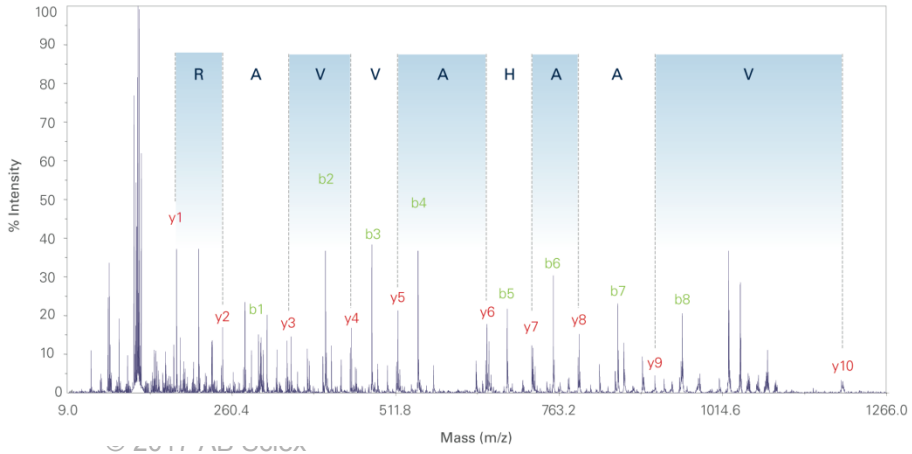
Q1	Q3	Sequence
615.4	631.4	GDVLTIVANVTK
763.9	814.5	LLYPPETGLRLVR
743.4	813.4	SIDEEVYFENLK
679.8	754.4	GEFGDVMLGDYR
…	…	…

MRM Detection in Biological Matrix



TripleTrap™ Scanning

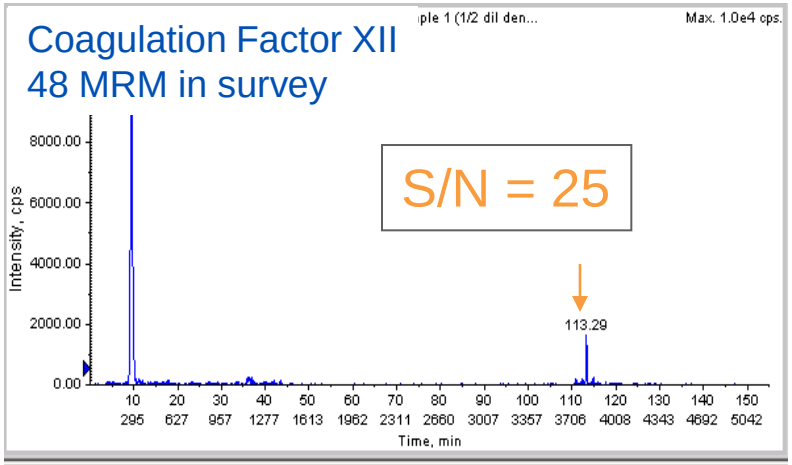
Linear _Accelerator_™ Trap MS/MS for Identification

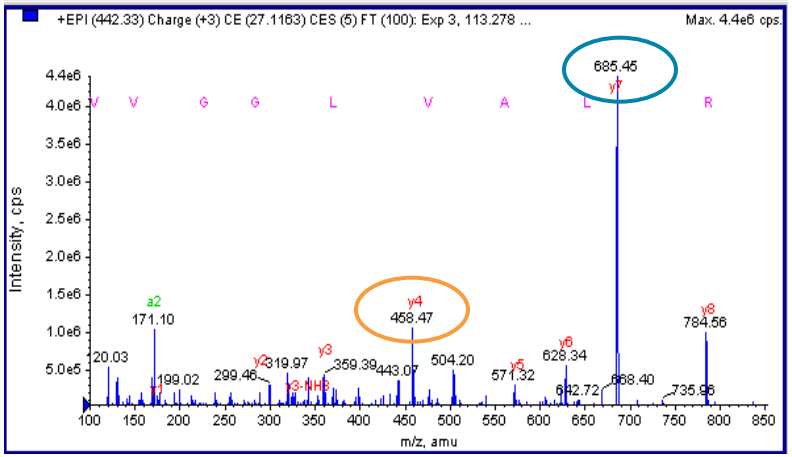


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Iterative MRM Design for Robustness

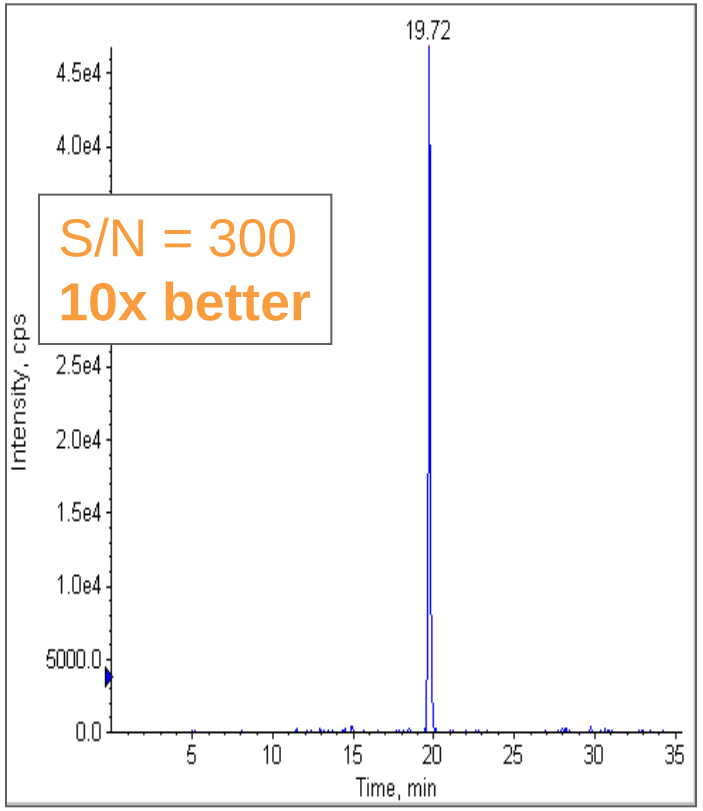
Maximize Sensitivity





Orange circle – *in silico* predicted -48 MRM

Blue circle – can we design a better MRM now?

