NARAYANA MEDICAL COLLEGE

Narayana Translational Research & Incubation Center (NTRIC), Conducted International Webinar entitled

Mass Spectrometry-based Proteomics & its Application in Medicine

on Monday, 5th July, 2021 at 3 pm

By

Dr. Srinivasan Yuvaraj, M.Sc., Ph.D.

Project Scientist, Institute of Genome Research,

Tokushima University, Tokushima, Japan.

Patron

Dr. Surya Prakasa Rao, MD
Professor and Dean, Narayana Medical College,
Nellore, Andhra Pradesh, India.

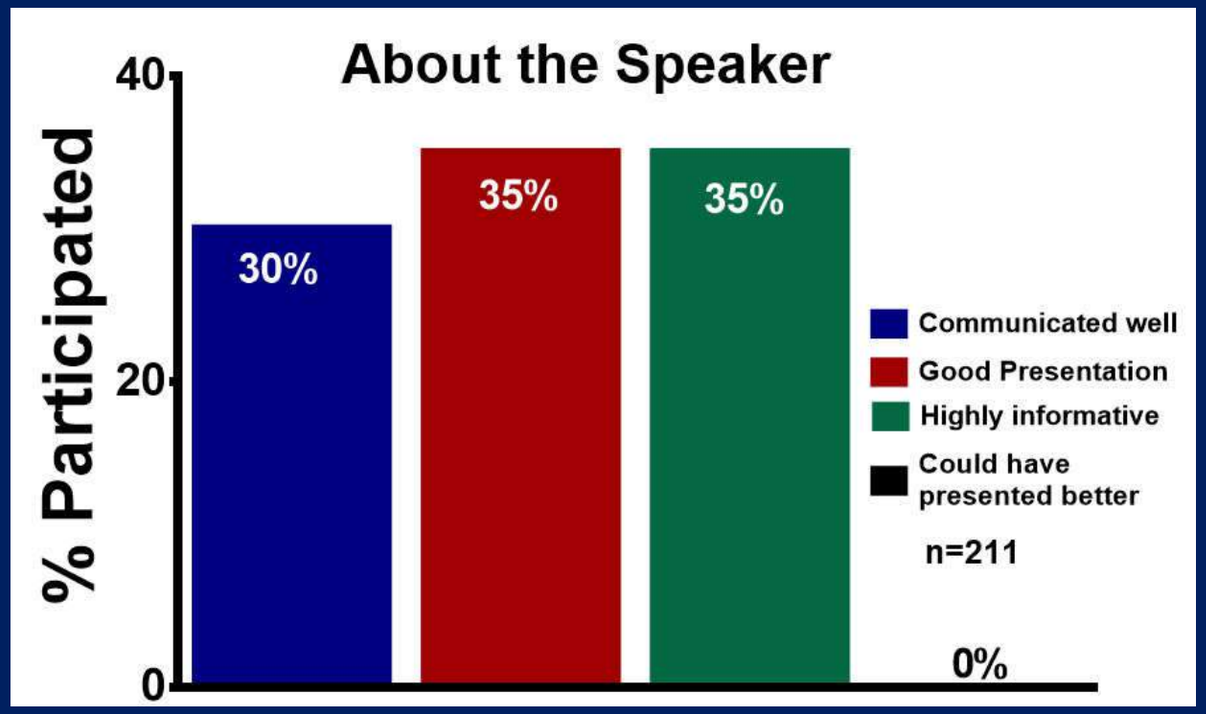
Convenor

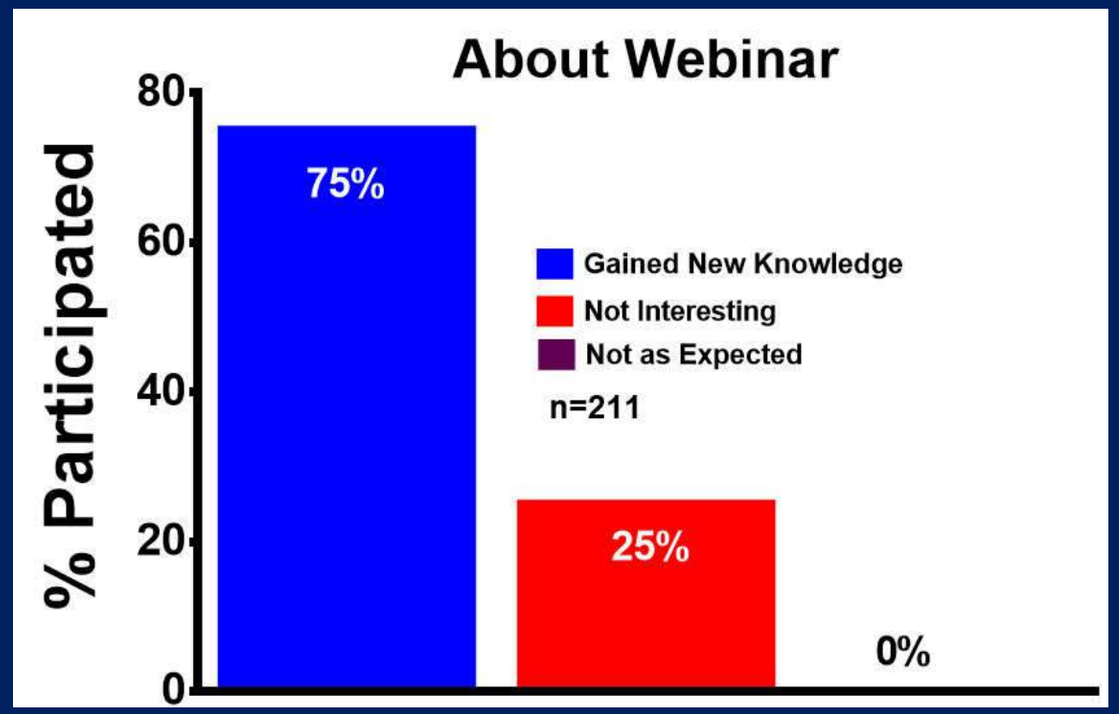
Dr. Sivakumar Vijayaraghavalu, Ph.D.
Professor and Head, NTRIC,
Mobile - 8925744196; Office - 0861-2355511 extn 2406,
E-mail - drvshiva@narayanamedicalcollege.com

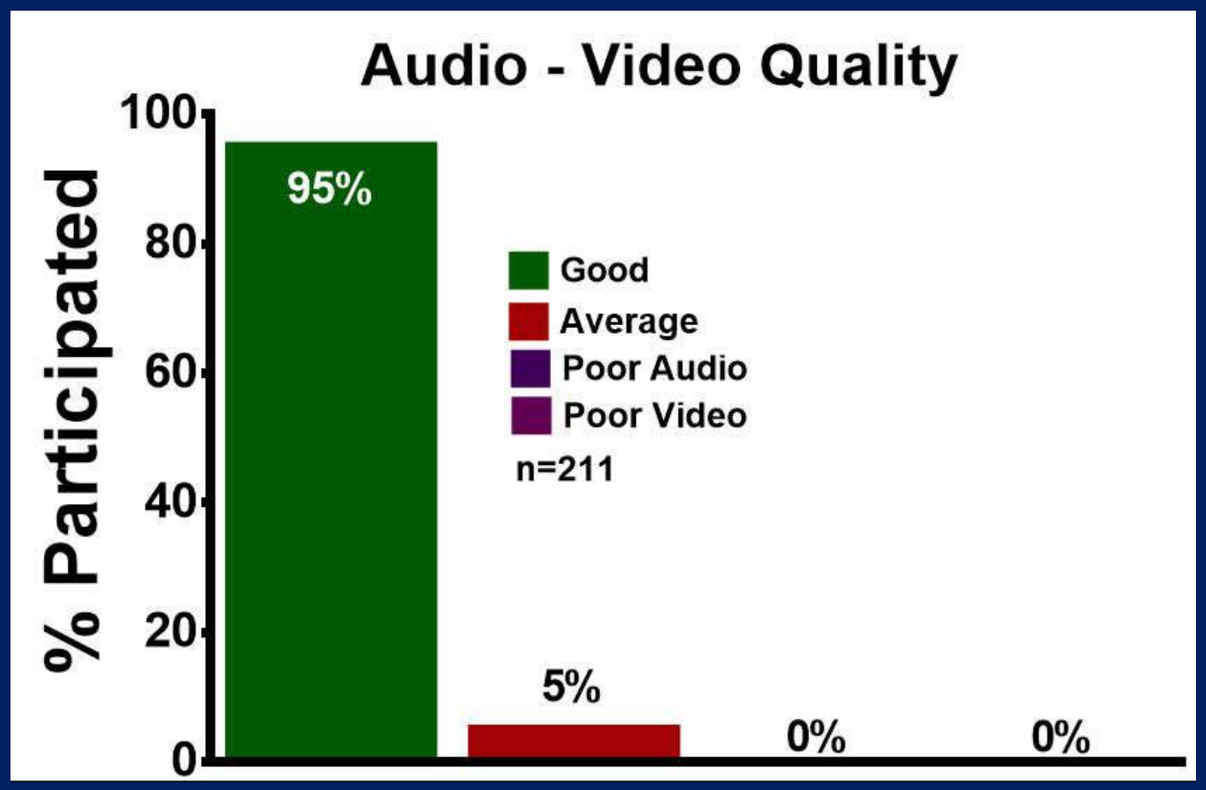
Registrants Profile:

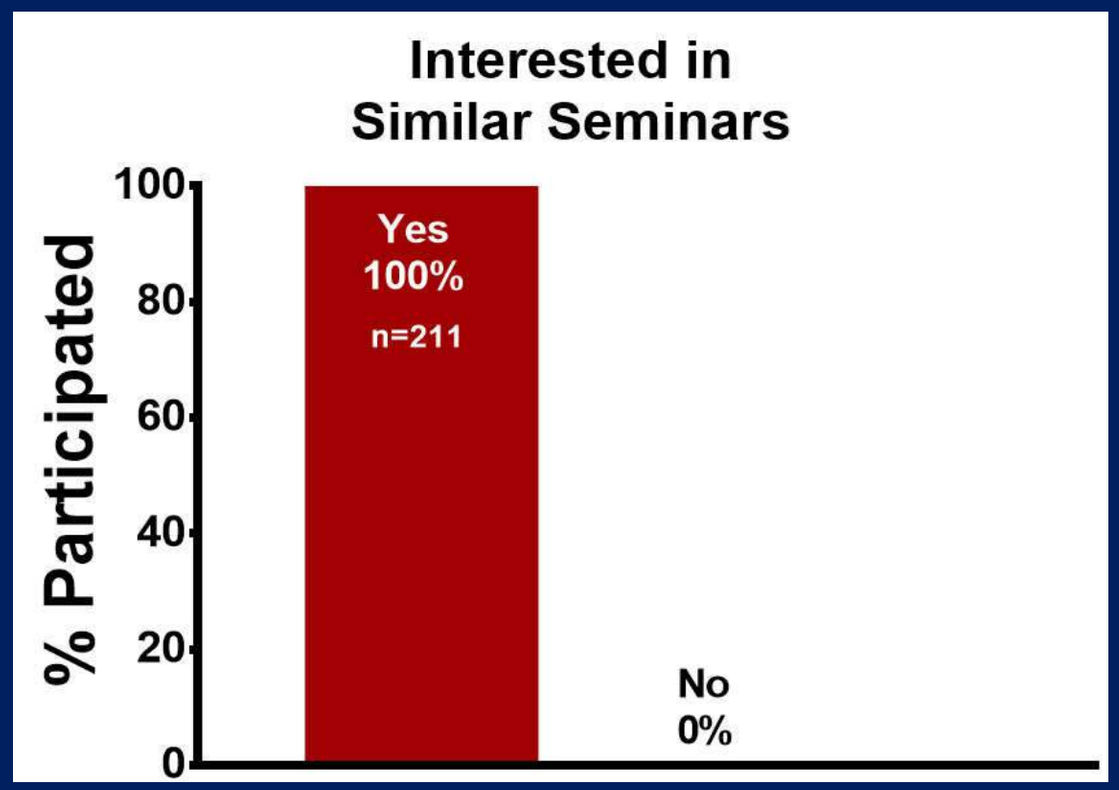
Registrants (211) were from all over the world. Six percent are from foreign countries which includes - Saudi Arabia, USA, Japan, Taiwan, Sweden, and Denmark. Ninety Four percent are from India; among them 63% are from Andhra Pradesh, followed by Tamil Nadu 34%; rest all are from the following states - Telangana, Karnataka, Uttar Pradesh and Kerala. Participants were even from corporate companies like ITC limited; Biocon; Roche Molecular Diagnostics, USA; IBM, USA; Immugenix.

Registrants Poll Survey









The Convenor Dr. Sivakumar Vijayaraghavalu, introduced the speaker to the audience as follows;

Good afternoon and Greetings to one and all, on behalf of our Institution and our honorable Dean, Dr. Surya Prakasaa Rao; I — Dr. Sivakumar Vijayaraghavalu, Professor and Head of Narayana Translational Research Center, with immense pleasure welcome you all to this International Webinar on Applications of proteomics in medicine; by the subject expert,

Dr. Srinivasan Yuvaraj.

He obtained his PhD in Medical Biochemistry from University of Madras in 2008. Then he joined RIKEN, one of the largest research institution in Japan with more than 3,000 researchers working at seven campuses pan across Japan. In that world-renowned institution; he got expertize in the field of proteomics. After completing three years of post-doctoral fellowship;

He came back to our country to establish and lead the proteomics core in ITC (India Tobacco Company), Limited, a corporate giant with turnover of 76,000 crores, even during this pandemic. He worked for 8 years in that company. Due to his passion towards academic research, he then moved back to Japan to join as a project scientist in the institute of Genome Research, Tokushima University in 2019 and continuing his research till now. He is a very versatile researcher; has experience in both academic and corporate sector. I am very honored and gifted to introduce my friend Dr. Yuvaraj.

Proteomics is very young field and is yet to explore in India. Hence, it will be good to hear from an eminent scientist like Dr. Yuvaraj. With this briefing, the convener requested the speaker to take over the session to deliver his talk and informed the audience that the Q & A will be at the end of the talk.

The speaker began his talk by thanking the organization and Dean Dr. SP Rao for giving him an opportunity and also thanked the convener for a nice introduction. He acknowledged his post-doc mentors; Dr. Yusuke Nakamura, University of Tokyo and doctors - H. Nakagawa & K. Ueda in RIKEN (largest research institution in Japan). He thanked them for training him in the field of proteomics. He informed the audience that his post-doc mentor Dr. Yusuke Nakamura is the pioneer in the field. Further he said that proteomics is very young field and it will remain young next two decades and advised youngsters to take proteomics as a career.

He also acknowledged the ITC (India Tobacco Company) Ltd., the company in which he established fully functional proteomics unit from the scratch. He also thanked his current mentor in Tokushima University, Tokushima, Japan for permitting him to deliver this webinar.

Then he defined the proteomics and mass spectrometry in a simple language. He described the lifecycle of proteomics with a good schematic diagram. In his comprehensive talk he also discussed about the establishment and management of proteomics core facility; which includes required plinth area, specifics about the room dimensions, infrastructure, instruments and man power. He emphasized the collaborative efforts of Biologists, Analytical chemists, Physicists, Bio-statisticians and Bioinformaticians role in development of proetomics as a field and scientific projects involving proteomics. He described about the commonly used methodologies in this multi-disciplinary field. Explained in detail about the 2D-gel electrophoresis and trypsin digestion of proteins, HPLC, ionization and sample analysis by Mass – spectrometer. He also discussed about the data pattern and how to interpret it.