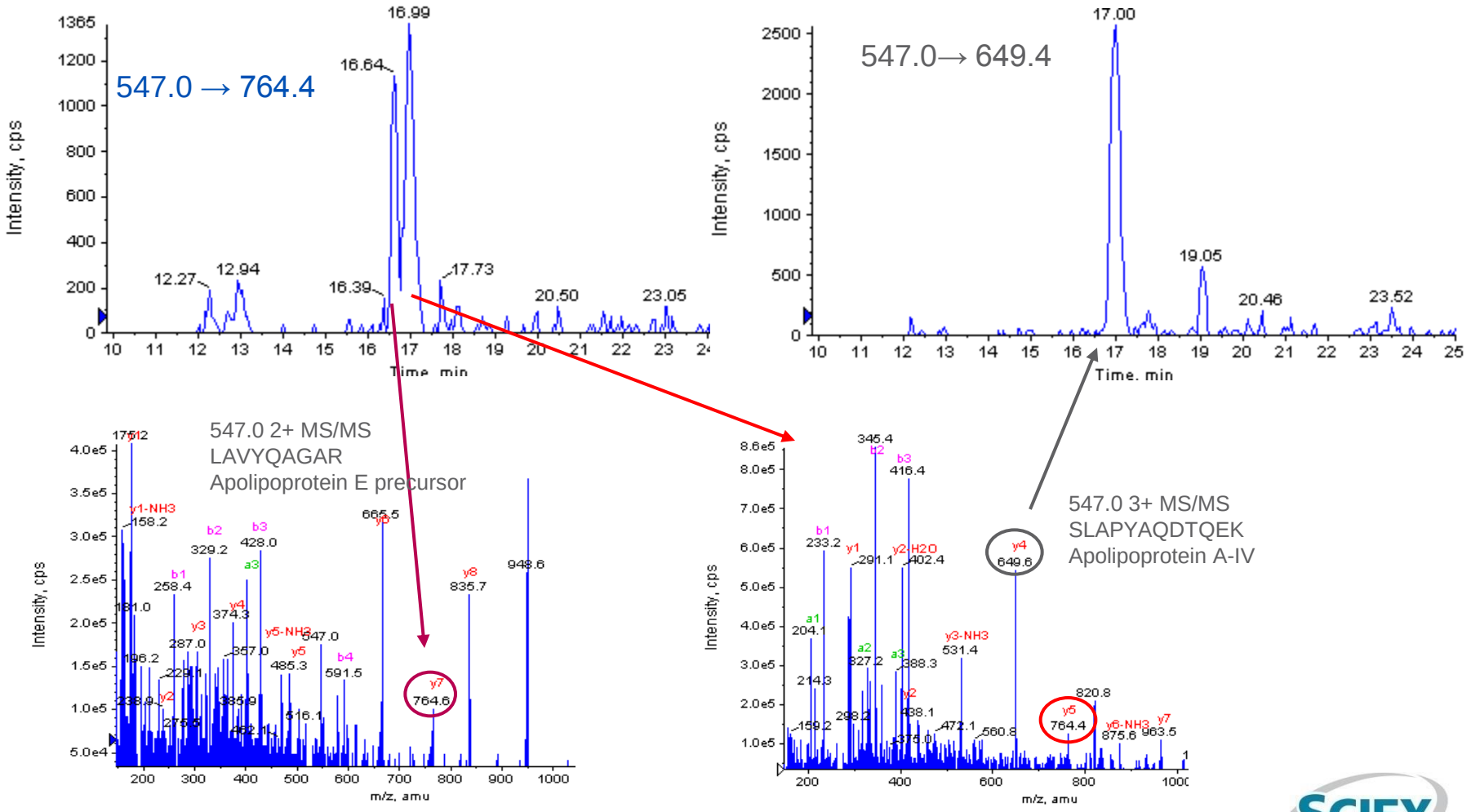


Final MRM peak in assay
- clean background, better S/N

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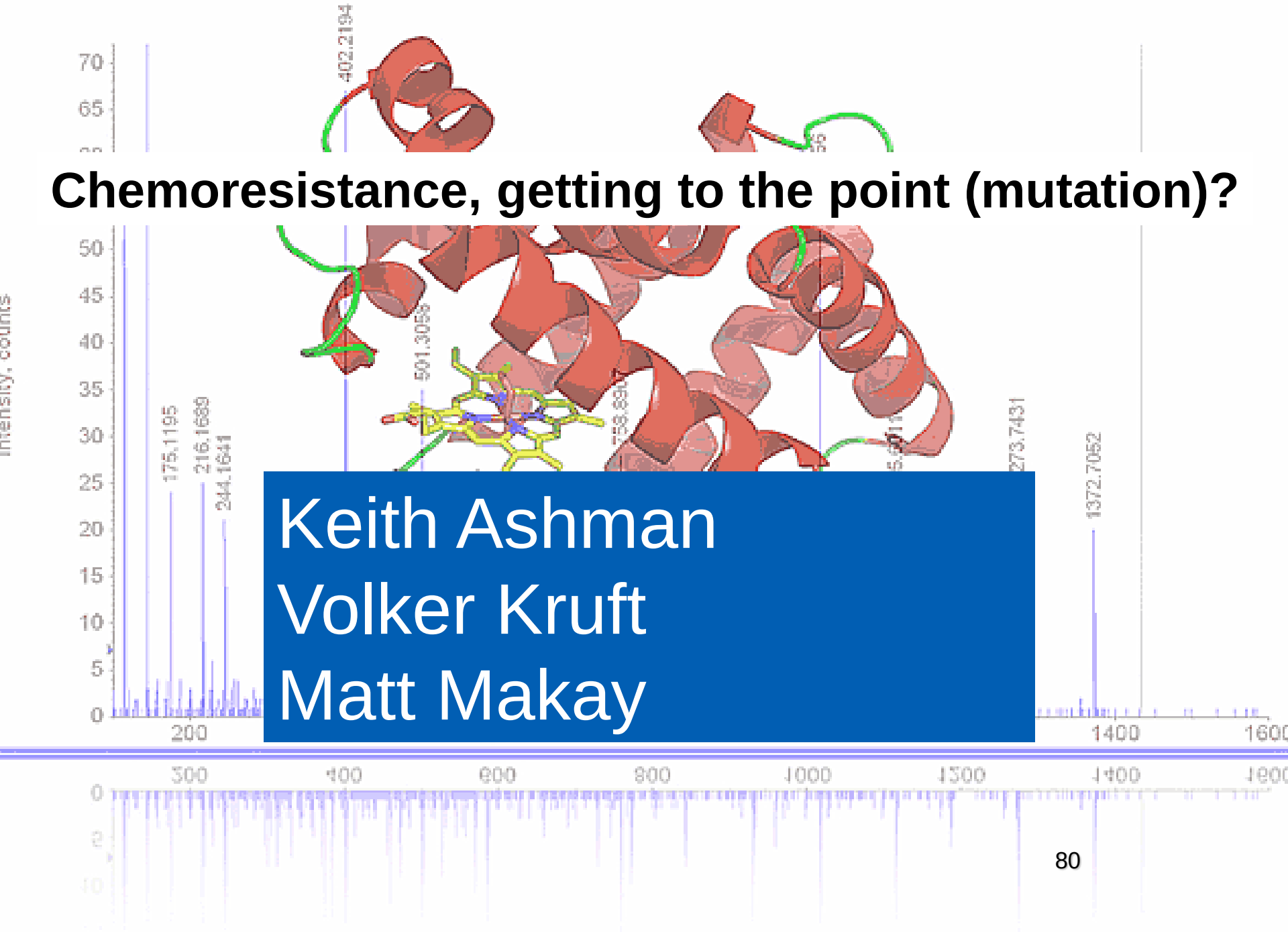
Iterative MRM Design for Robustness

Maximize Specificity



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Chemoresistance, getting to the point (mutation)?



Keith Ashman
Volker Kruft
Matt Makay

Proteomics 2012, *12*, 1319–1327 DOI 10.1002/pmic.201100611

RESEARCH ARTICLE

An improved quantitative mass spectrometry analysis of tumor specific mutant proteins at high sensitivity

Isabel Ruppen-Cañás[1], Pedro P. López-Casas[2]*, Fernando García[1], Pilar Ximénez-Embún[1], Manuel Muñoz[2], M. Pia Morelli[2], Francisco X. Real[3,4], Antonio Serna[5], Manuel Hidalgo[2,6] and Keith Ashman[1]*

[1] Proteomics Unit, Spanish National Cancer Research Center, Madrid, Spain
[2] Gastrointestinal Cancer Clinical Research Unit, Clinical Research Program, Spanish National Cancer Research Center, Madrid, Spain
[3] Molecular Pathology Programme, Spanish National Cancer Research Center, Madrid, Spain
[4] Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, Barcelona, Spain
[5] AB Sciex, Edificio Sotohenar, Madrid, Spain
[6] Centro Integral Oncólogico ''Clara Campal'', Hospital Universitario Madrid Sanchinarro, Madrid, Spain

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The importance of detecting chemosensitivity/-resistance (3)

"Although chemotherapy with gemcitabine is a common mode of treatment of pancreatic cancer, 75% of patients do not benefit from this therapy. It is likely that the sensitivity of cancer cells to gemcitabine is determined by a number of different factors. ...."

(http://clincancerres.aacrjournals.org/content/11/8/3094.full)

"In recent years considerable progress has been made in treatment strategies for chronic lymphocytic leukemia (CLL). However, the disease remains incurable because of the development of chemoresistance. Strategies to overcome resistance mechanisms are therefore highly needed. ...."

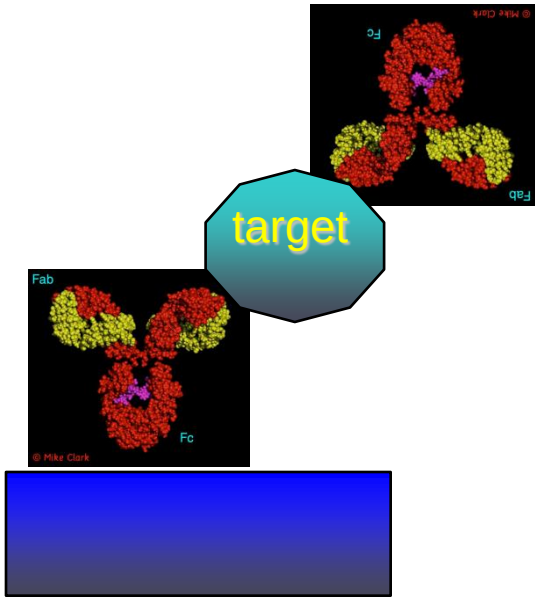
(Tonino et al., *Oncogene*, Oct 10, 2010)

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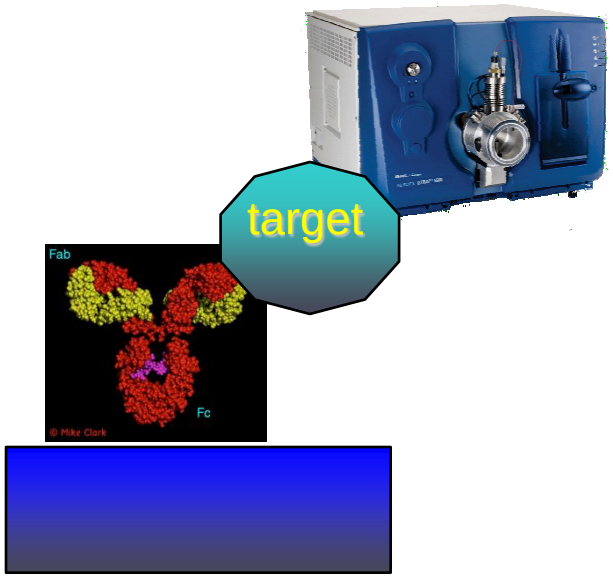
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Targeted LC-MS/MS: The Universal Antibody?

Sandwich ELISA



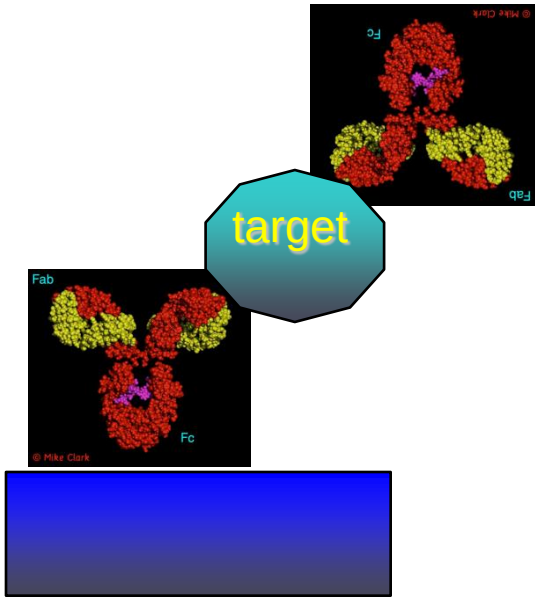
Antibody/MS/MS assay



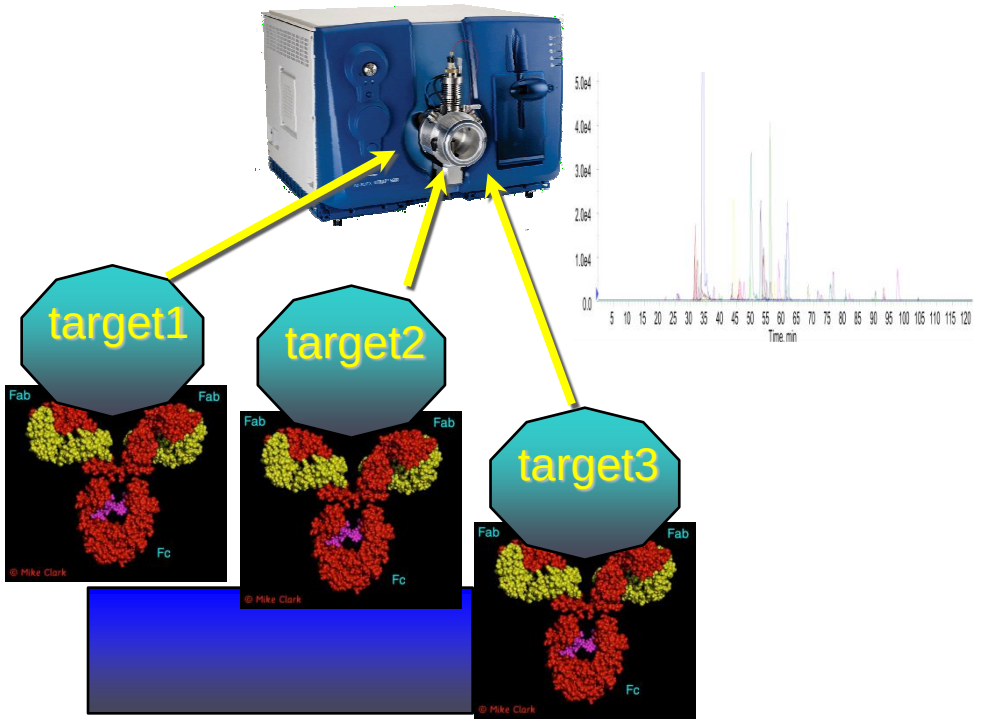
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Targeted LC-MS/MS: The Universal Antibody?