He further explained about different types of mass analyzers - such as Scanning and Ion beam mass spectrometers (TOF & Q); Trapping mass spectrometers (IT and Orbitrap); Whole protein mass analysis (time of flight (TOF) MS, or Fourier Transform Ion Cyclotron Resonance (FT-ICR) etc. He spoke about analyzing the isotopic labelled and label free samples. As well familiarized the audience about the proteomics algorithm software/ proteomics database. He comprehensively explained the entire process involved in proteomics, citing a project that resulted in discovery of tumor markers (BIG3/PHB2). Another example described was about finding a serum biomarker in the prostate cancer using proteomic approach. He also explained the challenges involved in serum proteomics. At the end of his talk; he answered the questions of the audience satisfactorily. The talk was then concluded with a vote of thanks by the Convener. The PPTs of Dr. Yuvaraj is in the following pages

Webinar title –

MASS SPECTROMETRY BASED PROTEOMICS

&

ITS APPLICATION IN MEDICINE

PPT s of Dr. Yuvaraj Srinivasan's presentation

Conflict of Interest

- **No direct conflict of interest.**
- **Have a small family business - (Saravana Clinical Laboratory / S-Finomics Financial Consultancy in Chennai).**
- **Investment / Shares -> Have investments in Listed Company in Indian Markets in Hospital, Pharma, Education / other Sectors (But not significant or > 0.1 % of Company holding)**

Acknowledgements

2008-2011

Dr. Yusuke Nakamura

2011-2019

2019- till now

Dr. H. Nakagawa

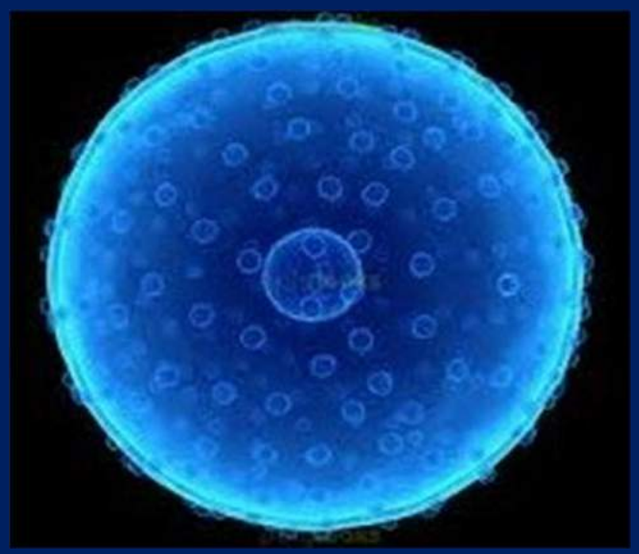
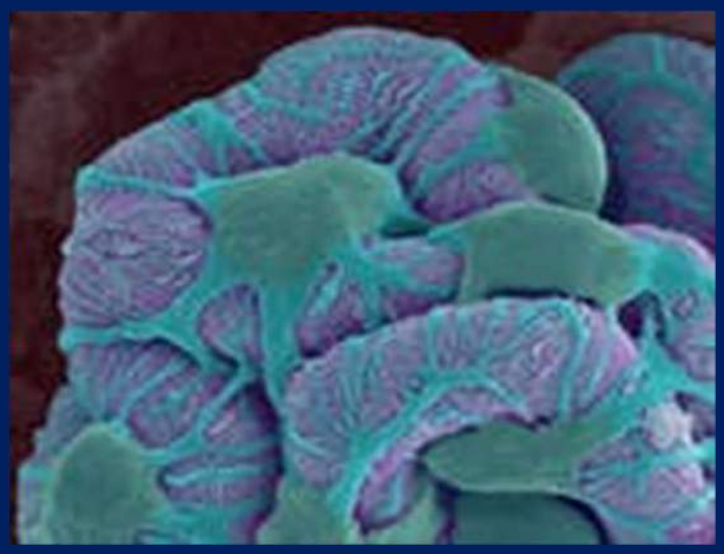
Dr. K. Ueda

MASS SPECTROMETRY BASED PROTEOMICS & ITS APPLICATION IN MEDICINE

- **OVERVIEW**
  - Proteomics & Mass Specs (MS)
  - Life Cycle of a Proteomics Project
  - Proteomics Core Facility -> Management
  - Different technologies to deal with different challenges
  - Clinical Proteomics Project (with Examples)

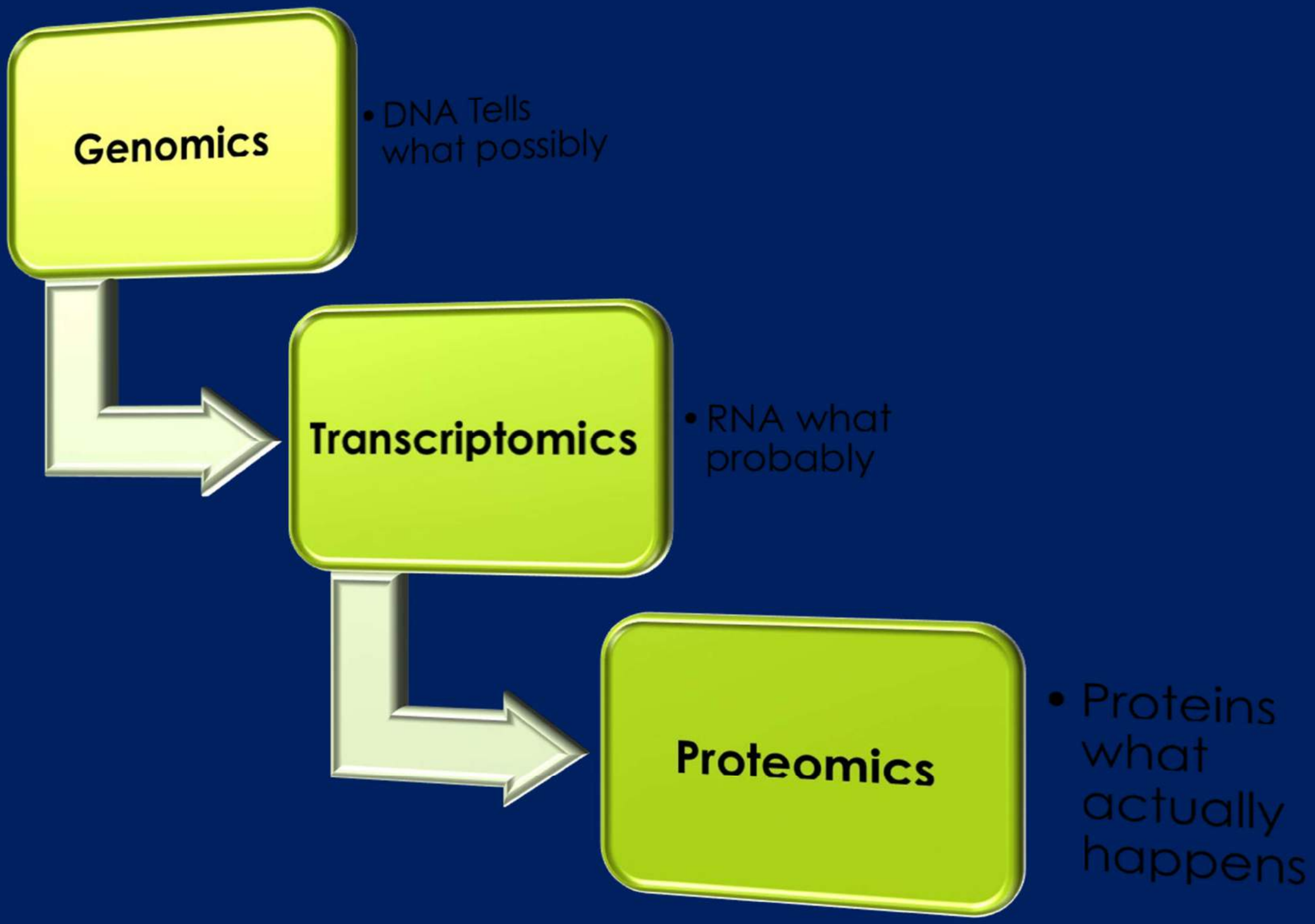
Proteome

- Entire set of proteins expressed by
  - Genome
  - Cell
  - Tissue
  - Organism


> 400.000 proteins, dynamic

Why proteomics? (1/2)



Why proteomics? (2/2)

- **Protein alterations cannot be fully deduced from DNA.**
- RNA expression does not always reflect protein levels:
  - Translational control
  - Degradation turnover
- Some tissues not suitable for RNA expression analysis.

- **Proteins are the physiological/pathological active key players.**
- General goal:
  - Better understanding of genesis and progression of diseases
- **Clinical goals:**

  Early disease detection (biomarkers)

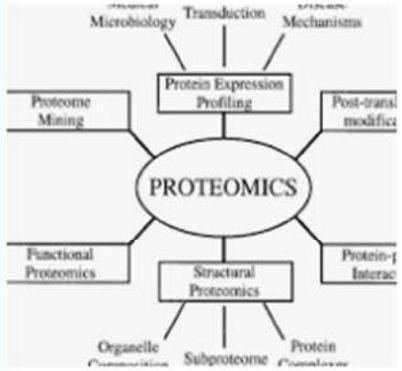
  Identification of therapeutic targets

  Therapy monitoring

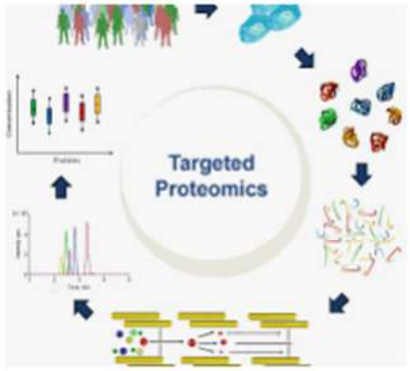
Mass Spectrometry Based Proteomics eg. Biomarker Discovery

A Pictorial Definition

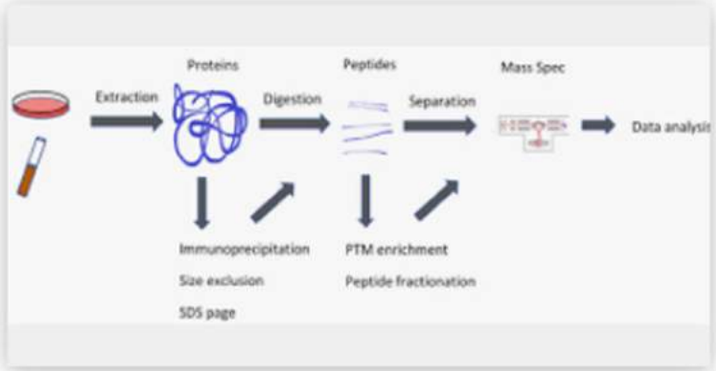
mass spectrometry | genomics | metabolomics | workflow | bioinformatics



Types of proteomics and their ...
researchgate.net

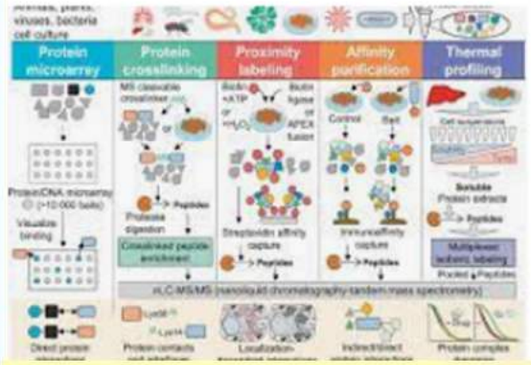


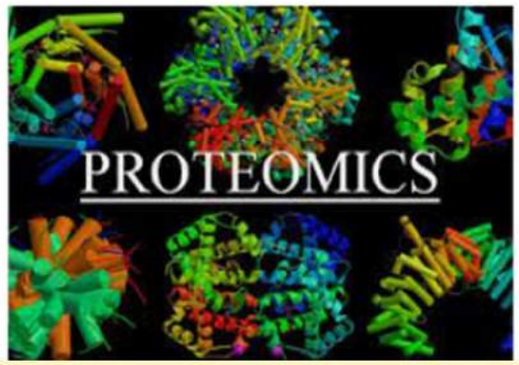
Targeted Proteomics | SpringerLink
link.springer.com

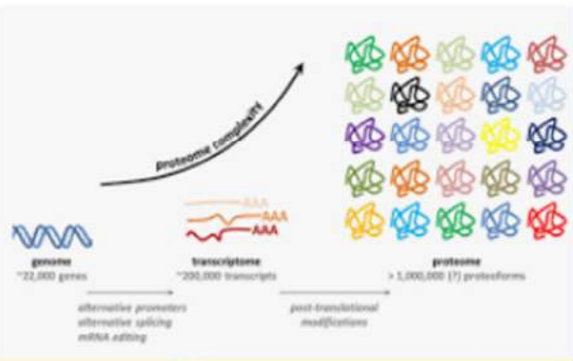


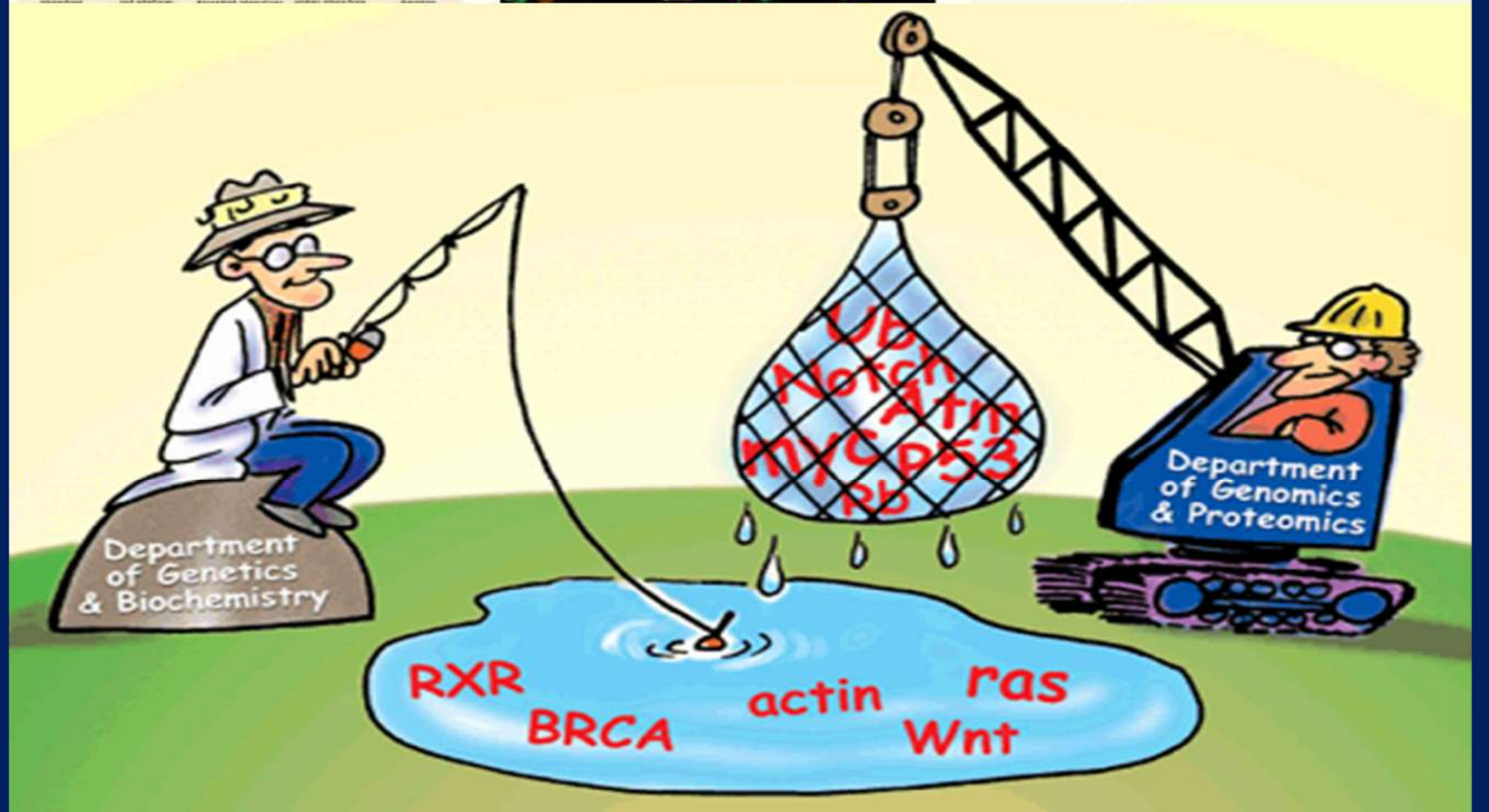
Introduction to Proteomics | USF Health
health.usf.edu

Subcel
nature.









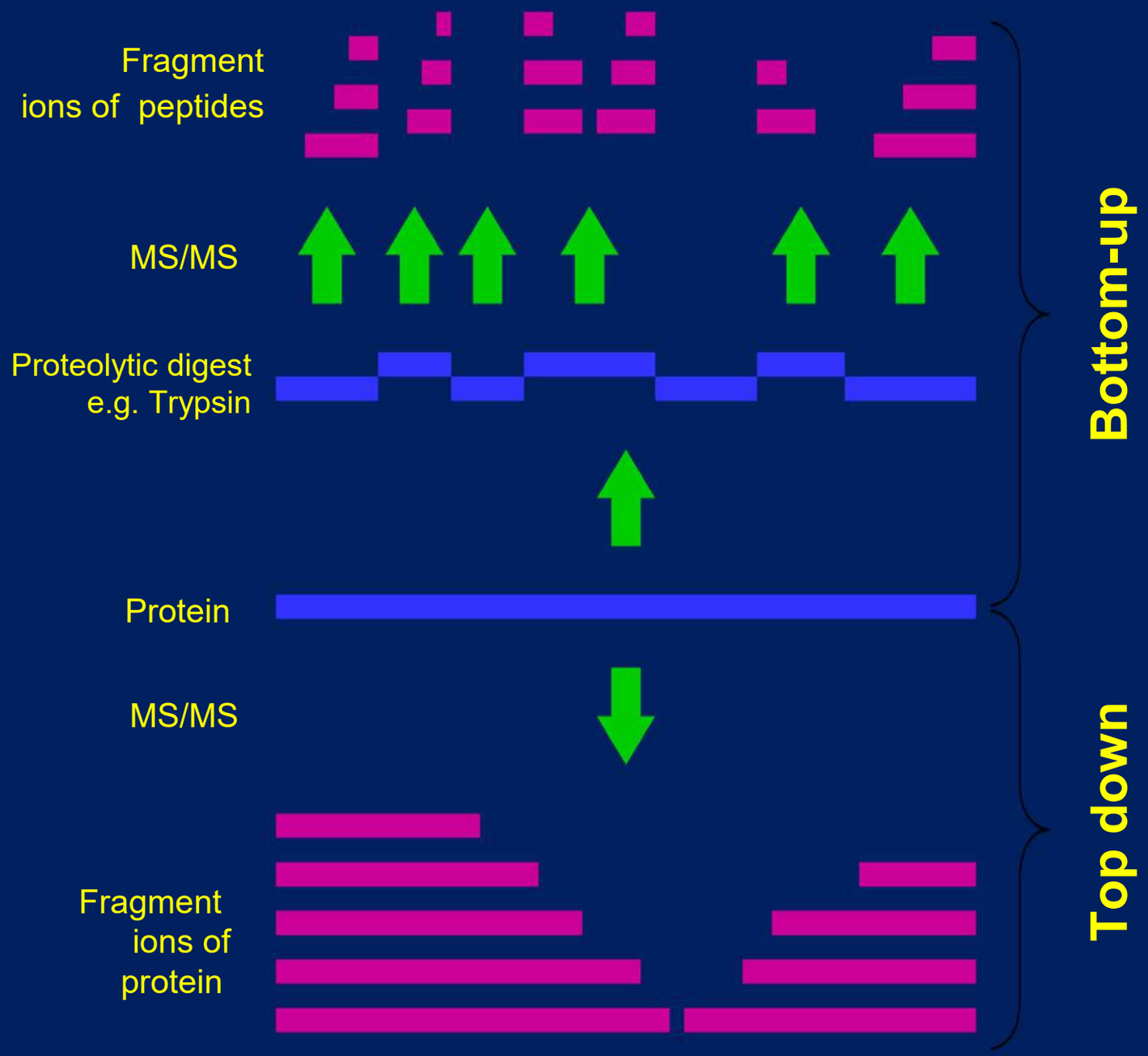
Top down or bottom up?

Bottom-up

- Most common
- Starting with proteolytic fragments
- Piecing the protein back together
- de novo repeat detection

Top down

- Tandem MS of whole protein ions
- Pulling them apart
- Electron capture dissociation
- Extensive sequence information



"Protein mass spectrometry" *Wikipedia, The Free Encyclopedia*. Wikimedia Foundation, Inc.