Sandwich ELISA

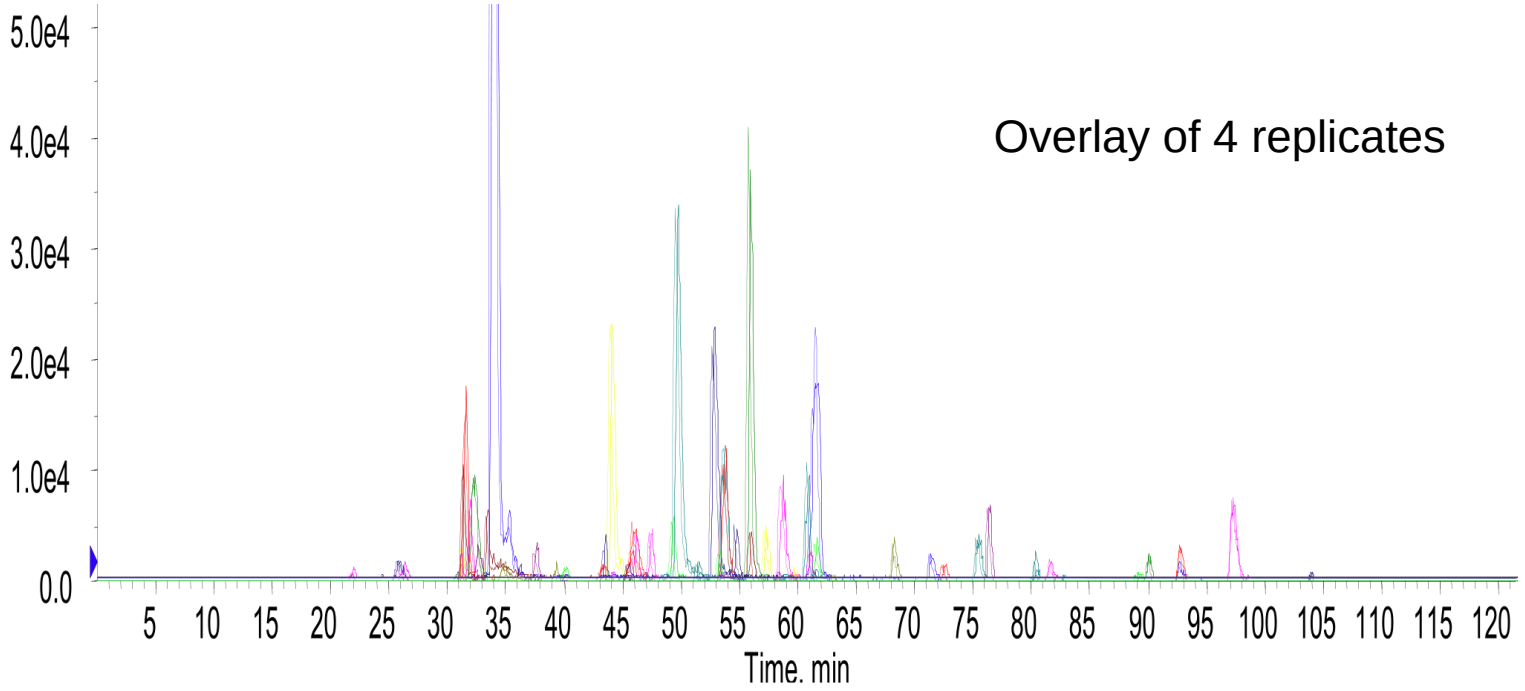


Antibody/MS/MS assay



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MRM/SRM Assays



α-1-antitrypsin, α-1-antichymotrypsin, α-1-acid glycoprotein, α-2-macroglobulin, antithrombin III, Apo-A1,Apo-B100, α-haptoglobin, β-haptoglobulin, ceruloplasmin, complement C3, complement C4, clusterin, fibrinogen α, fibrinogen β, fibrinogen γ, hemopexin, prothrombin, transferrin

Median CV (29 assays) = 4.6% (n=4) - CV Range 1.2-11.6%

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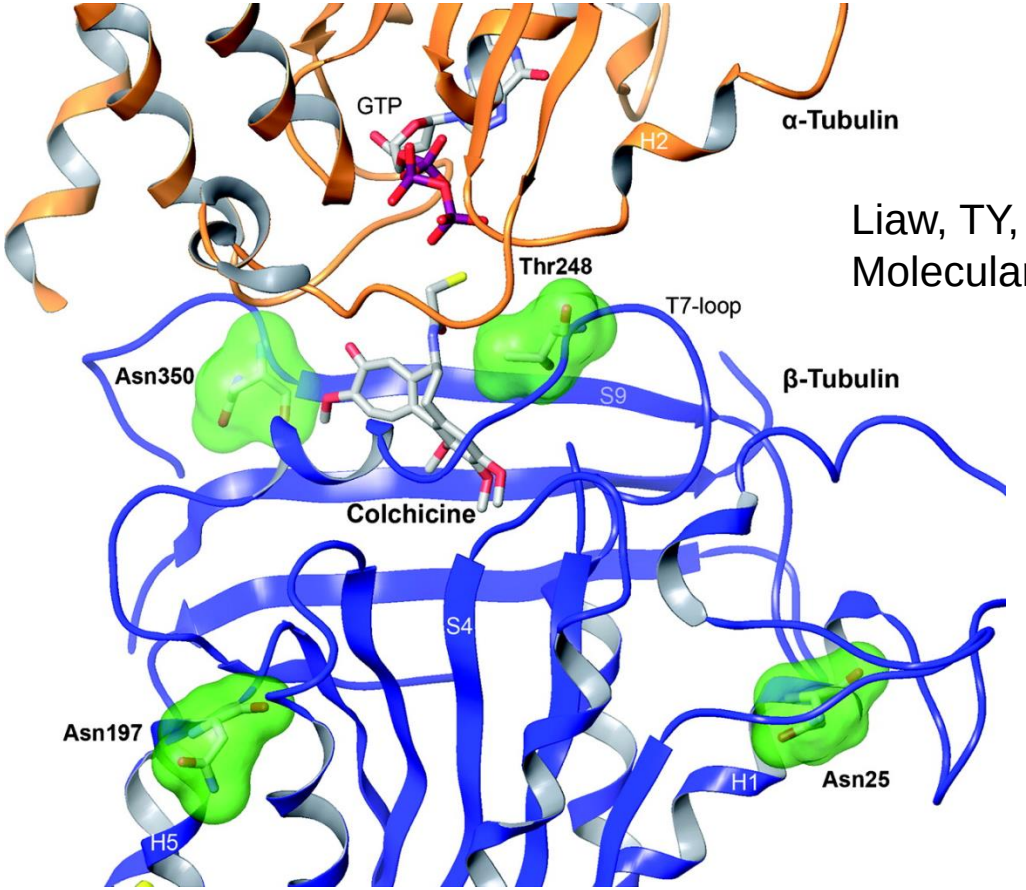
Detecting Amino Acid Substitutions in Chemoresistant Cells

- Chemoresistance cell sublines developed by stepwise exposure of cells to chemotherapeutic agent
- RT-PCR amplification and gene sequencing of the predominant, known isotype gene indicated 5 possible amino acid substitutions in chemoresistant cells
- Amino acid substitutions likely at positions:
  - 25 (Ser → Asn)
  - 197 (Asp → Asn)
  - 233 (Met → Val)
  - 248 (Ala → Thr)
  - 350 (Lys → Asn)
- Use MRM Initiated Detection and Sequencing (MIDAS™) to target peptides with and without amino acid substitutions

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SCIEX

Location of 4 mutated residues (Asn 25, Asn 197, Thr 248, Asn 352) in the tubulin heterodimer relative to the cholchicine binding site

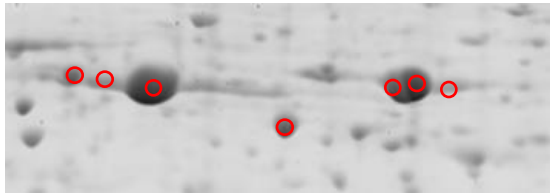


Liaw, TY, et al.,
Molecular Cancer Therapeutics, 2008, 7, 3150

©2008 by American Association for Cancer Research

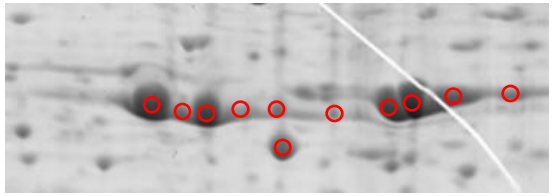
© 2017 AB Sciex

Detecting Amino Acid Sustitutions in Chemoresistant Cells



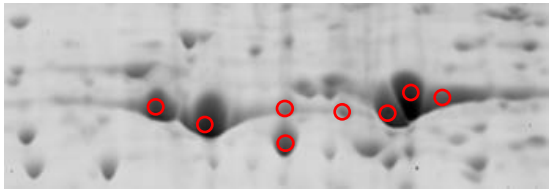
Cells able to grow in 0.9 µM chemotherapeutic drug

Stepwise exposure to chemotherapeutic drug



Cells able to grow in 3.6 µM chemotherapeutic drug

Stepwise exposure to chemotherapeutic drug



Cells able to grow in 28.8 µM chemotherapeutic drug

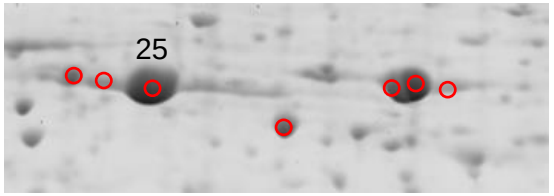
1. Excise gel spots
2. Use MRM to detect aa substitutions

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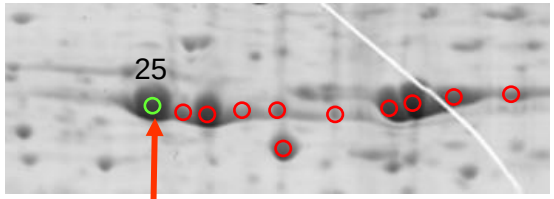
MIDAS Confirms Amino Acid Substitutions