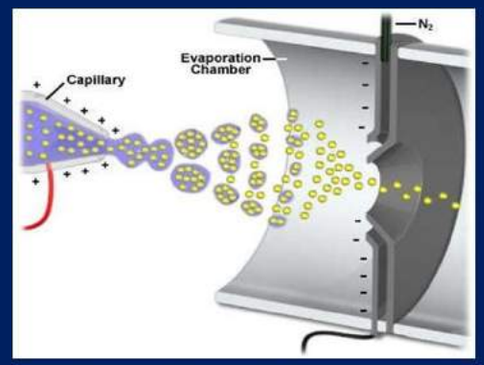
Multidisciplinary

Sample preparation

Cells, tissue

Separation

HPLC

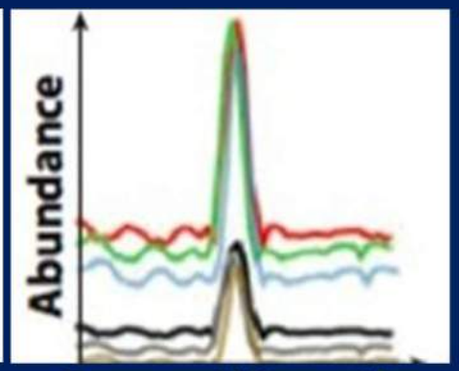


Ionisation

MALDI, ESI

Identification

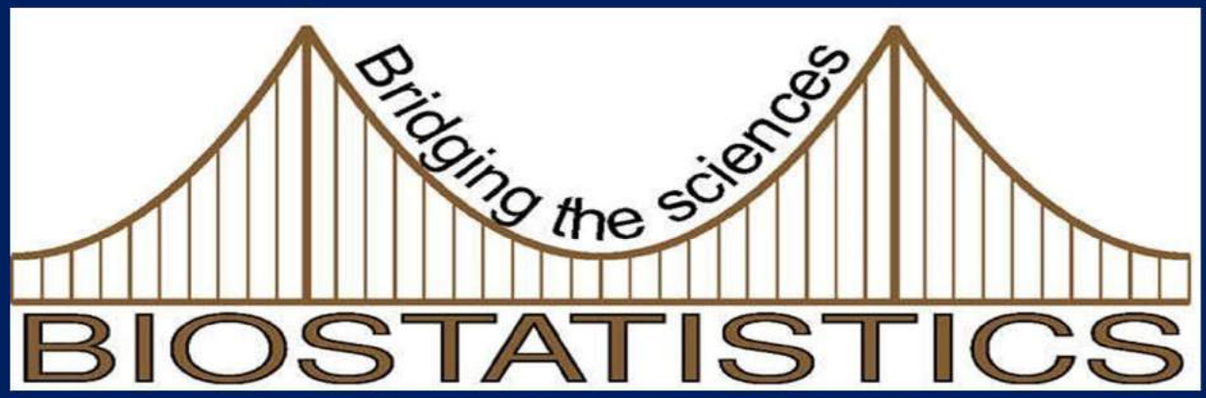
TOF, Q, IT



Quantification

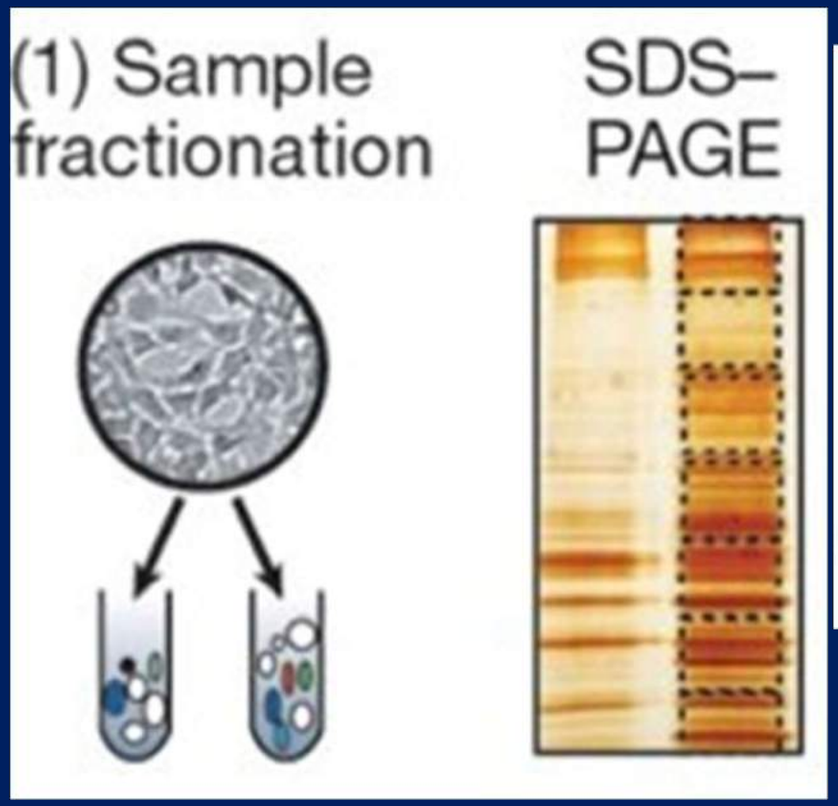
Algorithms

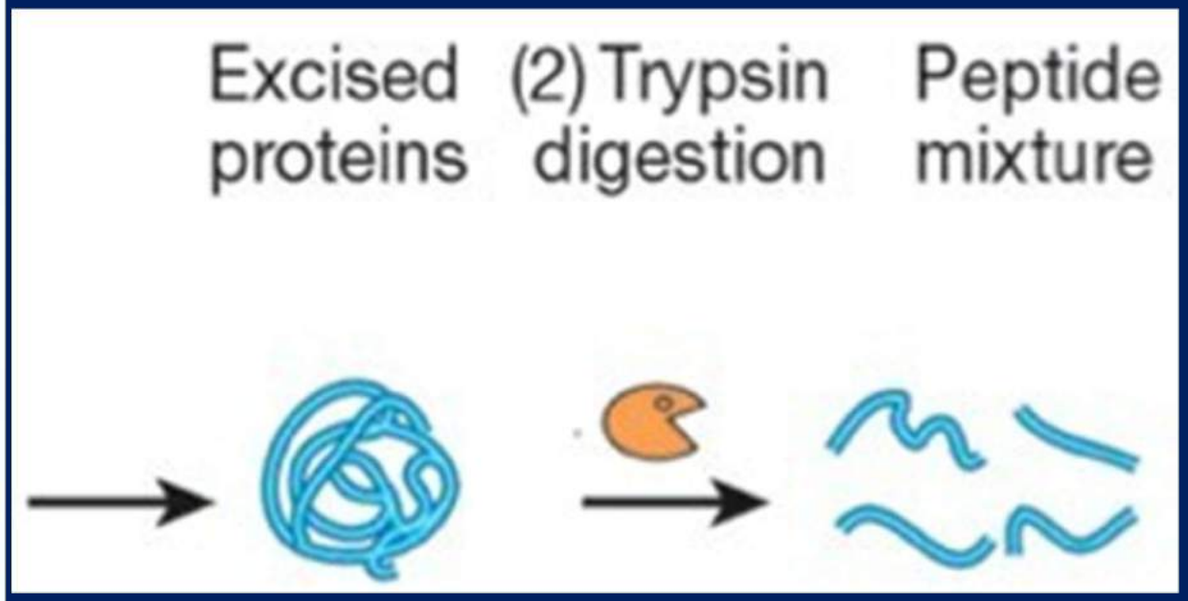
Bioanalytics

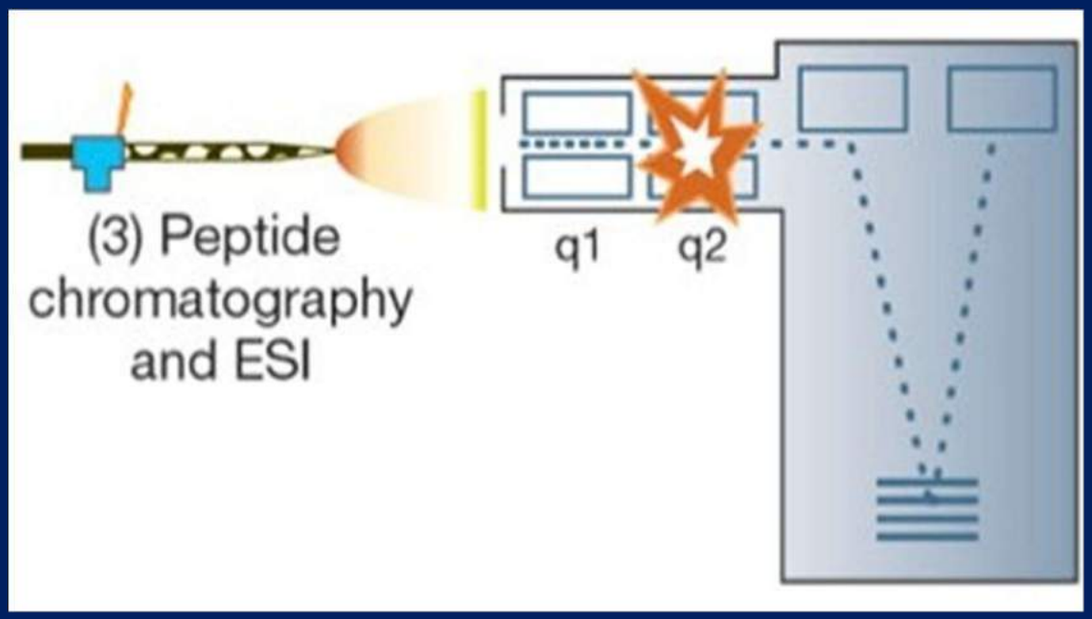


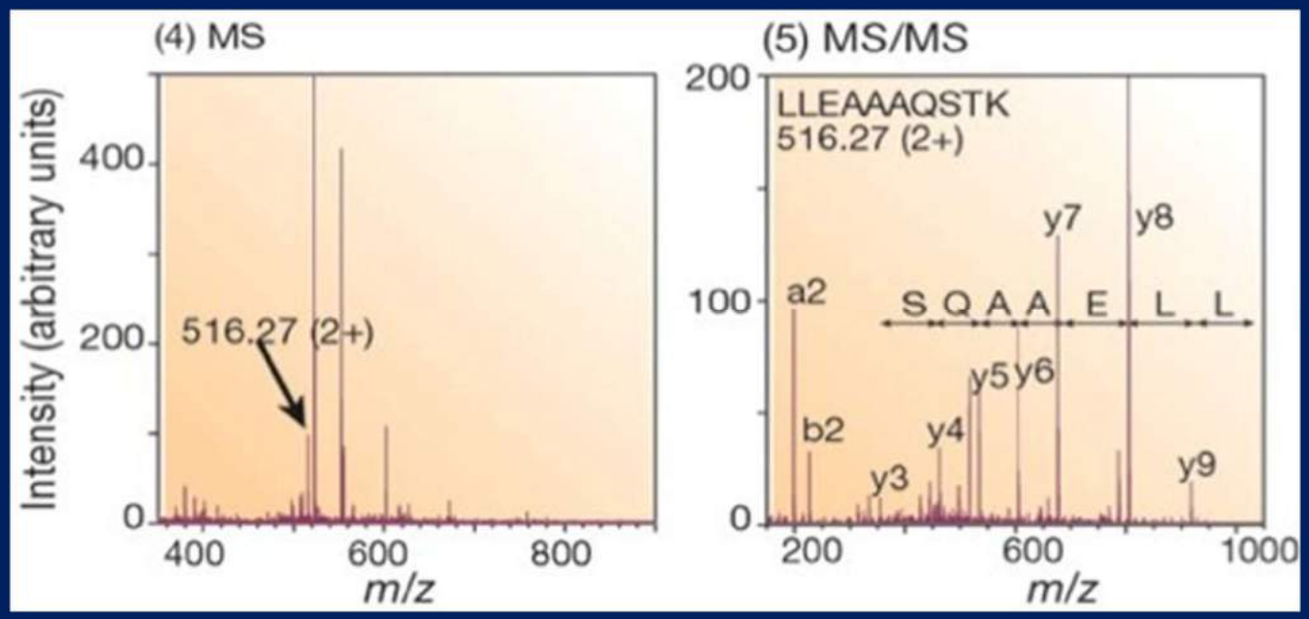
Bioinformatics

Typical stages - MS-Based Proteomics



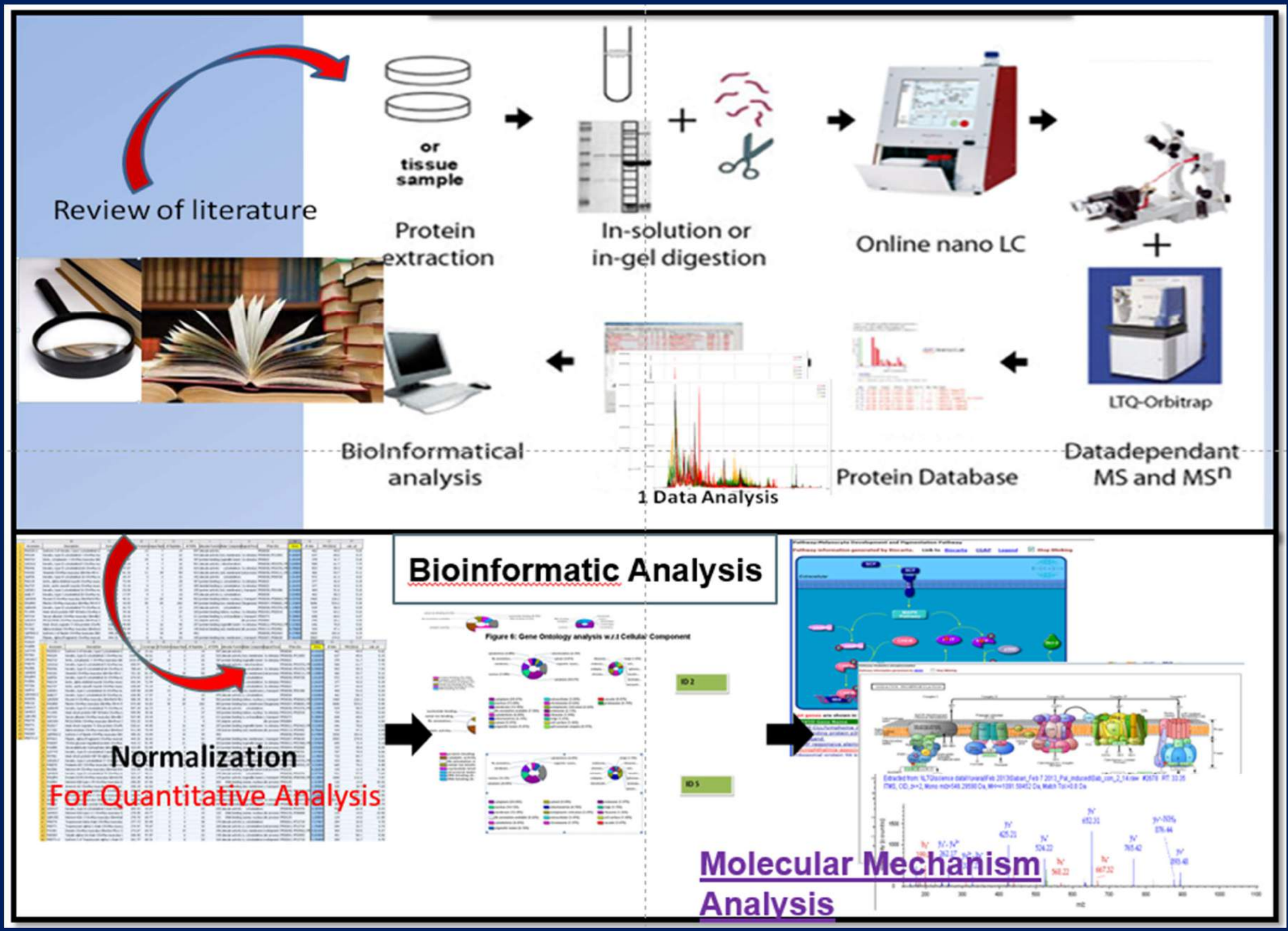




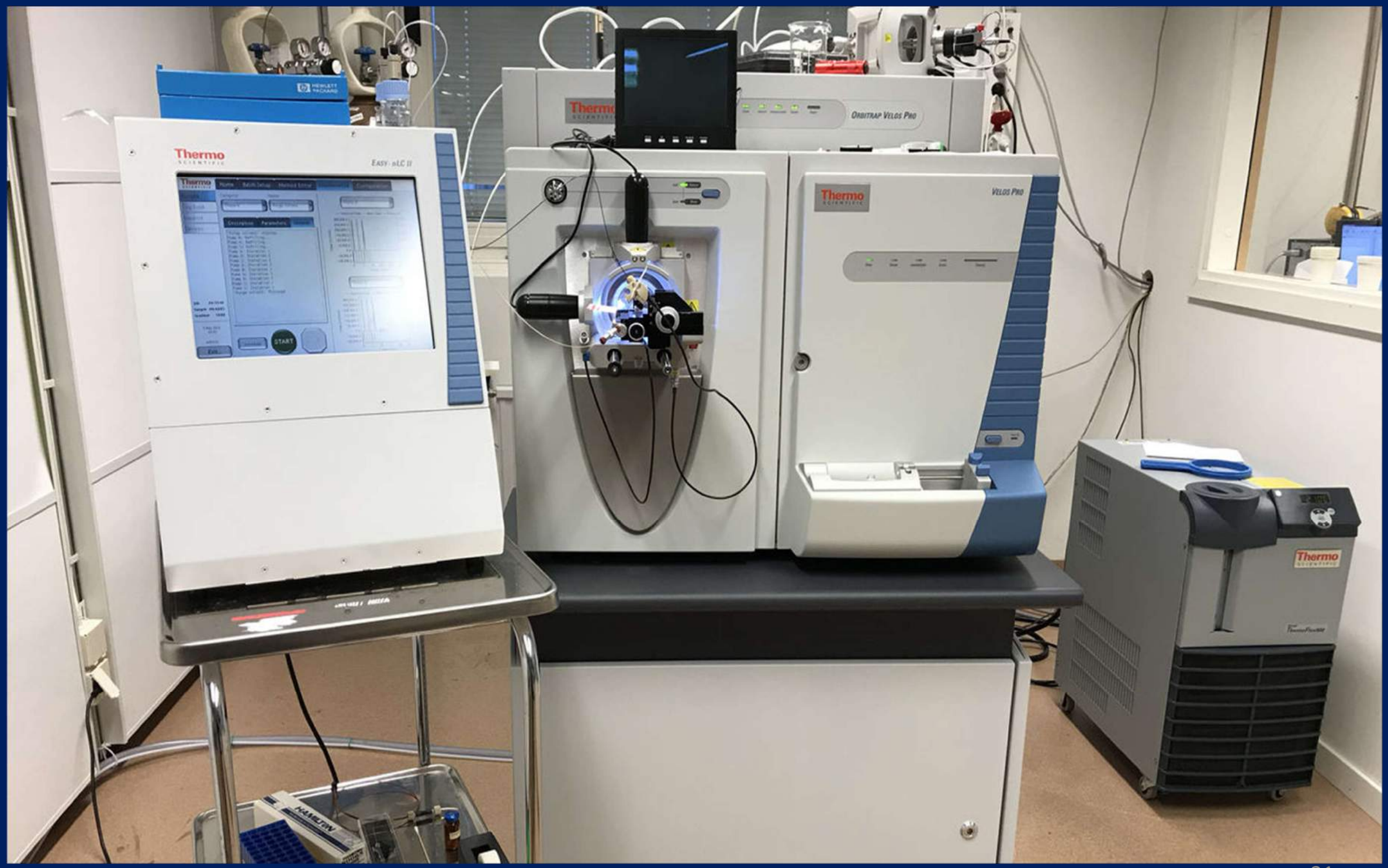


Aebersold R, Mann M, Nature. 2003

Broad Life Cycle of a Proteomics Project

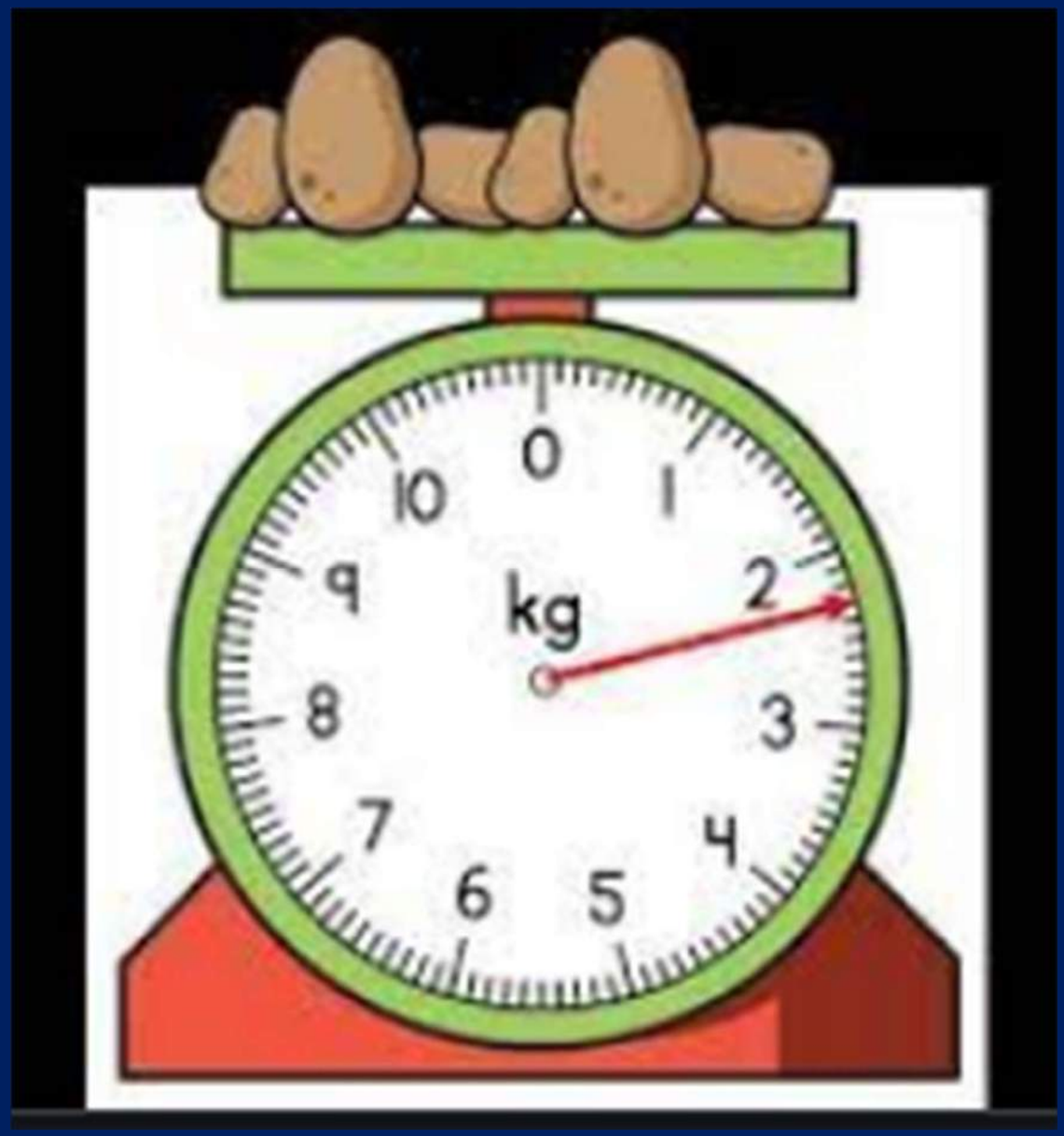


Proteomics Facility Set-up



Representative Image – Source -> Internet

A Simplified Visualization Mass Spectrometer



Flow / Aim of Doing an Omics Project

Data Driven Project

~ > 50 % is Biomarker Discovery

~ 30 % Lead Compound Molecular Mechanism

Proteomics / Genomics Studies
List of Genes / Protein ids With Fold Change Value
→ Pick the Best Biomarker from the list

Incubation and Translation *of Biomarker*
- **Functional Validation**

Validated Protein / Gene → Molecular Mechanism
Take Proteomics Help
- Interacting Protein to Biomarker

Molecular Mech. Cont.