resistant to 0.9 µM



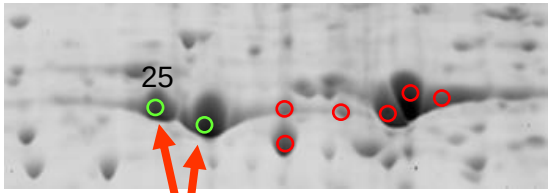
FPGQLN<u>A</u>DLR

resistant to 3.6 µM

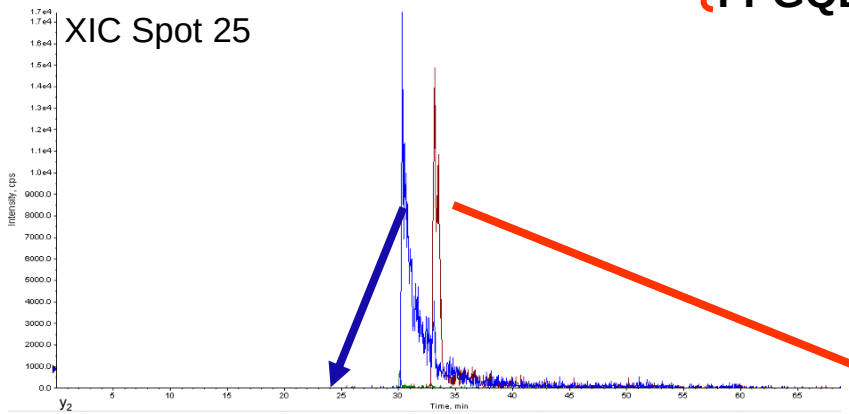


FPGQLN<u>A</u>DLR and
FPGQLN<u>T</u>DLR

resistant to 22.8 µM

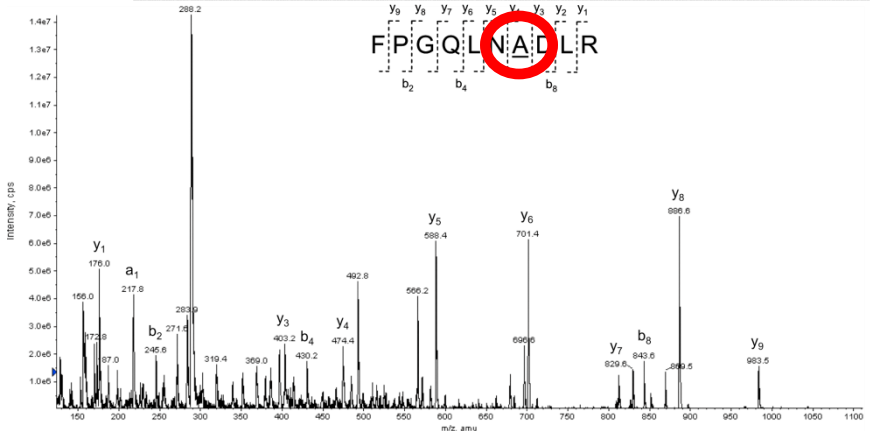


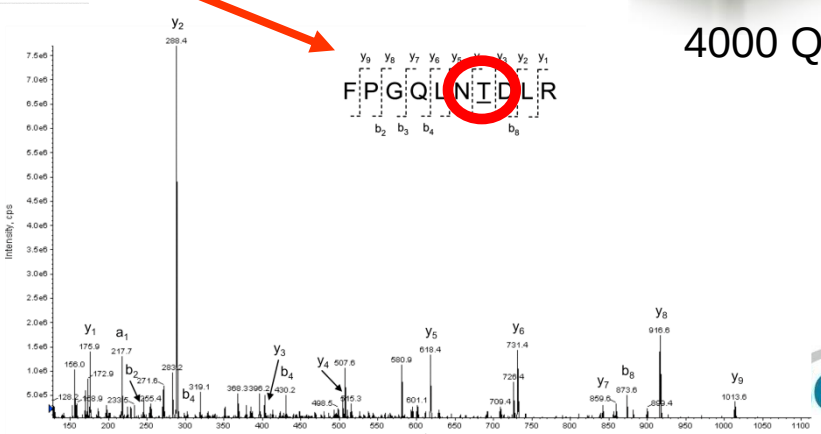
FPGQLN<u>A</u>DLR and
FPGQLN<u>T</u>DLR





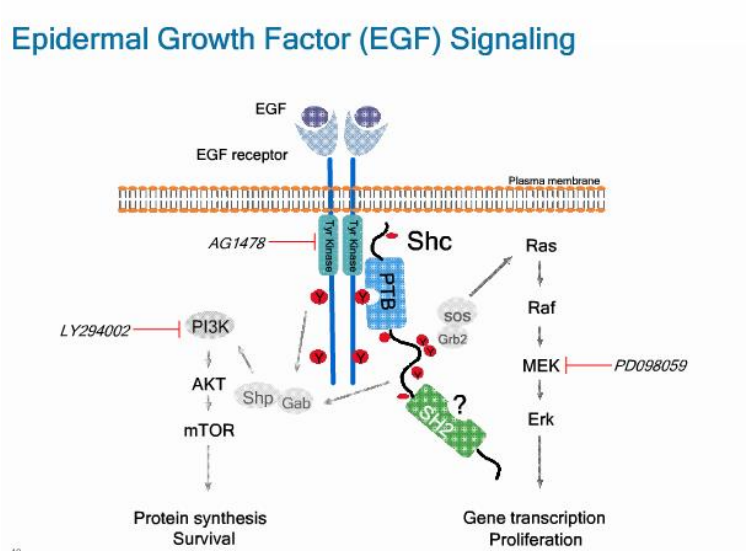
4000 QTRAP





CIEX

A Quantitative Method for Detecting Tumor Specific Signals in Mutated KRAS



EGFR/KRAS activation pathways. The external domain of EGFR is activated by circulating growth factors or inactivated by large monoclonal antibodies (cetuximab or panitumumab) while the internal EGFR tyrosine kinase domain is inactivated by small molecule inhibiitors (erlotinib, gefitinib). EGFR when activated will release downstream factors that activate KRAS. This stimulates growth and cell division

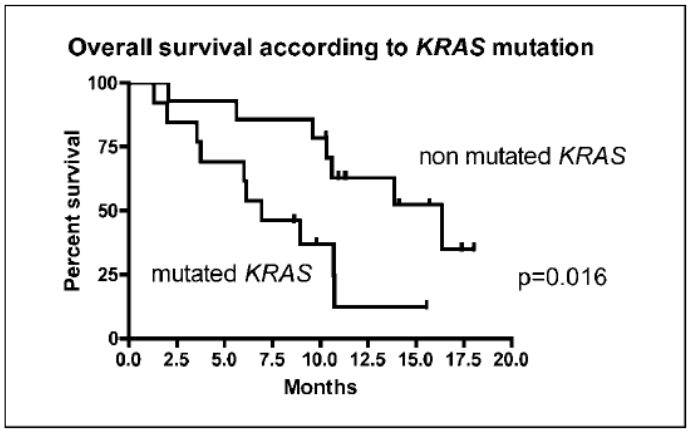
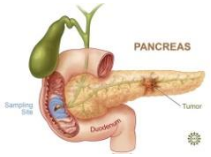


Figure 2. Overall survival curves of patients with a *KRAS*-mutated and nonmutated tumor.

- G12D is a mutation that constitutively activates the GTPase KRAS.
- The presence of G12D mutation is one of the most predictive factors of response in CRC and pancreatic cancer to EGFR blockers/inhibitors treatment.
- AIM: To develop a method to accurately quantify point mutations at the protein level in clinical samples at high sensitivity.

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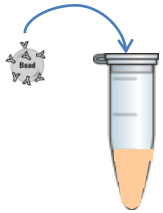
Workflow



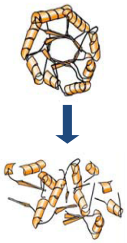
Tumour samples

Protein extraction





IP



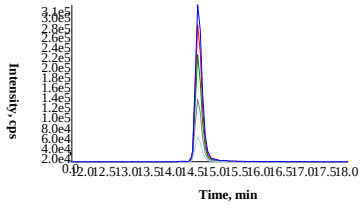
Digestion with trypsin



Addition of heavy peptide IS



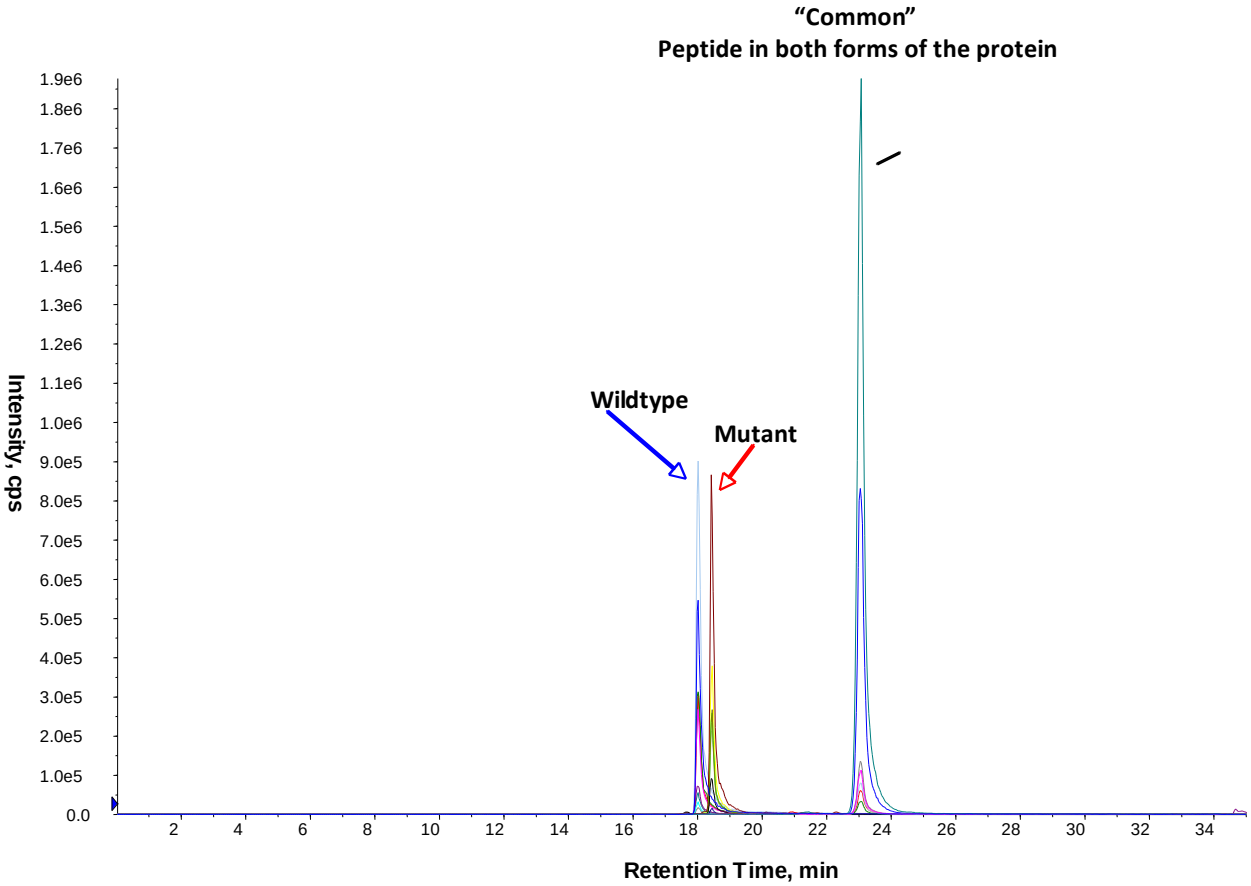
nanoLC-MS/MS by SRM (QTRAP MS)



Data analysis and quantification

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MRM-based quantitation of wild-type and mutant peptides