Functional Studies

Working with existing pathways / Novel Pathway

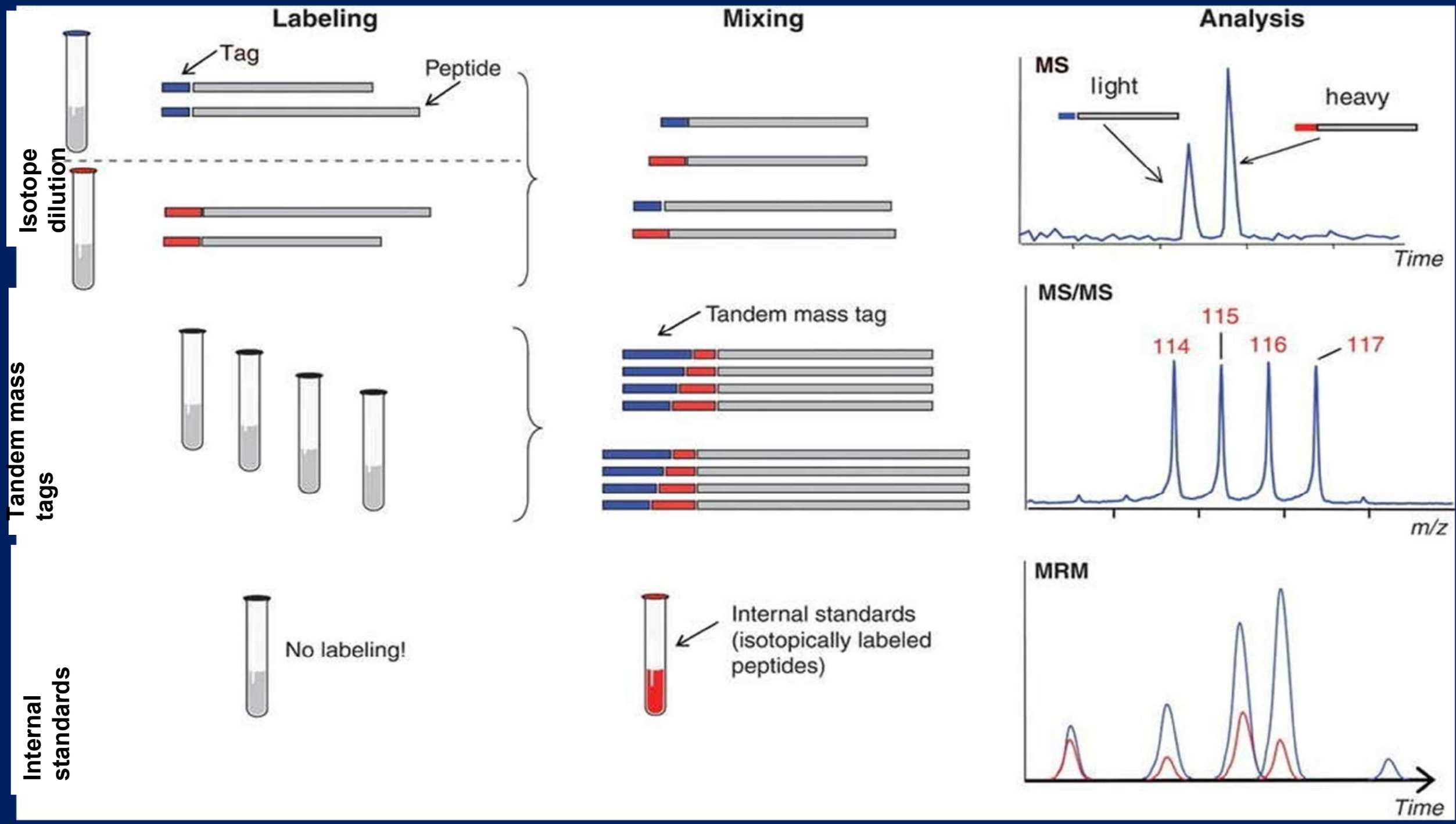
Targeted Therapy

Novel / existing Drug → Target the Biomarker-interacting protein / Pathway → Screening

Drug-Biomarker Molecular Mechanism

Take Proteomics Help if needed / Other Functional Studies

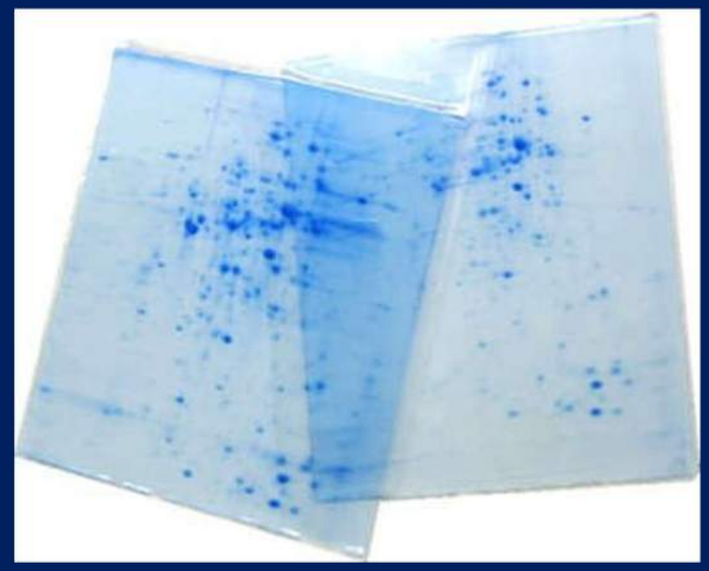
Quantification strategies



Domon B, Aebersold R. Science. 2006

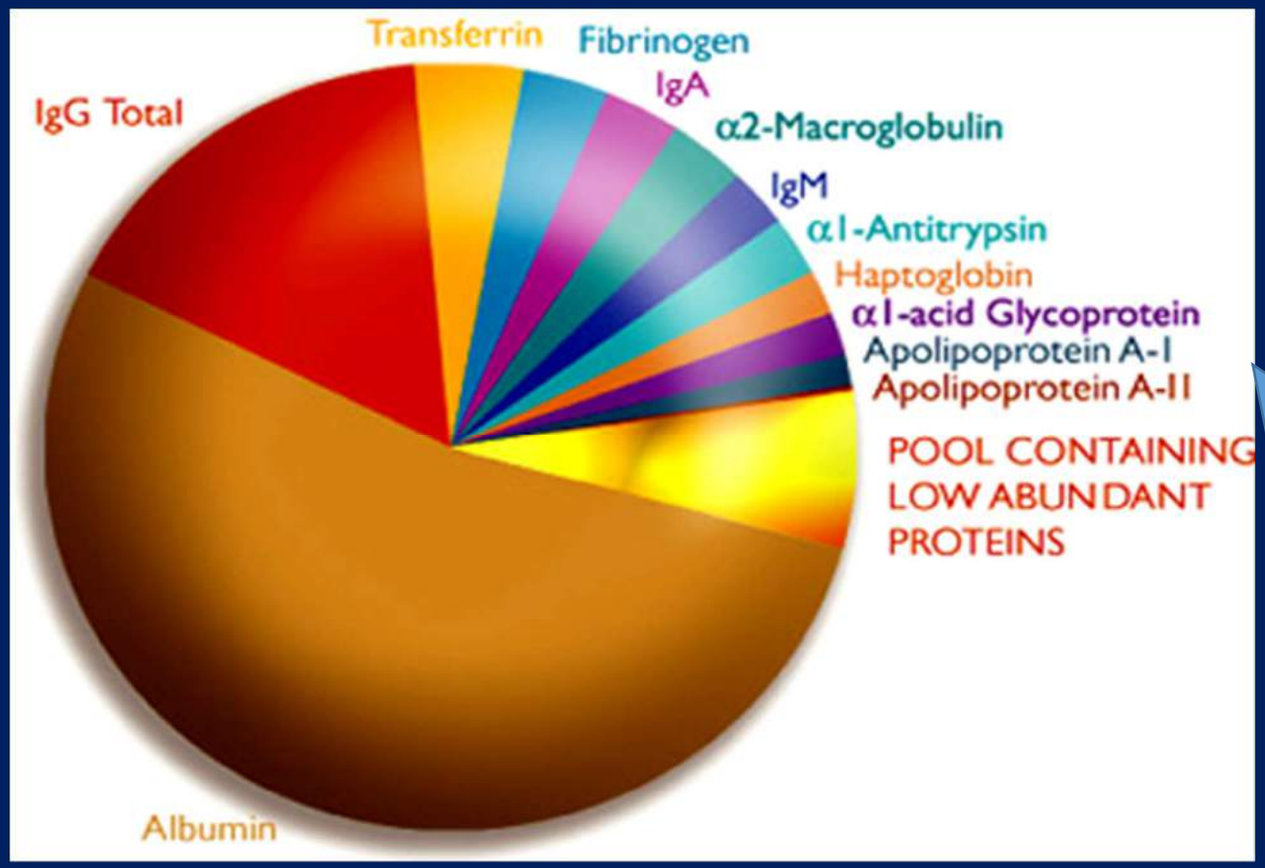
Protein Sample Preparation Strategies / Separation

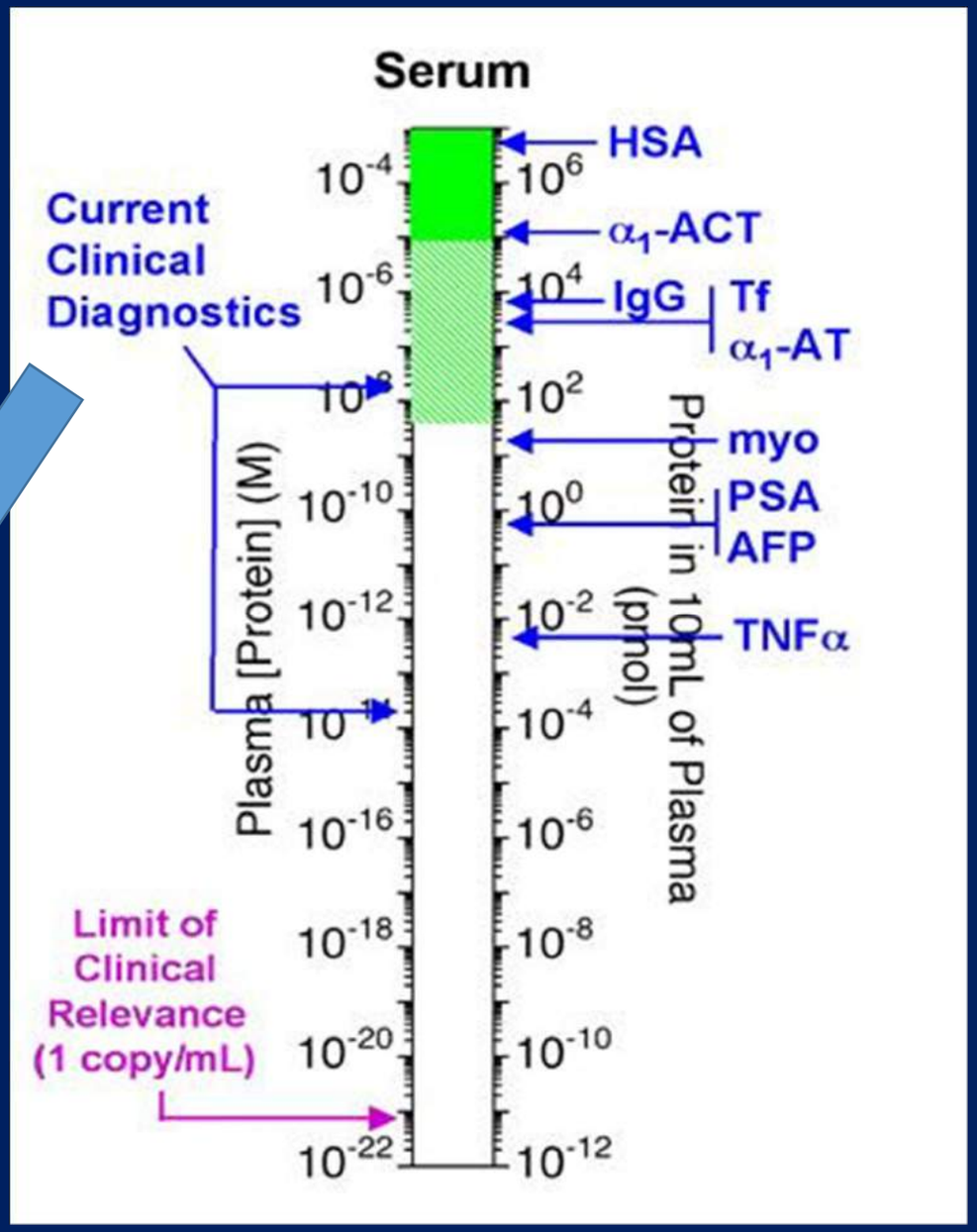
- Specific protein biophysical parameters
  - Isoelectric point
  - Molecular weight
  - Affinity
- Chromatographic methods
  - HPLC
  - 2D-HPLC
  - ProteinChips
- Electrophoretic methods
  - SDS-PAGE
  - 2-D E
- Reverse phase (RPLC) – Hydrophobicity



Yates JR, et al. Annu Rev Biomed Eng. 2009

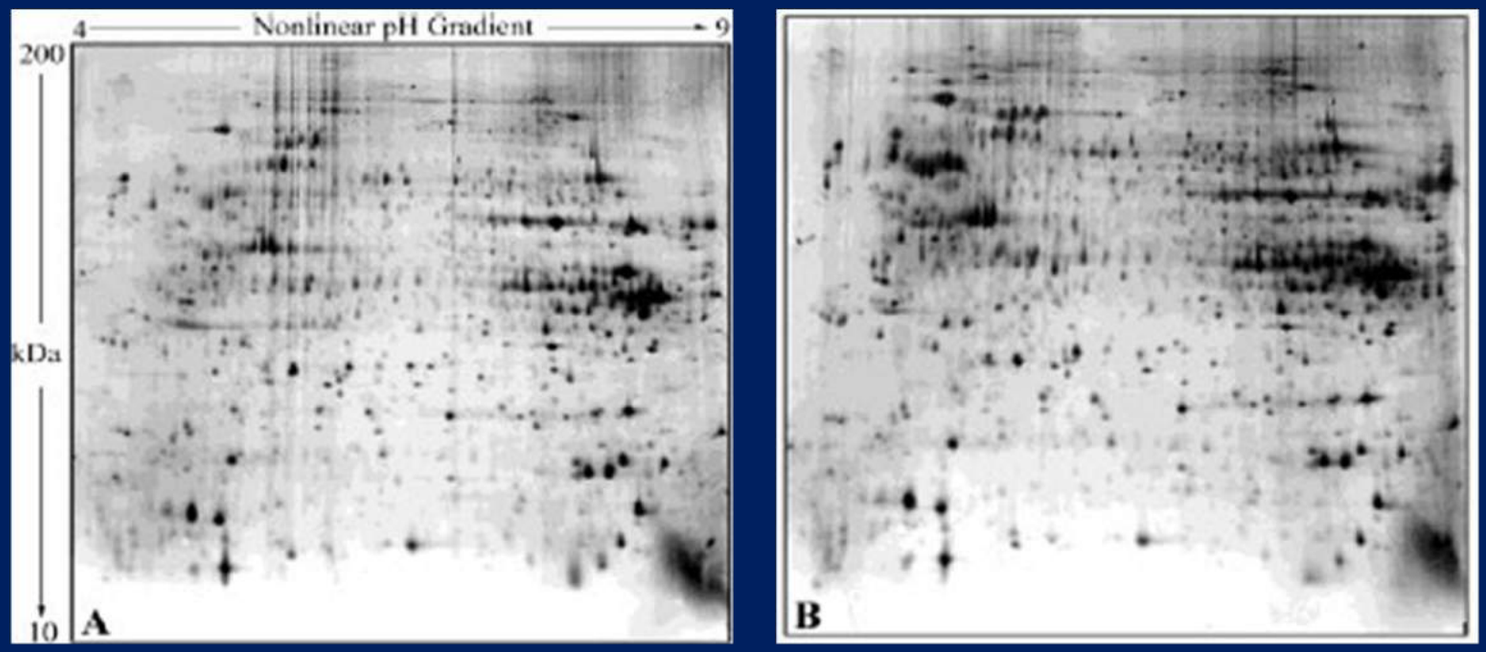
Protein as a Sample / SERUM as a Sample and its Complexity





2D Gel electrophoresis (History)

- 1D: isoelectric focussing (IEF) separation by IP
- 2D: dimension: SDS-PAGE separation by MW
  - staining > 1000 proteins /gel
- molecular analysis by
  - MS
  - HPLC
  - Westernblot
- Pitfalls
  - very basic / acidic;
  - large / small;
  - hydrophobic;
  - low-abundance proteins