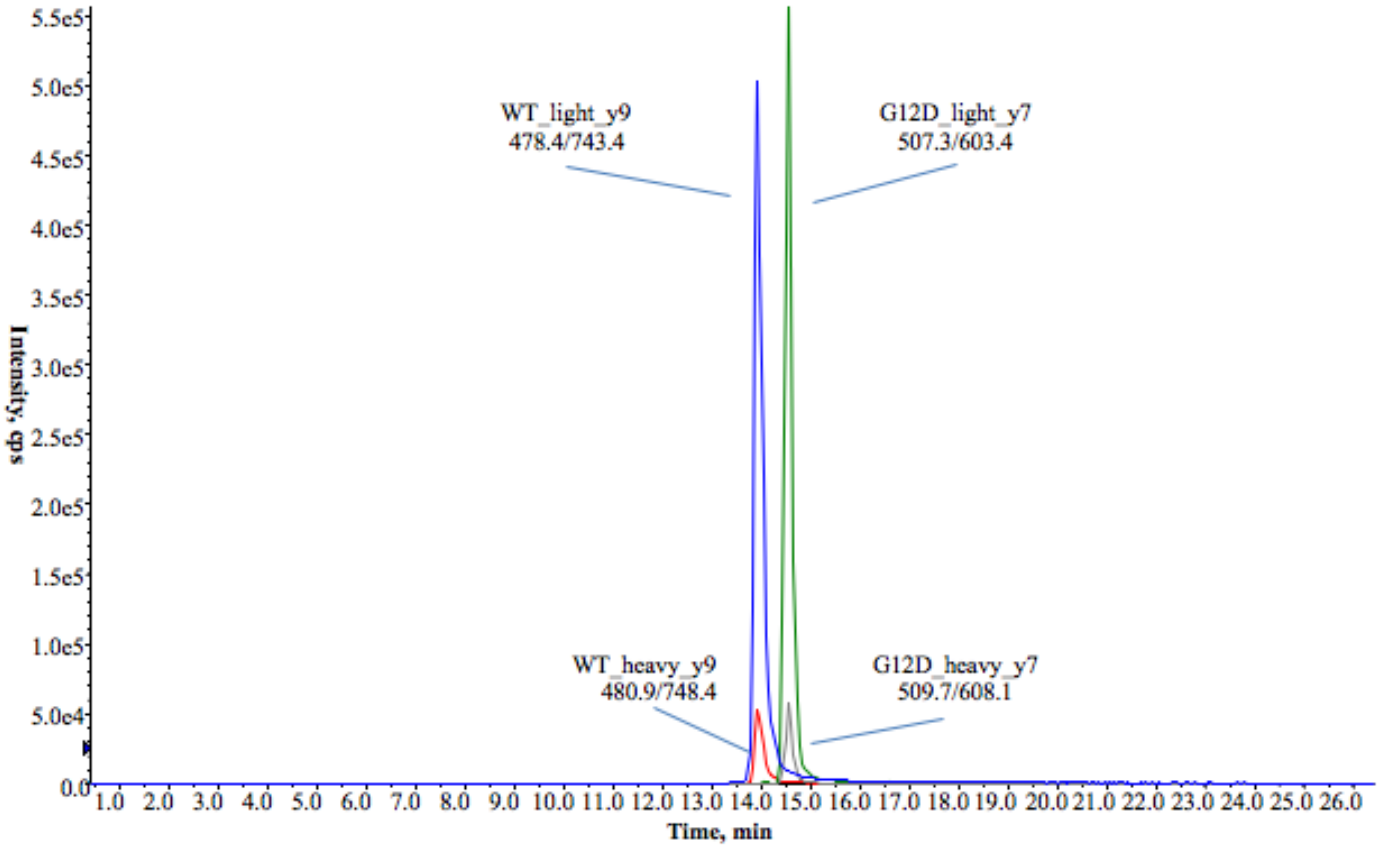


QTRAP 5500
(with Eksigent UPLC/
50µ Chromolith)

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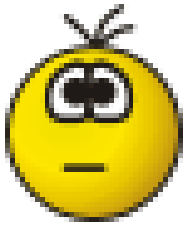
Quantitation via MRM with isotopically labeled standard peptides

(5 fmol/µl + internal standard 0.5 fmol/µl)



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So what about human serum or plasma?

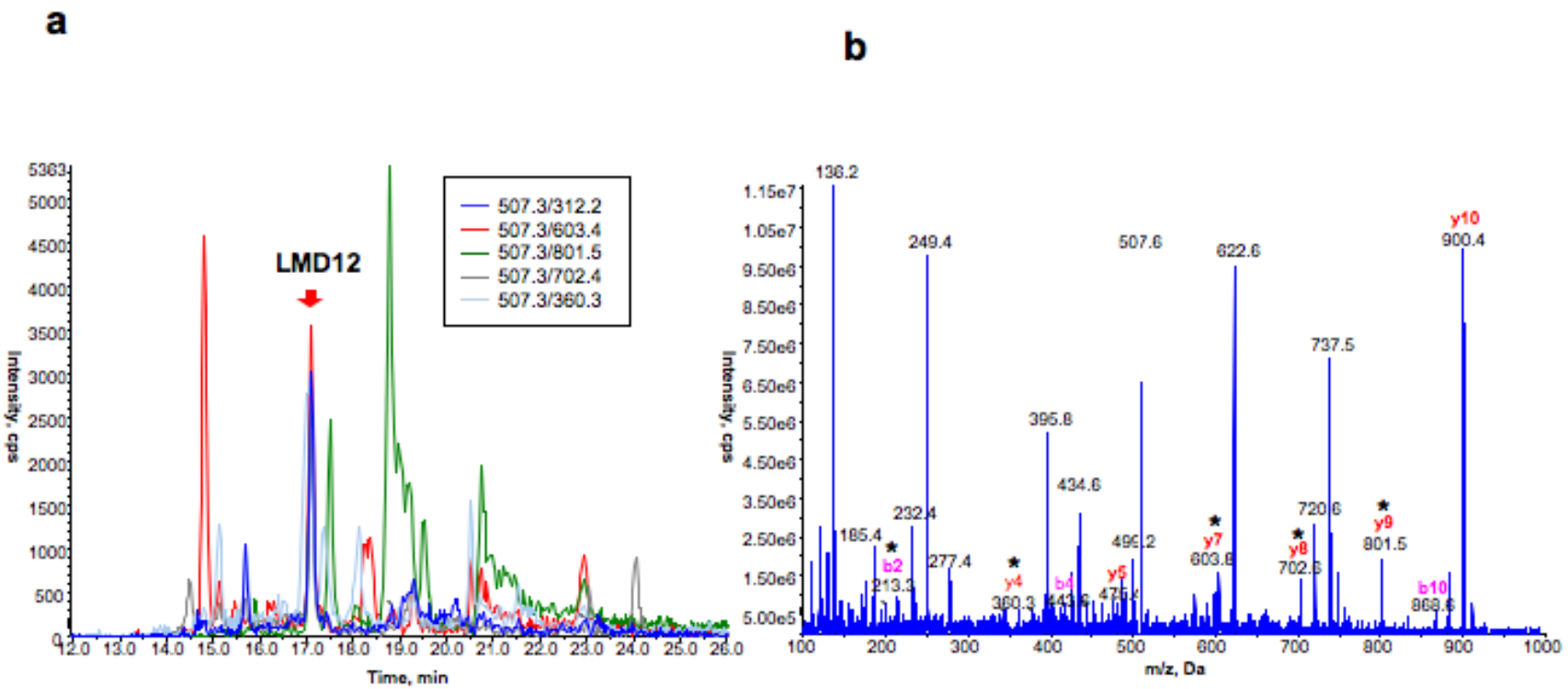


- sensitivity
- interferences
- ??

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SCIEX

Human Mutant KRAS in Mouse Plasma



Extracted ion chromatograms derived from the injection of 250 µl plasma-equivalent protein extract on-column, showing the five transitions monitored for LMD12. *B,* MS/MS spectrum of peptide fragments taken at the time of the peak (17.13 min), showing peptide sequencing performed using BioAnalyst software 1.5 (AB Sciex) (* marks the fragments monitored in the SRM method) (b). *cps*, counts per second.

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RAPID COMMUNICATIONS IN MASS SPECTROMETRY
Rapid Commun. Mass Spectrom. 2007; **21**: 2585–2593
Published online in Wiley InterScience (www.interscience.wiley.com) DOI: 10.1002/rcm.3130

RCM

The application of ultra-performance liquid chromatography/tandem mass spectrometry to the detection and quantitation of apolipoproteins in human serum

Richard G. Kay*, Barbara Gregory, Philip B. Grace and Steve Pleasance
HFL Ltd., Newmarket Road, Fordham, Cambridge CB7 5WW, UK

Received 2 May 2007; Revised 4 June 2007; Accepted 11 June 2007

The detection and quantitation of apolipoproteins, important markers for coron
serum by liquid chromatography/tandem mass spectrometry (LC/MS/MS) usi
monitoring (MRM) is reported. A tryptic digest of depleted human serum was a



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ANALYSIS

nature biotechnology

Multi-site assessment of the precision and reproducibility of multiple reaction monitoring–based measurements of proteins in plasma

Terri A Addona[1], Susan E Abbatiello[1], Birgit Schilling[2], Steven J Skates[3], D R Mani[1], David M Bunk[4], Clifford H Spiegelman[5], Lisa J Zimmerman[6], Amy-Joan L Ham[6], Hasmik Keshishian[1], Steven C Hall[7], Simon Allen[7], Ronald K Blackman[1,18], Christoph H Borchers[8], Charles Buck[9], Helene L (
Michael P Cusack[2], Nathan G Dodder[4], Bradford W Gibson[2], Jason M Held[2], Tara Hiltk
Eric B Johansen[7], Christopher R Kinsinger[11], Jing Li[6], Mehdi Mesri[11], Thomas A Neube
Trenton C Pulsipher[3], David Ransohoff[12], Henry Rodriguez[11], Paul A Rudnick[4], Derek S
Tony J Tegeler[13], Asokan M Variyath[5], Lorenzo J Vega-Montoto[5], Åsa Wahlander[10], Sofia

s reserved.