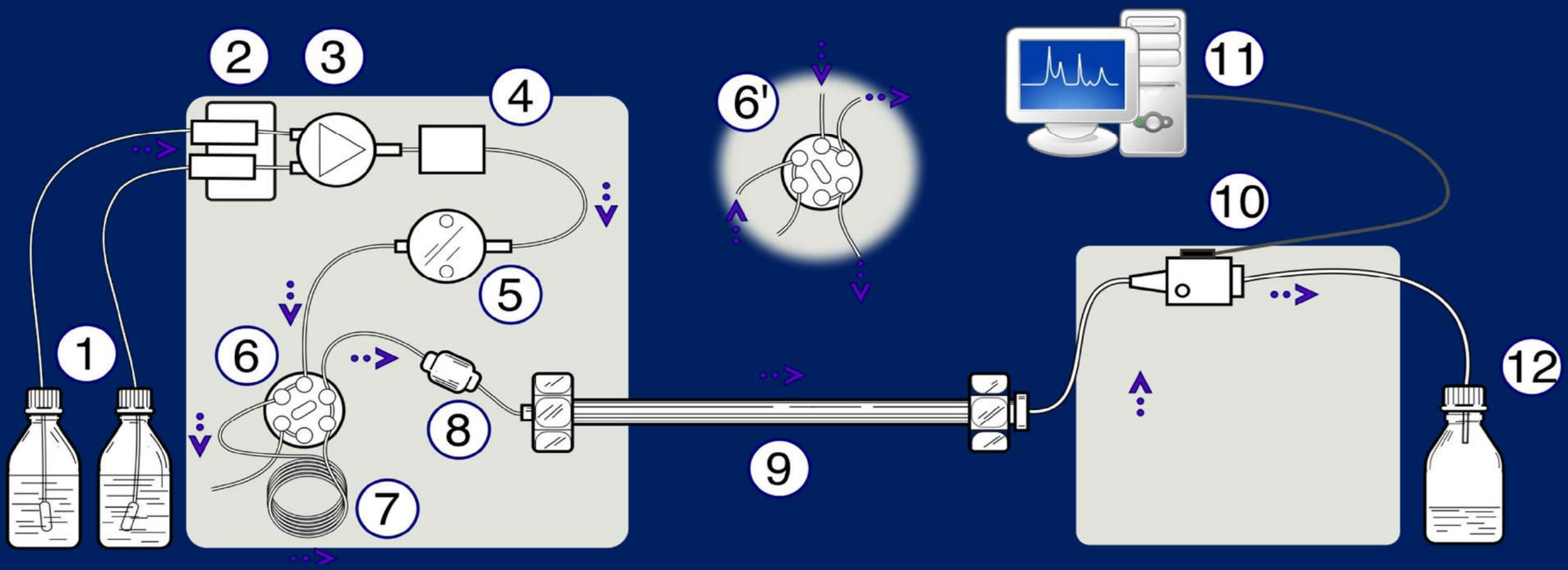


Fontana et al. Proteomics 2004

Robotic Isolation

”Protein mass spectrometry" *Wikipedia, The Free Encyclopedia*. Wikimedia Foundation, Inc.

Separation -> HPLC



”HPLC" *Wikipedia, The Free Encyclopedia*. Wikimedia Foundation, Inc.

Separation -> HPLC

- HPLC/MS
- Peptides from protein mixture fractionated in steps
- Eluent
  - ESI-MS
  - MALDI: series

Representative Image – Source -> Internet ”HPLC" *Wikipedia, The Free Encyclopedia*. Wikimedia Foundation, Inc.

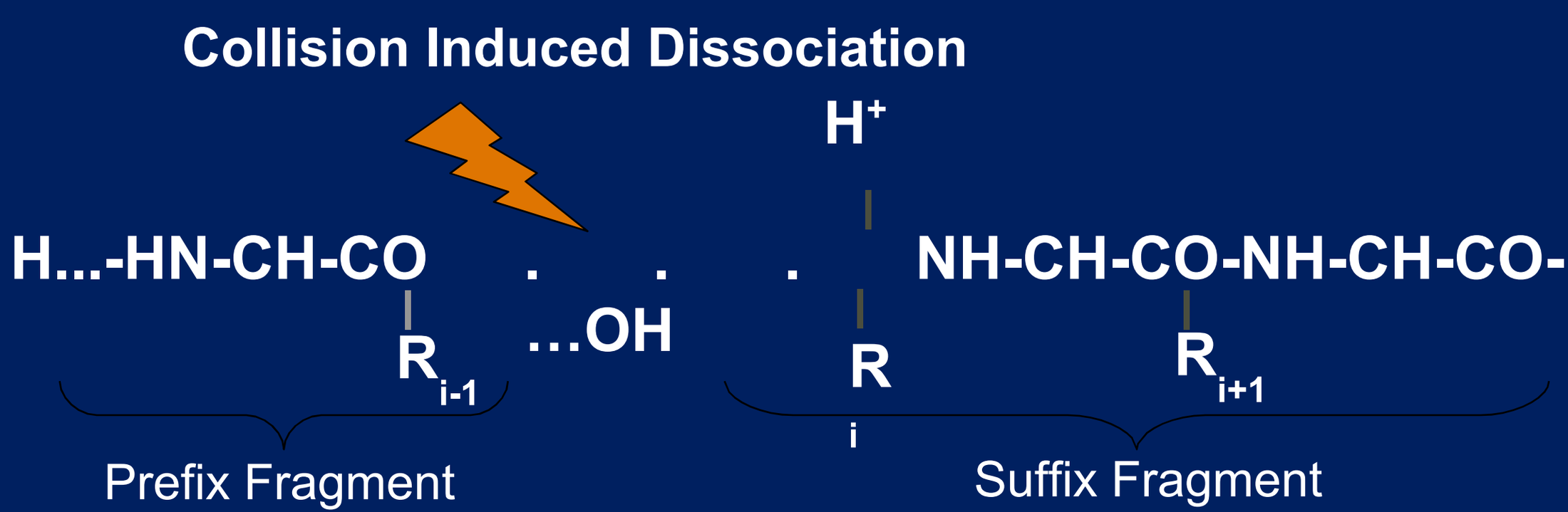
Protein & peptide fractionation

- Complex mixtures
  - Proteins
  - Molecules
- Works only if mixture has equal amounts
  - **Abundant species suppress signals from less abundant ones**
  - Difficult to interpret
  - Enyzmatic digestion -> many peptide products
- 2-D electrophoresis
  - Protein fractionation
- High performance liquid chromatography
  - Peptide fractionation

”Protein mass spectrometry" *Wikipedia, The Free Encyclopedia*. Wikimedia Foundation, Inc.

Proteins Peptides Fragments

- Proteases, e.g. trypsin, break protein into peptides
- Tandem MS breaks peptides into fragment ions
  - Measures the mass of each piece.
  - MS accelerates the fragmented ions;
  - heavier ions accelerate slower than lighter ones
  - MS measure mass/charge ratio of an ion

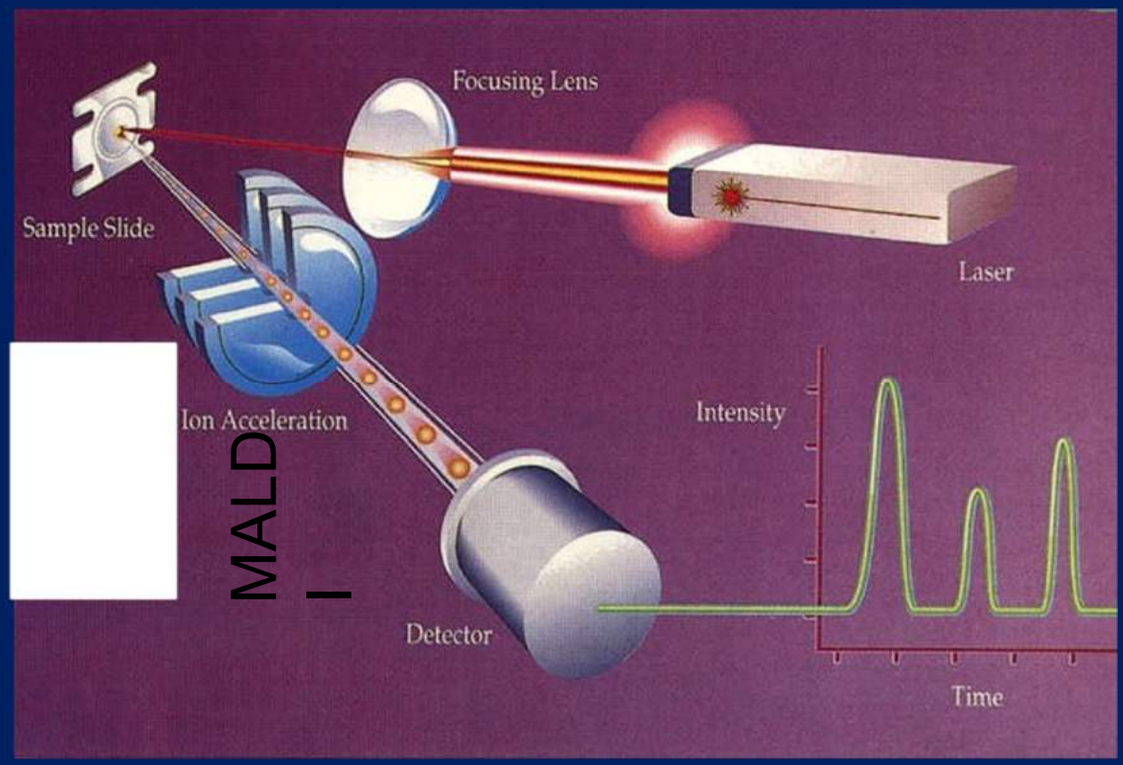


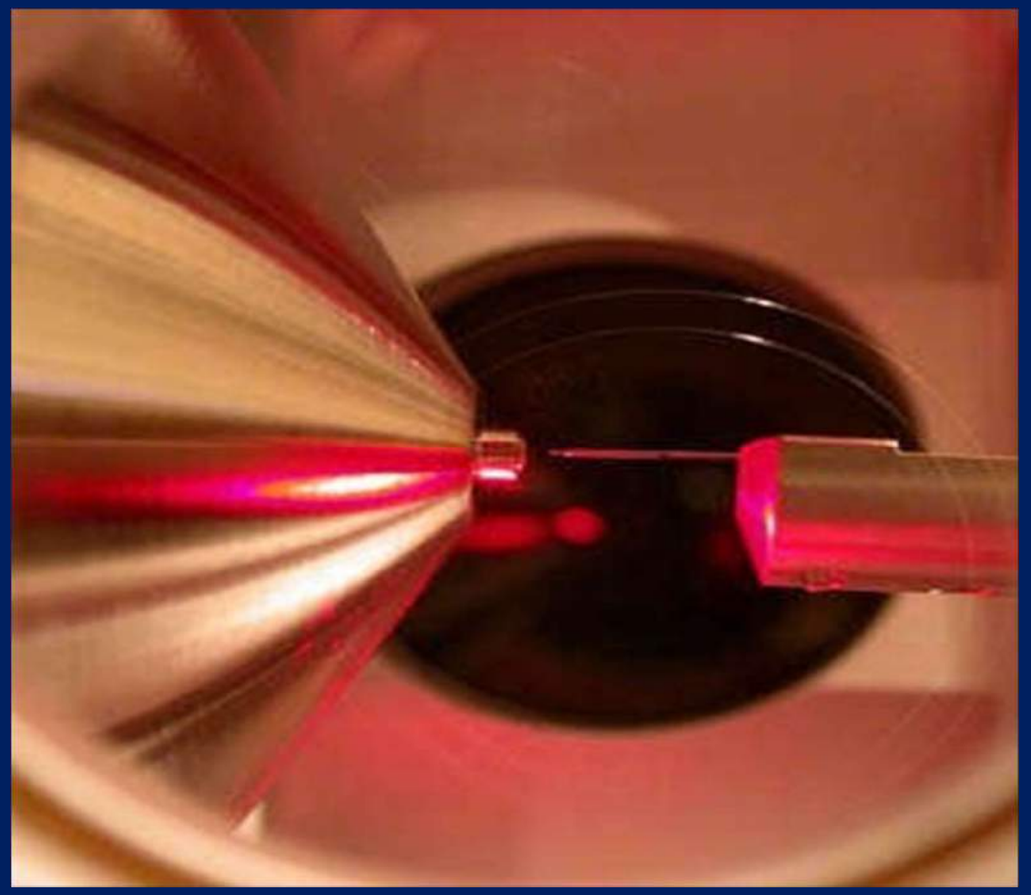
Peptides tend to fragment along the backbone.

Fragments can also loose neutral chemical groups like NH_3 and H_2O.

Ionisation

- Proteins
  - Polar
  - Nonvolatile
  - Thermally unstable
- Ionisation transfers analyte into gas phase
  - No degradation
- **Matrix-assisted laser desorption ionization** (MALDI)
  - Laser nitrogen beam (soft)
  - Matrix protection (Sinapinic acid)
- **Electrospray ionization** (ESI)
  - No fragmentation

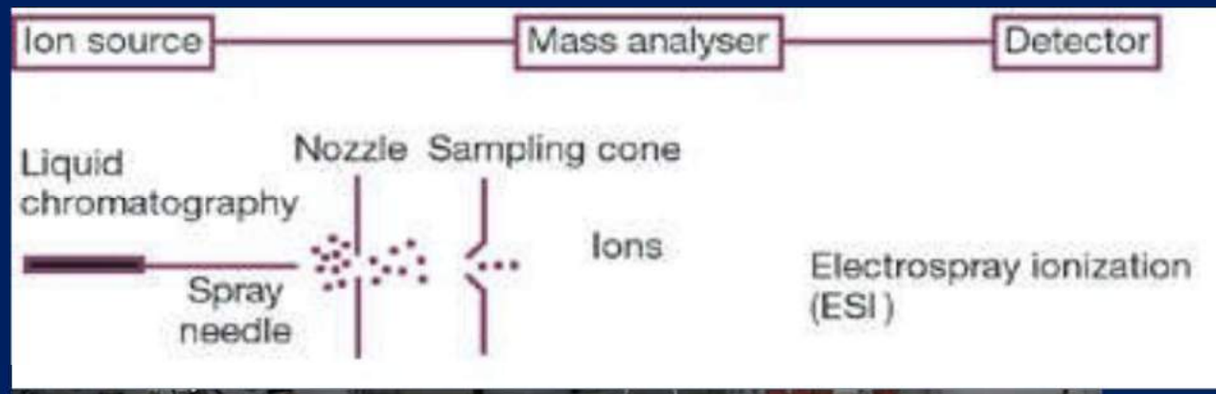


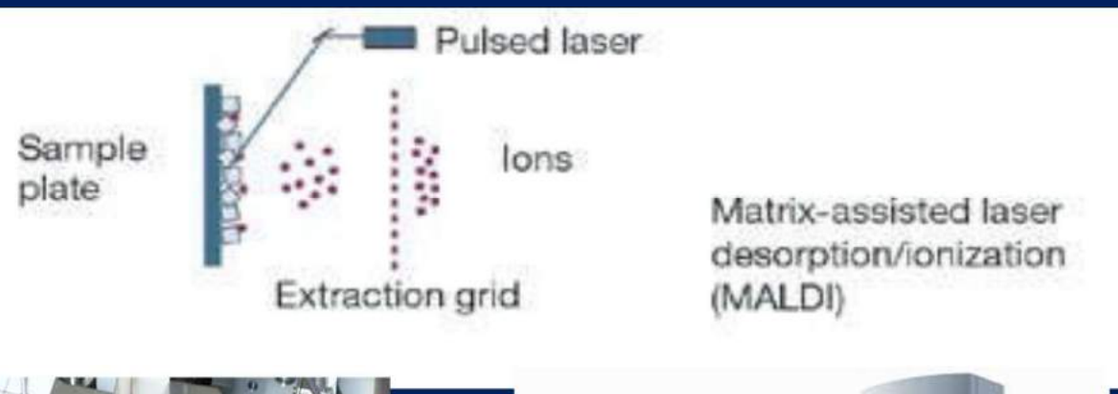


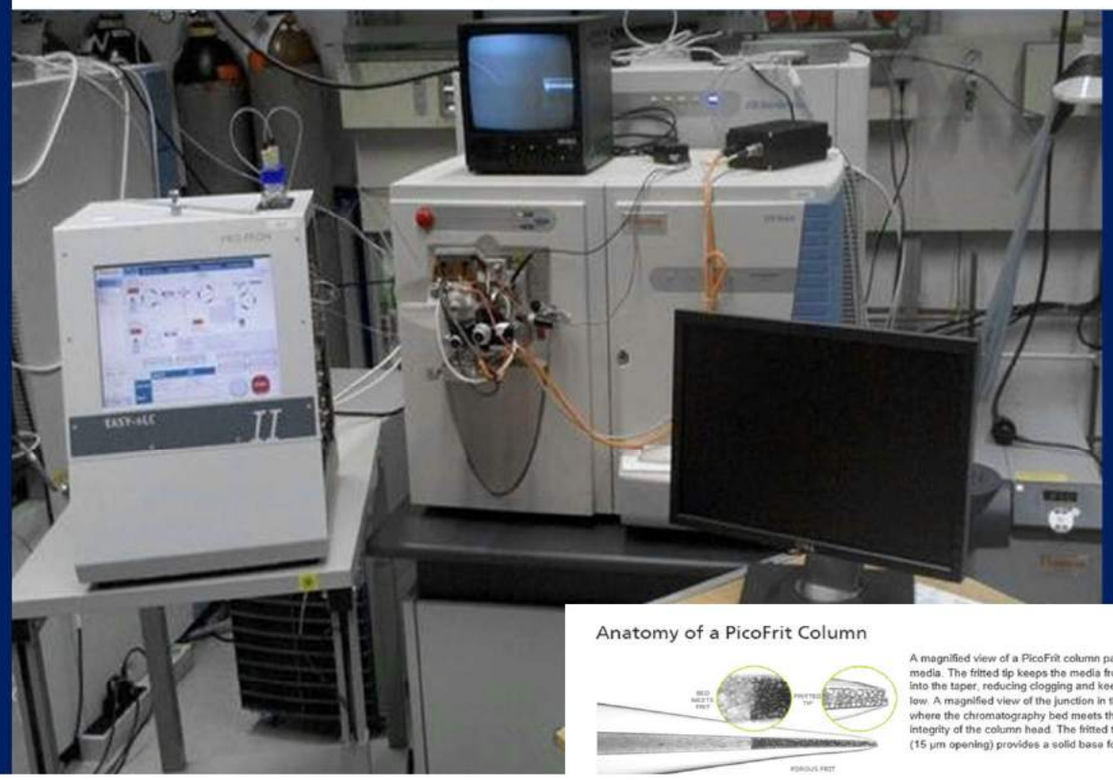
Mass analysers

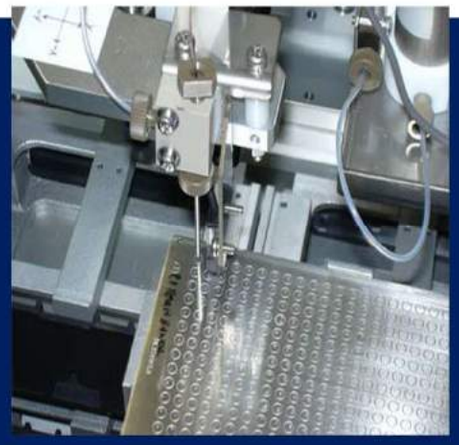
- **Scanning and ion-beam** mass spectrometers
  - TOF and Q
- **Trapping** mass spectrometers
  - IT and Orbitrap
- **Whole protein** mass analysis
- time-of-flight (TOF) MS, or
- Fourier transform ion cyclotron resonance (FT-ICR).
- Mass analysis of **proteolytic peptides** more popular
  - Low costs
  - Sample preparation is
  - MALDI time-of-flight instruments
  - Multiple stage quadrupole-time-of-flight and
  - quadrupole ion trap also find use in this application.