Nature Biotech, published online 28 June 2009

Clinical Proteomic Technology Assessment for Cancer Network of the National Cancer Institute (NCI-CPTAC)

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ASBMB

lular Proteomics

Clinical quantitation of prostate specific antigen biomarker in the low nanogram/mL range by conventional bore liquid chromatography-tandem mass spectrometry (MRM) coupling and correlation with ELISA tests

Author list

T. Fortin[1,2], A. Salvador[2*], J.P. Charrier[1], C. Lenz[3], X. Lacoux[1], A. Morla[1], G. Choquet-Kastylevsky[1], J. Lemoine[2*]

[1]-R&D Proteomique, bioMérieux SA, Marcy l'Etoile, France

[2]-UMR 5180 Sciences Analytiques, Université de Lyon, Lyon1, France

[3]-PSM Support, Applied Biosystems, Darmstadt, Germany



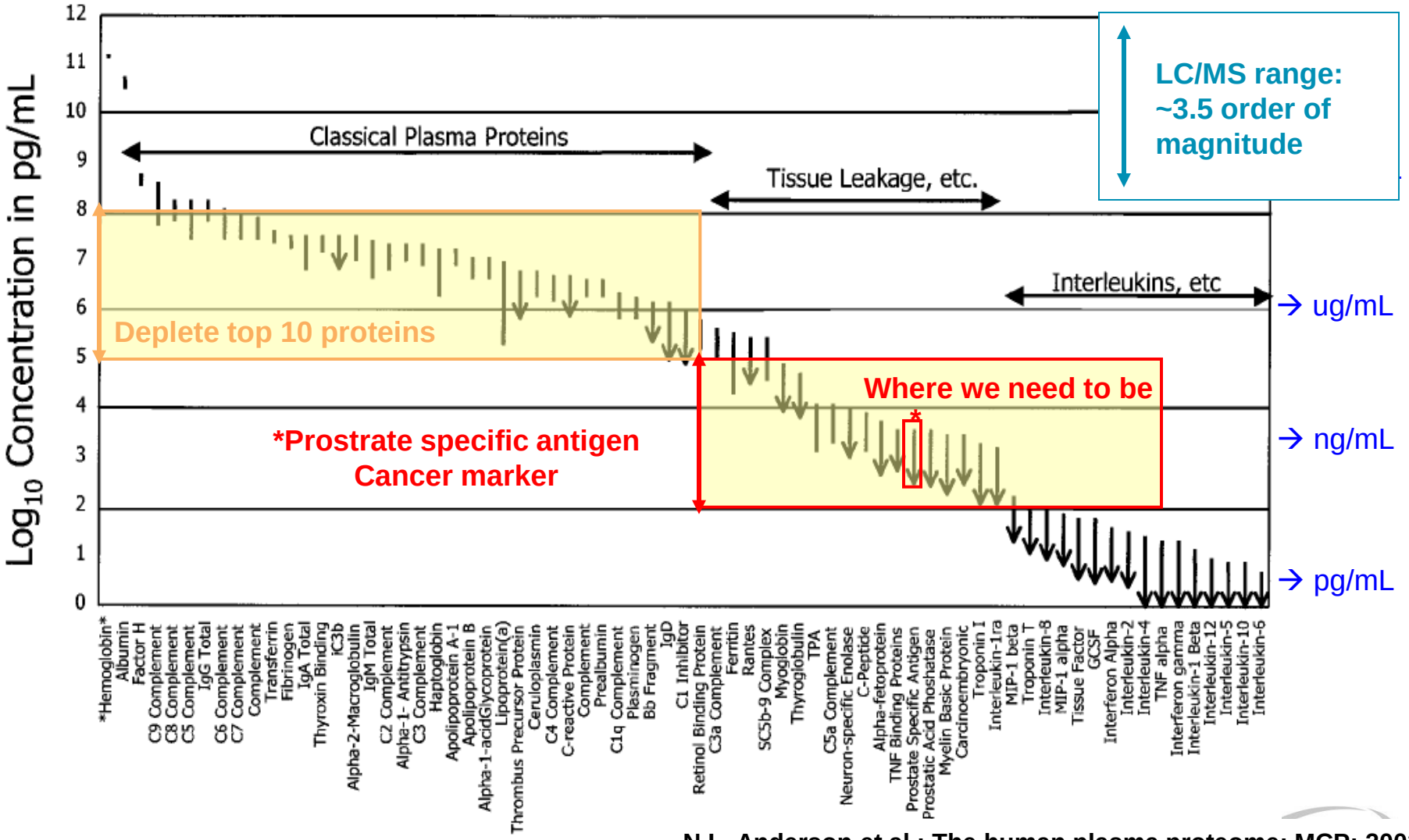


MCP, Dec 2008

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SCIEX

The Dynamic Range Challenge: Plasma Proteomics

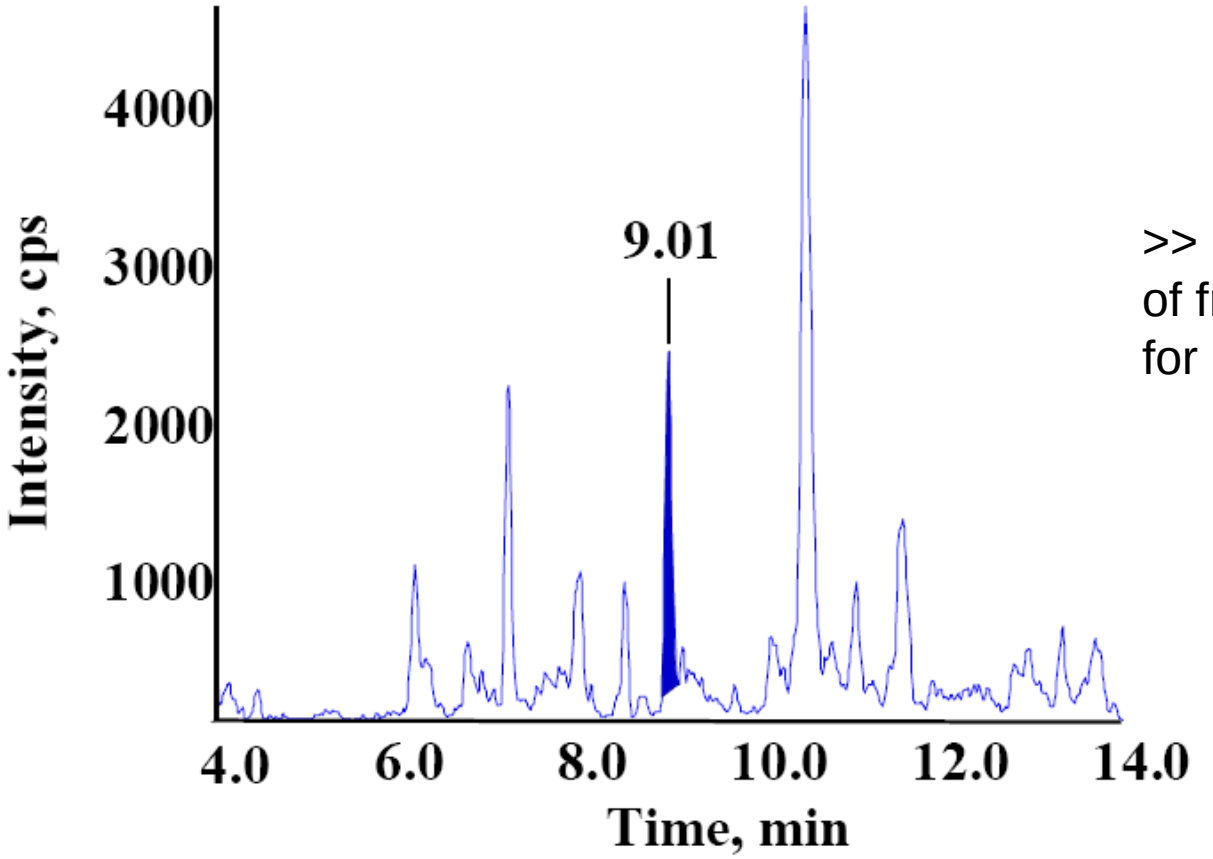


N.L. Anderson et al.; The human plasma proteome; MCP; 2002

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How can we increase MRM quantitation specificity?

LSAPAELTDAVK from PSA in human serum @ 10 ng/ml, MRM 636.8/943.5



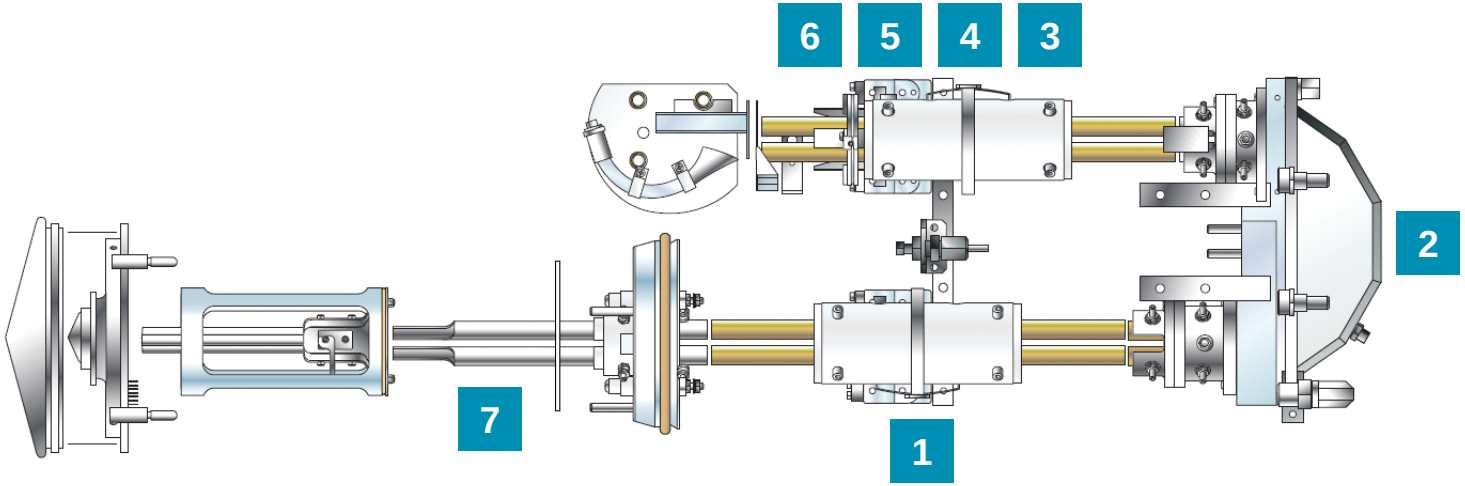
>> Use an additional stage of fragmentation (i.e. MS^3) for MRM-like quantitation

„MRM³“

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SCIEX

MS^3 on the QTRAP® 5500 LC/MS/MS System

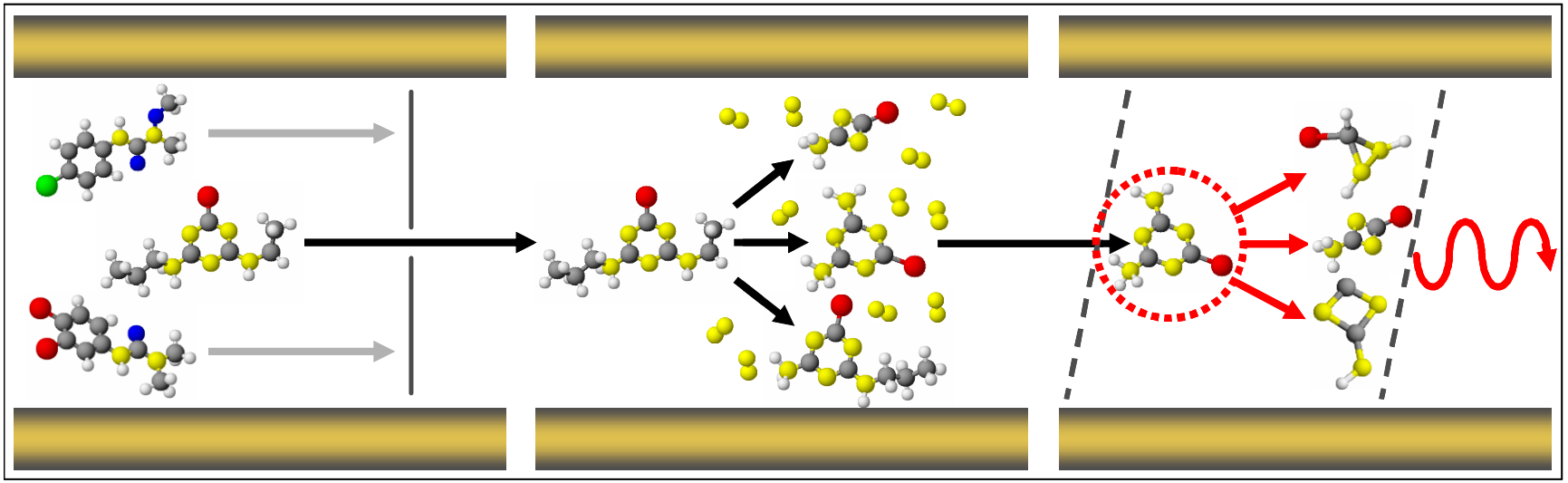


1. Precursor selection in Q1.
2. Collisional activation in the Q2 Qurved LINAC collision cell
3. Trapping of first-generation fragment ions in the Linear Accelerator Trap
4. Isolation of 2nd precursor in the LIT by RF/DC isolation.
5. Collisional activation of 2nd precursor by Single Frequency Excitation
6. 2nd generation fragments are scanned out of the Linear Accelerator Trap
7. During steps 3-6, ions for the next cycle are accumulated in Q0 for enhanced sensitivity (Q0 Trapping).

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MRM^3 Scan for Quantitation

Above and Beyond MRM



Isolation of 2nd precursor ion in LIT

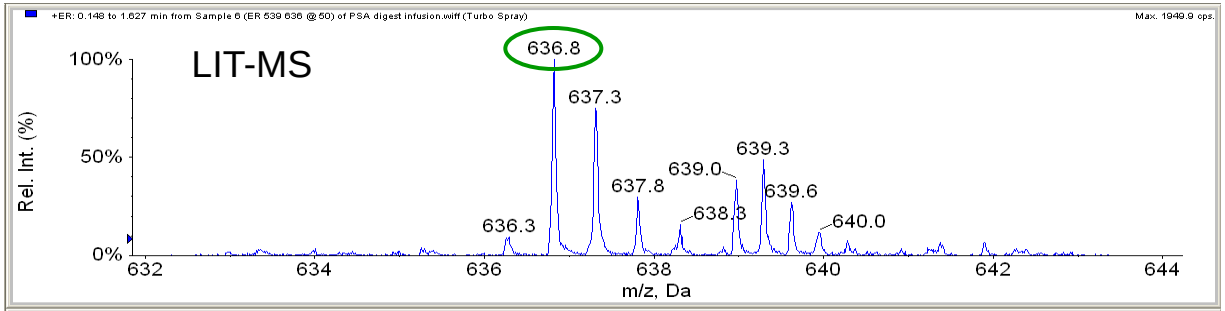
Fragmentation in LIT

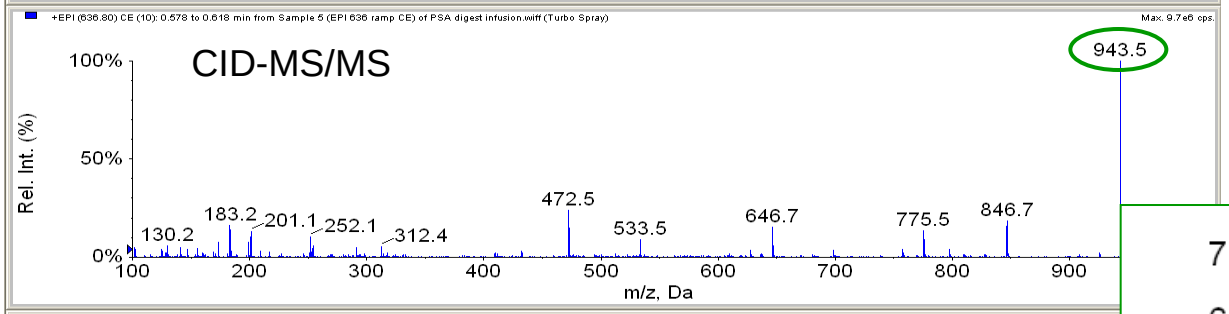
2nd generation of product ions scanned out

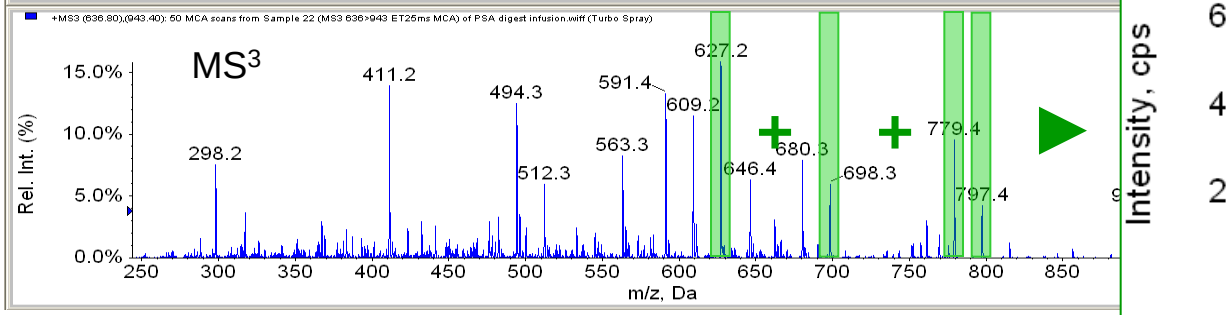
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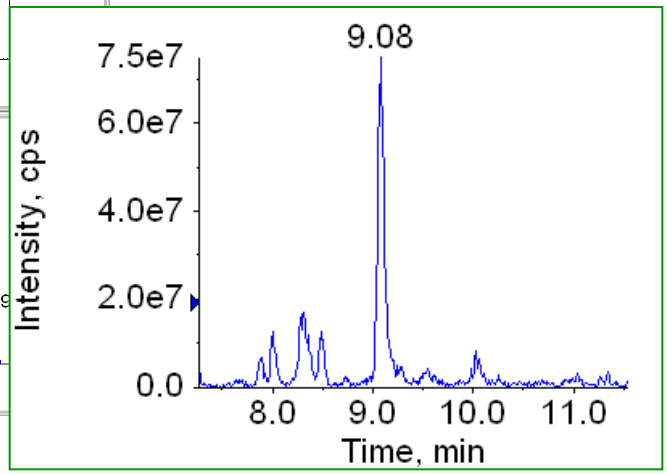
MRM³: Quantitation of PSA in human serum

MRM³ method development on the PSA peptide LSEPAELTDAVK









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MRM^3: Selectivity vs. Normal MRM

Prostate Specific Antigen (PSA) from Human Serum

Anal. Chem. **XXXX,** *xxx,* 000–000

Multiple Reaction Monitoring Cubed for Protein Quantification at the Low Nanogram/Milliliter Level in Nondepleted Human Serum

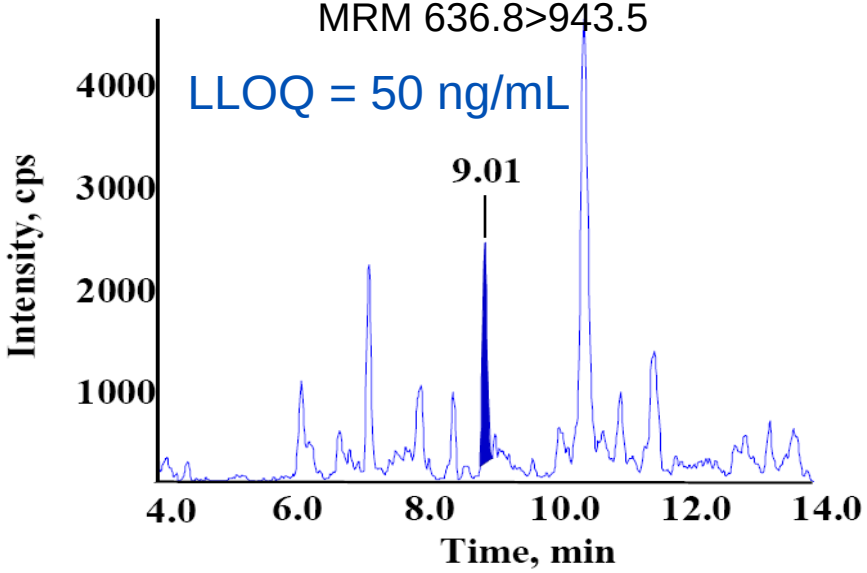
T. Fortin,[†,‡] A. Salvador,[‡] J. P. Charrier,[†] C. Lenz,[§] F. Bettsworth,[†] X. Lacoux,[†] G. Choquet-Kastylevsky,[†] and J. Lemoine*[,‡]

R&D Proteomique, bioMérieux SA, Marcy l'Etoile, France, UMR 5180 Sciences Analytiques, Université de Lyon, Lyon1, France, and PSM Support, Applied Biosystems, Darmstadt, Germany

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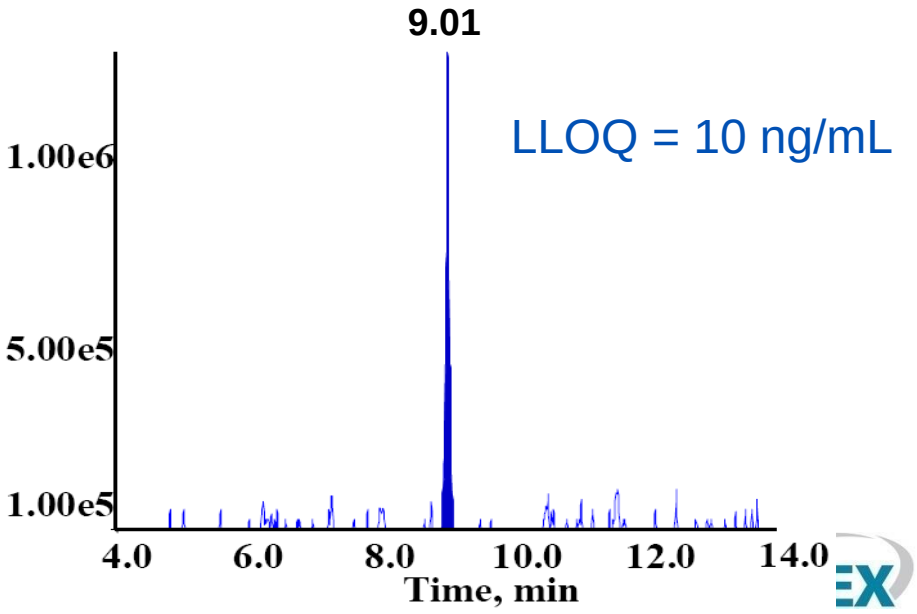
MRM 636.8>943.5

LLOQ = 50 ng/mL



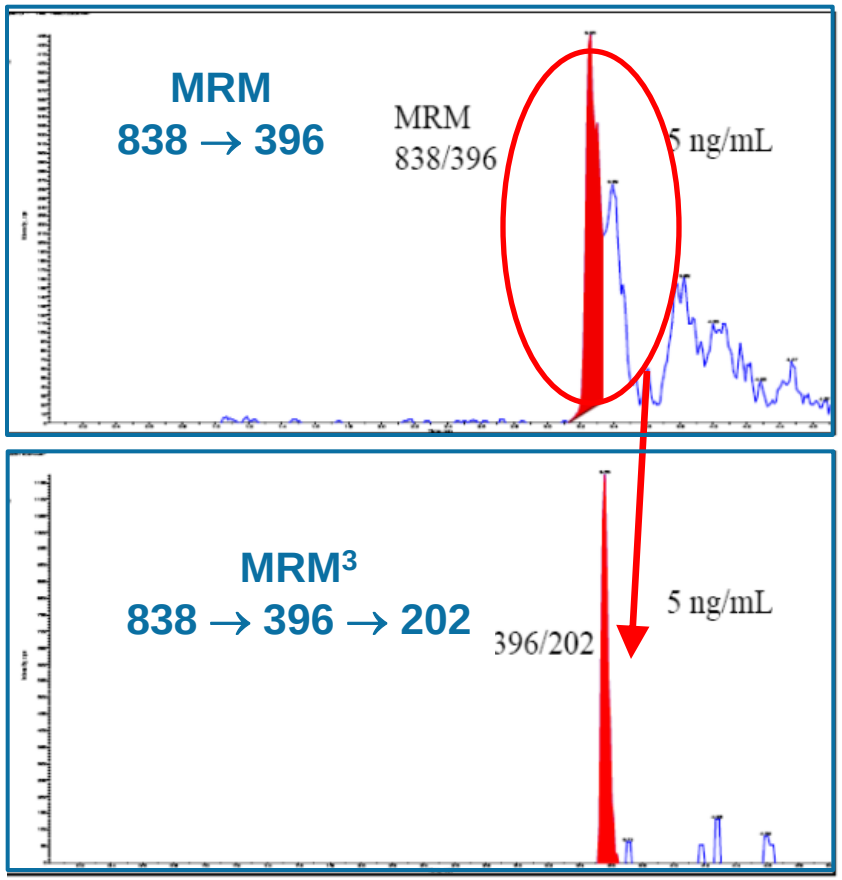
MRM^3 636.8>943.5> (627.3+698.3+779.4+797.4)

LLOQ = 10 ng/mL



Quantitation of Exenatide in Plasma

MRM on API 5000™ System vs. MRM³ on QTRAP® 5500 System



- MRM was not selective enough and suffered from large interferences in plasma
- MRM³ completely eliminated the interferences and improved both the LOQ and the linearity

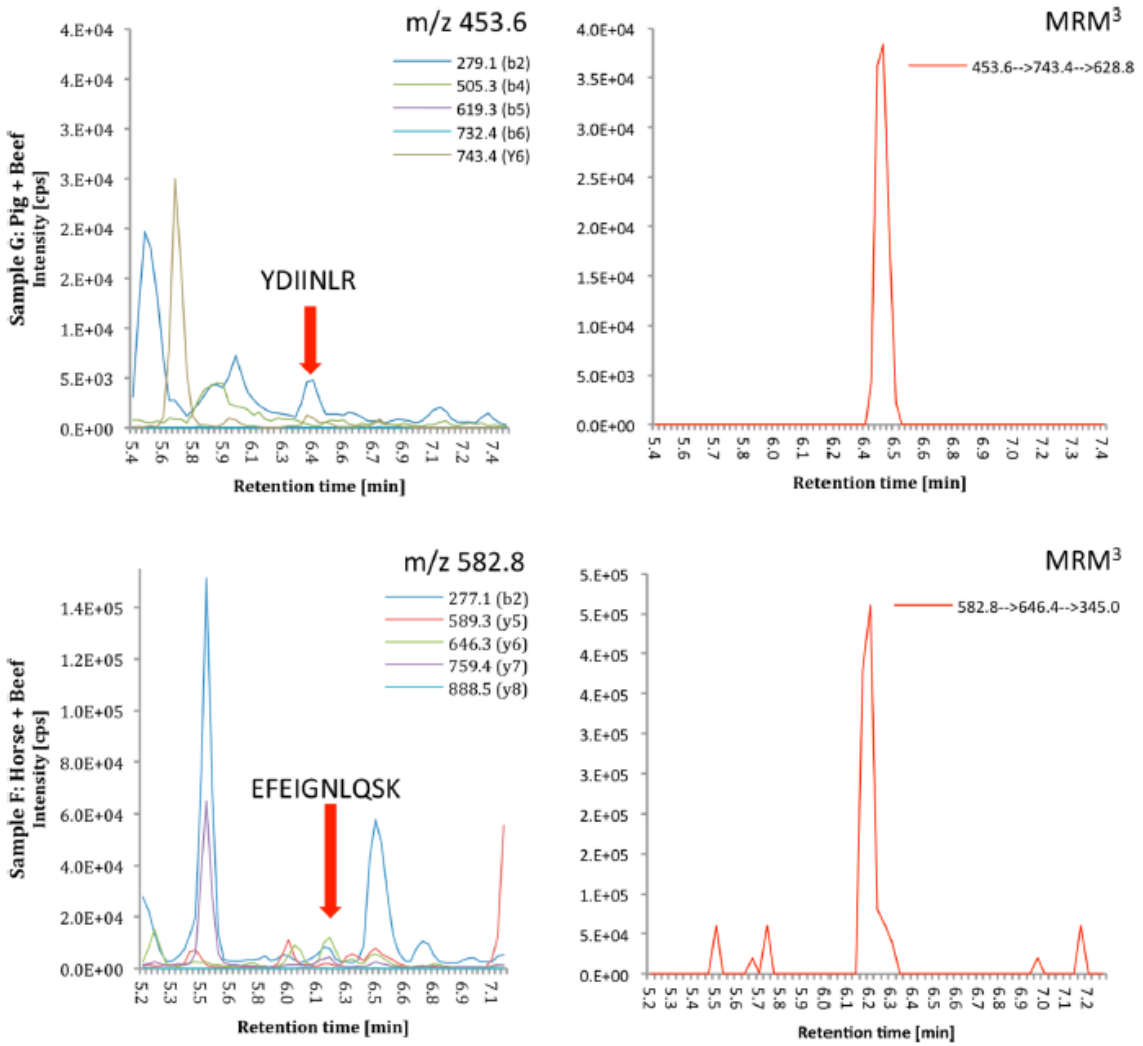
Yan Xu et al, "Quantitation of Exenatide in Human Plasma Using LC-MS/MS/MS on a Linear QTRAP 5500 System" Medpace Bioanalytical Laboratories, APA 2009 Poster

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The effect of moving to using MRM3 in the detection of pork at a concentration of 0.13 % pork spiked into beef

Journal of Agricultural and Food Chemistry Article



© 2017 AB Sci

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Bivalirudin (FPRPG GGGNG DFEEI) catabolism

For Research Use Only. Not for Use In Diagnostic Procedures.

Drug Discovery and Development

SCIEX

Therapeutic Peptide Catabolite Identification using MetabolitePilot™ 2.0 Software and the TripleTOF® 6600 System

Find, Identify and Confirm Peptide Catabolites Using MetabolitePilot™ 2.0 Software and the Advanced Biotransform Solution

Eva Duchoslav[1], Mark Woodward[2], Jinal Patel[1], Suma Ramagiri and Ian Moore[1]
[1]SCIEX, Concord, ON, Canada and [2]Qodon Consulting LLC, Raleigh-Durham, NC, USA

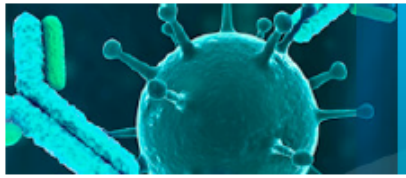
Key Challenges in Peptide Catabolism Studies

- Therapeutic peptides are susceptible to unspecific proteolysis along the peptide backbone and therefore the number of potential catabolites to be considered is much larger than those from a small molecule MetID study



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Peptide quant with ion mobility separation



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Drug Discovery and Development

SCIEX

Rapid Quantitation of Substance P in Plasma Using Differential Mobility Spectrometry and Micro flow Liquid Chromatography

An exceptionally selective and sensitive strategy for high throughput peptide quantification is presented by coupling DMS (Differential Mobility Spectrometry) technology with µLC/MS/MS analysis.

Daniel M. Warren and Sushmit Maitra
AB SCIEX, 500 Old Connecticut Path,
Framingham, MA 01701

Key challenges of multiple-reaction monitoring for peptide quantification

- **Impaired sensitivity in complex matrices** – Very low-level peptide detection (sub-pg/mL) can be suppressed by high background and competing ions in biological samples.
- **Lack of throughput** – Complex biological matrices hamper



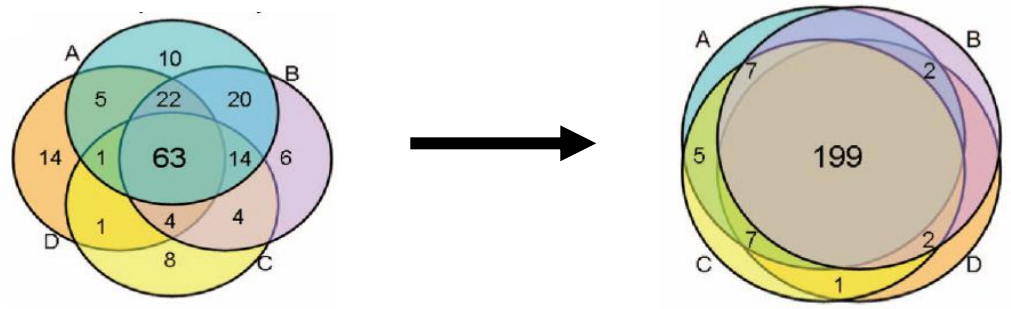
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SCIEX

MRM as a “Mass Western Blot“?

Conceptually, our use of the MRM method to investigate the EGFR signaling network is analogous to performing, in a single 2-h analysis, 206 simultaneous, highly specific, quantitative, reproducible, and high-throughput Western blots with antibodies targeted to specific phosphorylation sites. This technique should significantly improve our ability to accurately quantify signaling networks across a variety of conditions, bringing routine application of phosphoproteomics closer to reality.

Wolf-Yadlin et al., PNAS 104, 5860-5865 (2007)



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감사

ขอบคุณ

متشكرم

Благодаря!

Tak

Tesekkurler

Kiitos

Danke

Dziękuję

Děkuji

Thanks

Niżżik ħajr

Salamat po

Gracias

ありがとう

Спасибо

cảm ơn

Paldies

дєкую

धन्यवाद

Thank you for your attention

谢谢

ধন্যবাদ

Гялайлаа

Tapadh leibh

Hvala

Obrigado

Ačiu

תודה רבה

Köszönöm

Merci

(بهت) شکریه

Mulţumesc

감사

Ευχαριστω

Dank

Tack

Děkuij

Grazie

Þakka þér

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