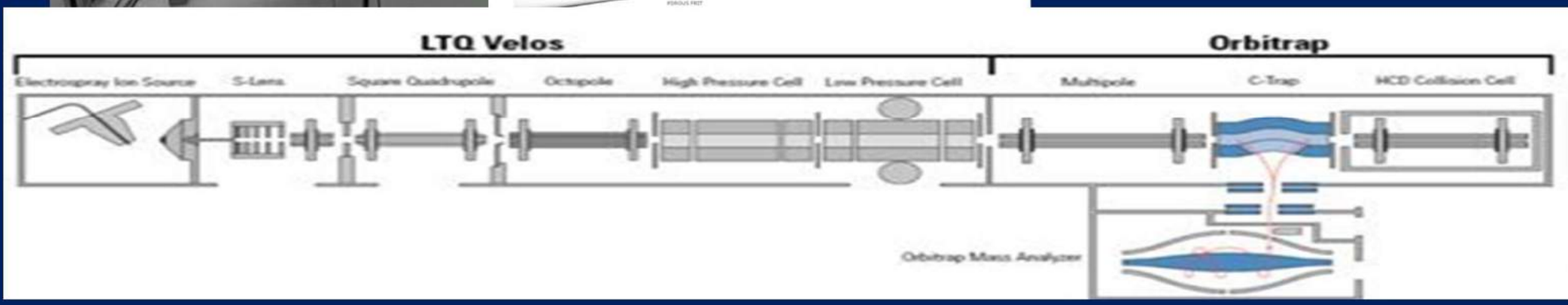
Mass spectrometers

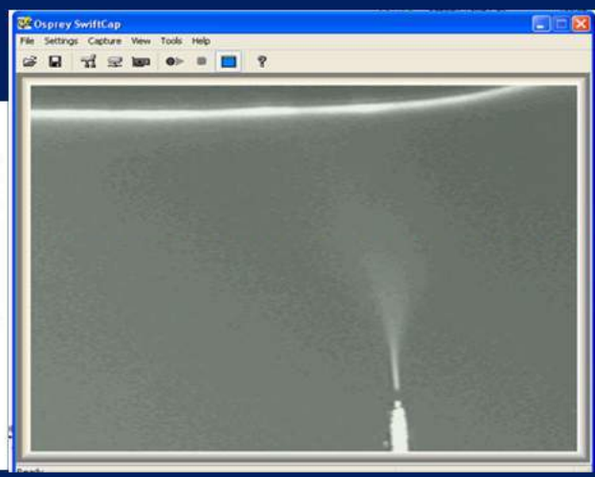




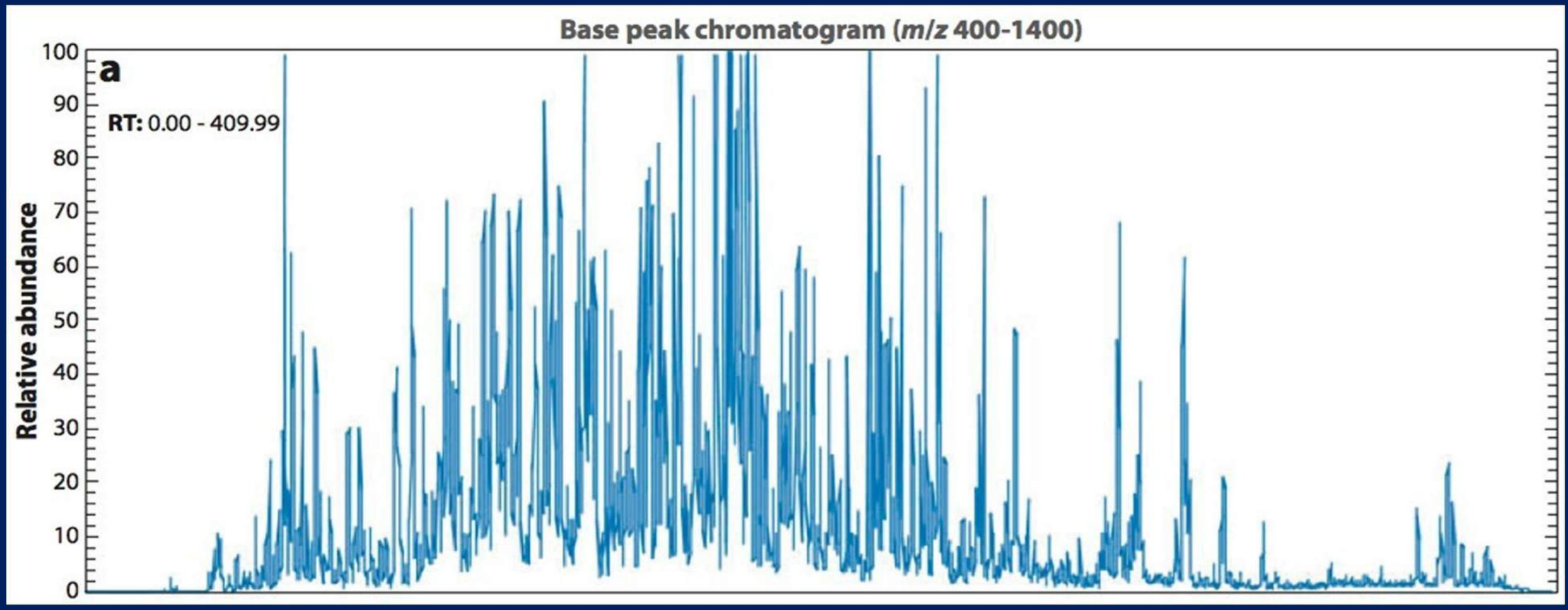






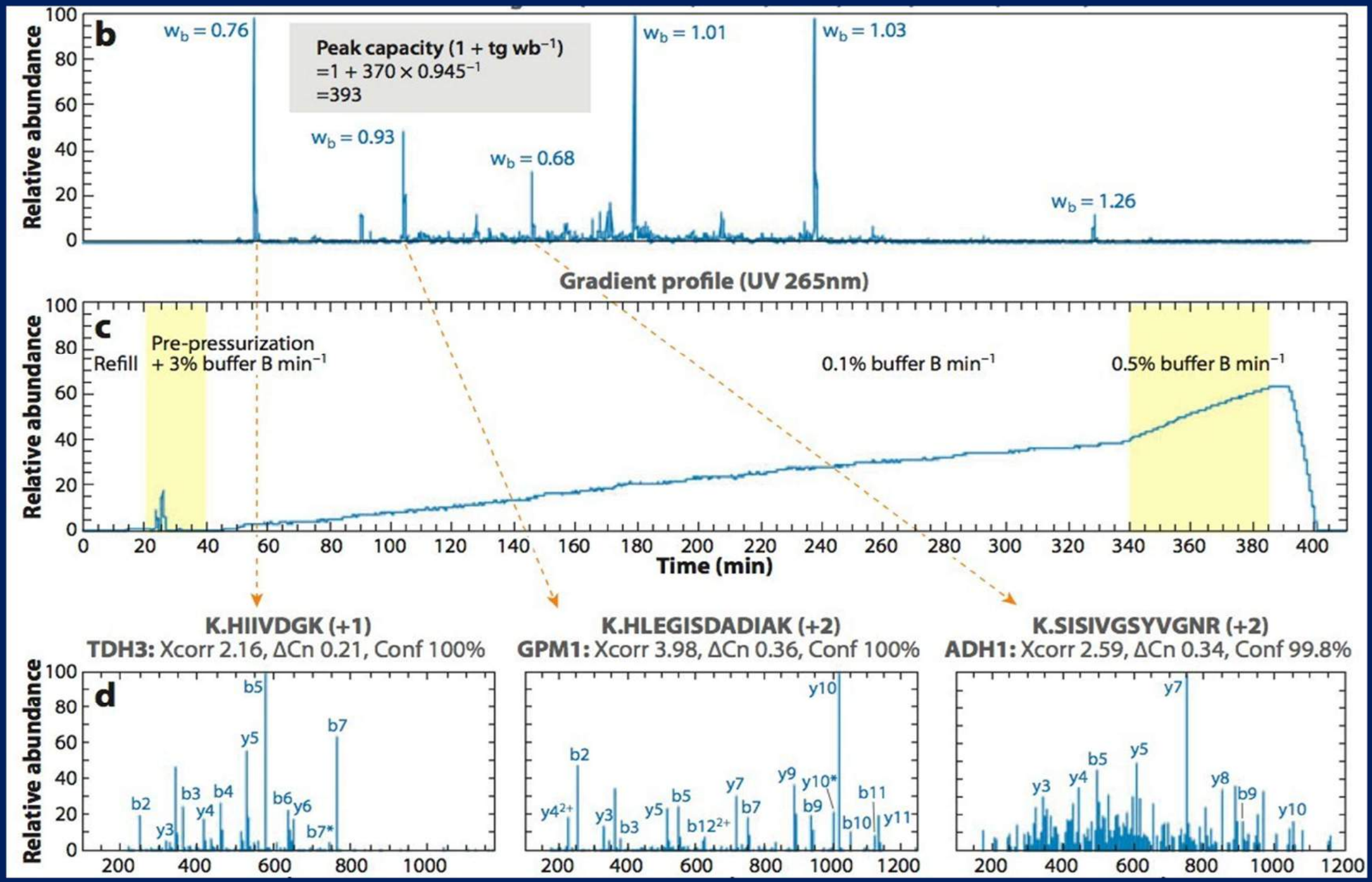


Base peak chromatogram



Yates JR, et al. Annu Rev Biomed Eng. 2009

Mass chromatogram

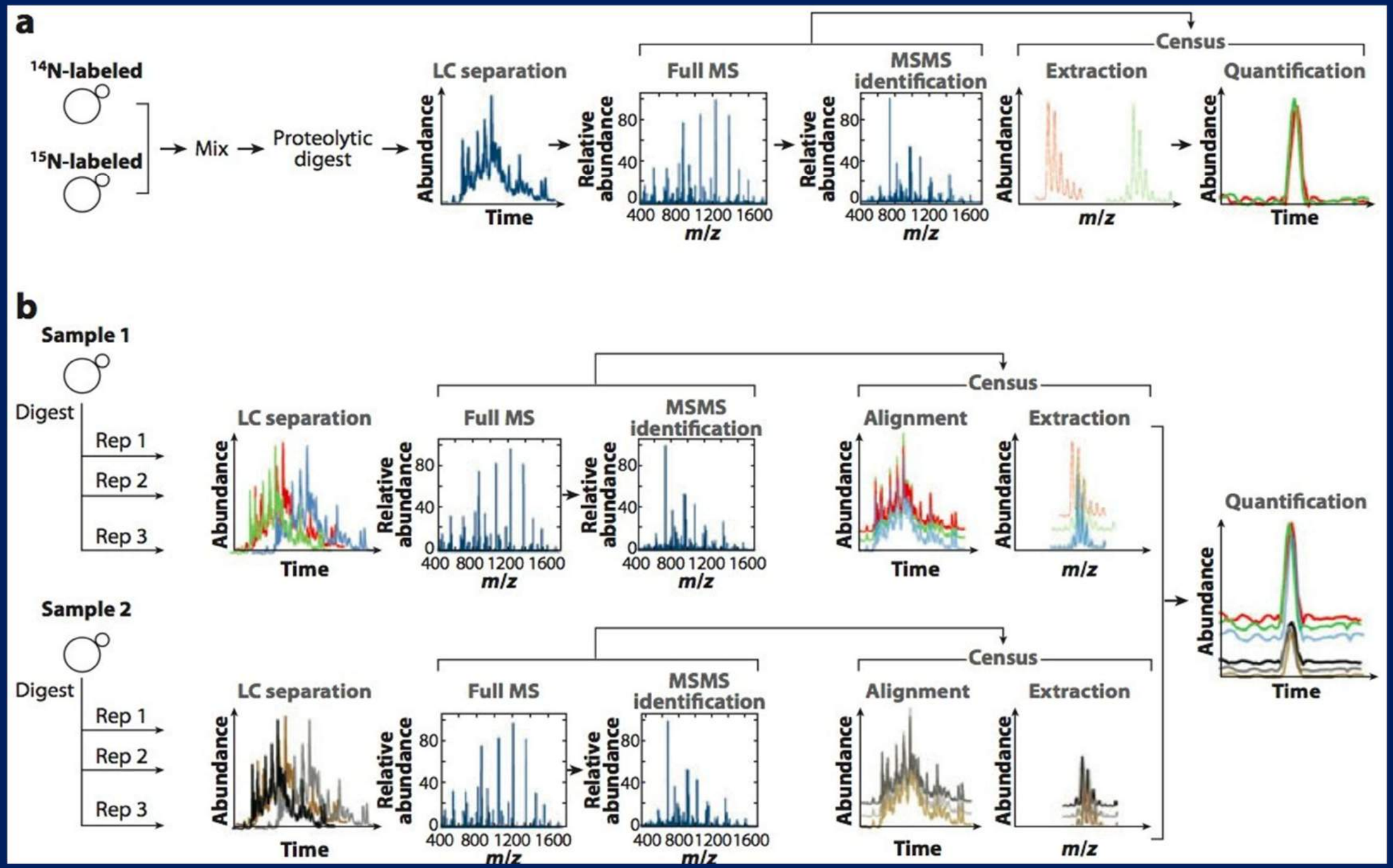


Yates JR, et al. Annu Rev Biomed Eng. 2009

Quantitative analysis

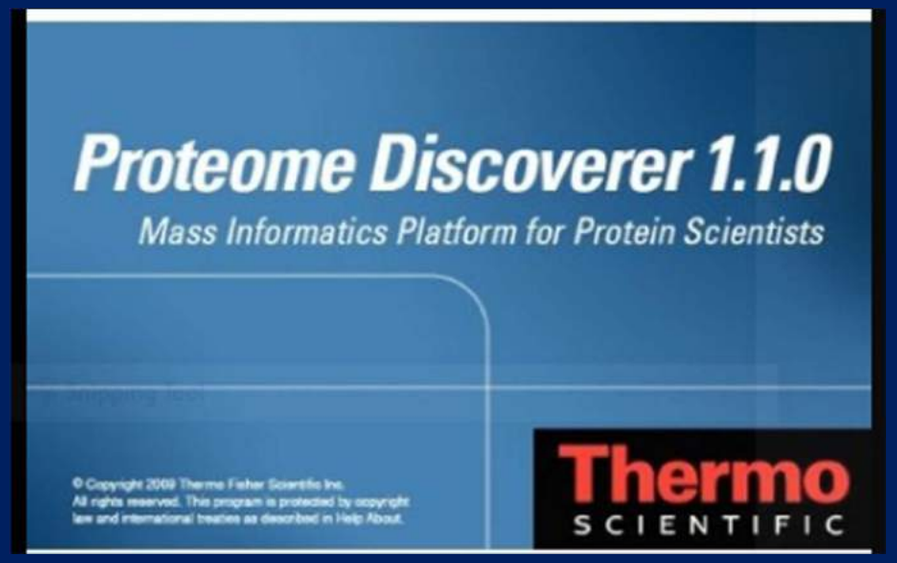
Isotopic labeling

Label-free analysis



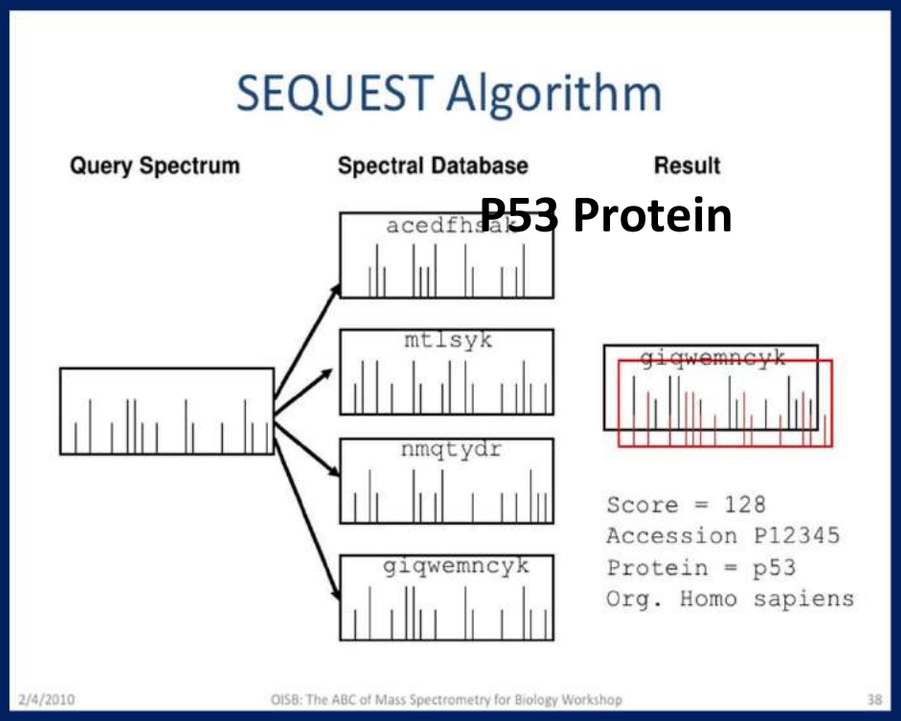
Yates JR, et al. Annu Rev Biomed Eng. 2009

Protein Identification -> Proteomics Algorithms Software / Proteomics Database



Uniprot Protein Database

Orbitrap Fusion ™ Lumos ™ Tribrid ™ Mass Spectrometer



P53 Protein

A Quantitative Omics (Proteomics) Data Analysis Tool Development

Prolyzer

Prolyzer – One Excel File with Results (Fold Change)

	S. No.	ID	Protein Name	a	Fold Change_Treat_1	Fold Change_Treat_2	Fold Change_Treat_3
235	13	P52480-2	Isoform M1 of Pyruvate kinase PKM OS=Mus musculus GN=Pkm - [KPYM_M	a	0.057716207	-0.064484186	-2.674260387
236	315	P03975	IgE-binding protein OS=Mus musculus GN=Iap PE=2 SV=1 - [IGEB_MOUSE]	a	-0.987791525	-1.872998839	-2.783797763
237	592	Q64521	Glycerol-3-phosphate dehydrogenase, mitochondrial OS=Mus musculus G	a	0.202964704	-1.505621876	-2.794208816
238	927	Q3UB60	Putative uncharacterized protein OS=Mus musculus GN=Ppid PE=2 SV=1 -	a		-0.42854941	-2.823144929
239	26	P11499	Heat shock protein HSP 90-beta OS=Mus musculus GN=Hsp90ab1 PE=1 SV=	a	0.19703084	-0.37225035	-2.947053245
240	84	O35737	Heterogeneous nuclear ribonucleoprotein H OS=Mus musculus GN=Hnrnp	a	-0.312362726	-1.013617405	-2.98391434
241	79	Q02053	Ubiquitin-like modifier-activating enzyme 1 OS=Mus musculus GN=Uba1	a	-0.172703539	-0.504424141	-3.042752565
242	342	Q9D0M3-2	Isoform 2 of Cytochrome c1, heme protein, mitochondrial OS=Mus muscu	a	-0.024258004	-0.903866095	-3.054217051
243	126	P63242	Eukaryotic translation initiation factor 5A-1 OS=Mus musculus GN=Eif5a P	a	0.099339126		-3.057161075
244	49	P68040	Guanine nucleotide-binding protein subunit beta-2-like 1 OS=Mus muscu	a	-0.228441657	-0.939018931	-3.224270082
245	24	P07901	Heat shock protein HSP 90-alpha OS=Mus musculus GN=Hsp90aa1 PE=1 SV	a	0.163132894	-0.369801614	-3.22592239
246	1	P17182	Alpha-enolase OS=Mus musculus GN=Eno1 PE=1 SV=3 - [ENOA_MOUSE]	a	-0.057919829	0.219316752	-3.273911453
247	122	P99027	60S acidic ribosomal protein P2 OS=Mus musculus GN=Rplp2 PE=1 SV=3 - [	a	-0.215881197	-0.41106497	-3.388217624
248	337	F6SVV1	Protein Gm9493 OS=Mus musculus GN=Gm9493 PE=4 SV=1 - [F6SVV1_MO	a	0.104594034	-0.247282588	-3.55126338
249	583	Q3UW83	40S ribosomal protein S10 OS=Mus musculus GN=Rps10 PE=1 SV=1 - [Q3UV	a	1.330383356	2.195486523	-3.627557881
250	94	Q3UDA7	Putative uncharacterized protein (Fragment) OS=Mus musculus GN=Npm	a	-0.554440394		-3.67857946
251	42	E9PZF0	Nucleoside diphosphate kinase OS=Mus musculus GN=Gm20390 PE=3 SV=	a	0.200067683	0.214625391	-3.752891241
252	107	P10639	Thioredoxin OS=Mus musculus GN=Txn PE=1 SV=3 - [THIO_MOUSE]	a	-0.077021746	-0.738038307	-3.983588685
253	599	A2AGN7	26S protease regulatory subunit 6A OS=Mus musculus GN=Psmc3 PE=1 SV	a	-0.468684976		-4.174017059
254	158	Q9CZD3	Glycine--tRNA ligase OS=Mus musculus GN=Gars PE=1 SV=1 - [SYG_MOUSE	a	-1.702262117	-1.403889337	-4.348487176
255	499	Q9R089	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 2 OS=Mus musculus GN	a	-2.10443884	-2.845231167	-6.698475267

Less than 5 to 10 Minutes, Quantitative Data in Fold Change format, irrespective number of Samples or its Replicates

Acknowledgment
Mr. Suresh / Mr. Vinay

Eg. 1 Omics Biomarker Discover (Cancer) & Drug Development

2003-2004

Proteomics / Genomics Studies
Omics-Based Biomarker Discovery for Breast Cancer
→ Pick the Best Biomarker from the list

Incubation and Translation of Biomarker
- **Functional Validation**
- BIG3-> Brefeldin A-inhibited guanine nucleotide-exchange **protein** 3

Validated Protein / Gene → Molecular Mechanism
Take Proteomics Help
- Interacting Protein to Biomarker

Brefeldin A-inhibited guanine nucleotide-exchange **protein** 3

BIG3 **PHB2**

Molecular Mech. Cont.

Functional Studies

Working with existing pathways / Novel Pathway

2013-2016

Targeted Therapy

Novel / ~~existing Drug~~ → Target the Biomarker-interacting protein / Pathway → A peptide based drug to dissociate BIG3-PHB2 Interaction

2017-2018

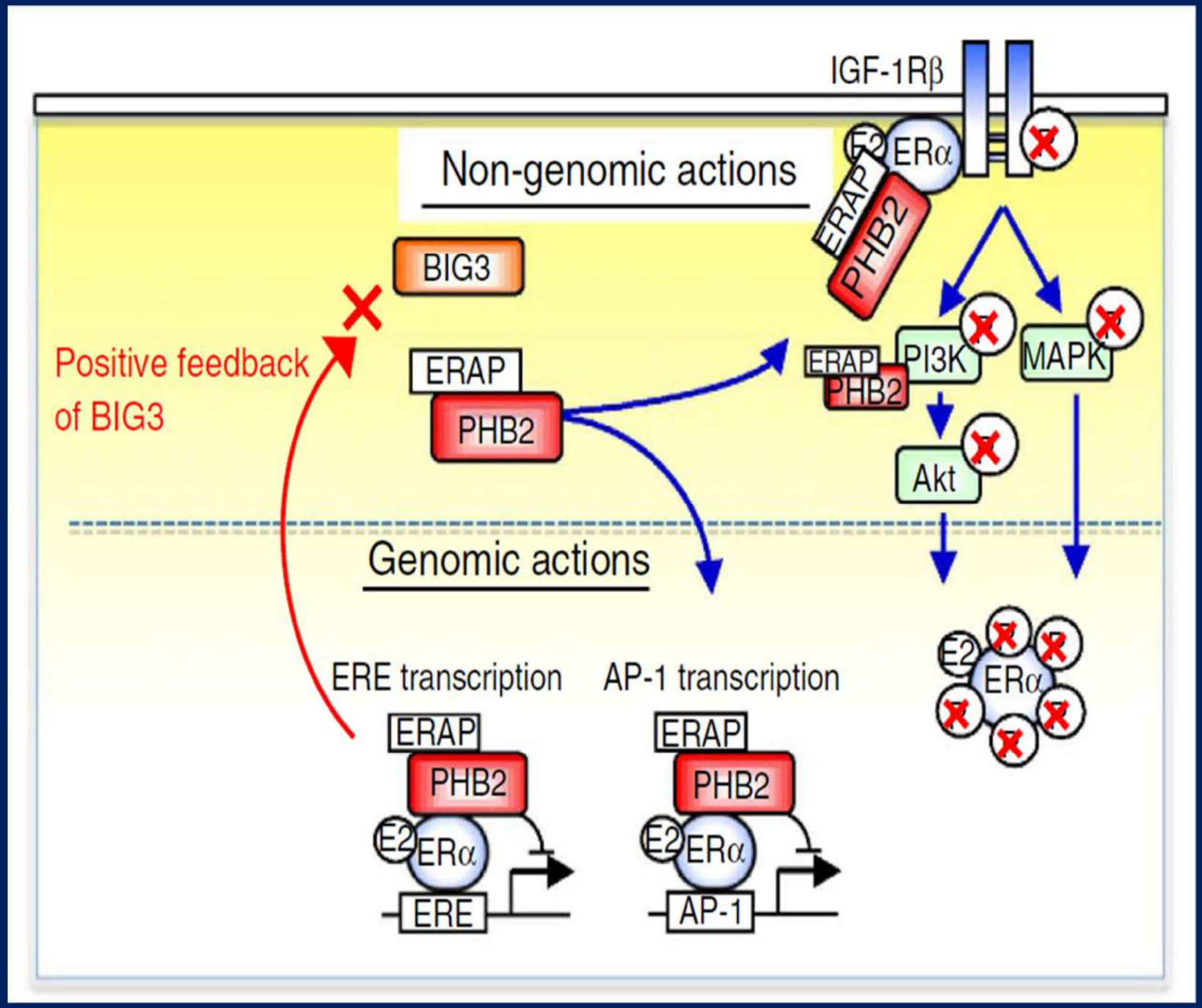
Drug-Biomarker Molecular Mechanism

Take Proteomics Help if needed / Other Functional Studies

StERAP

Dr. Yusuke Nakamura / Dr. Toyomasa Katagiri -> Lab Publication 2004 - Present

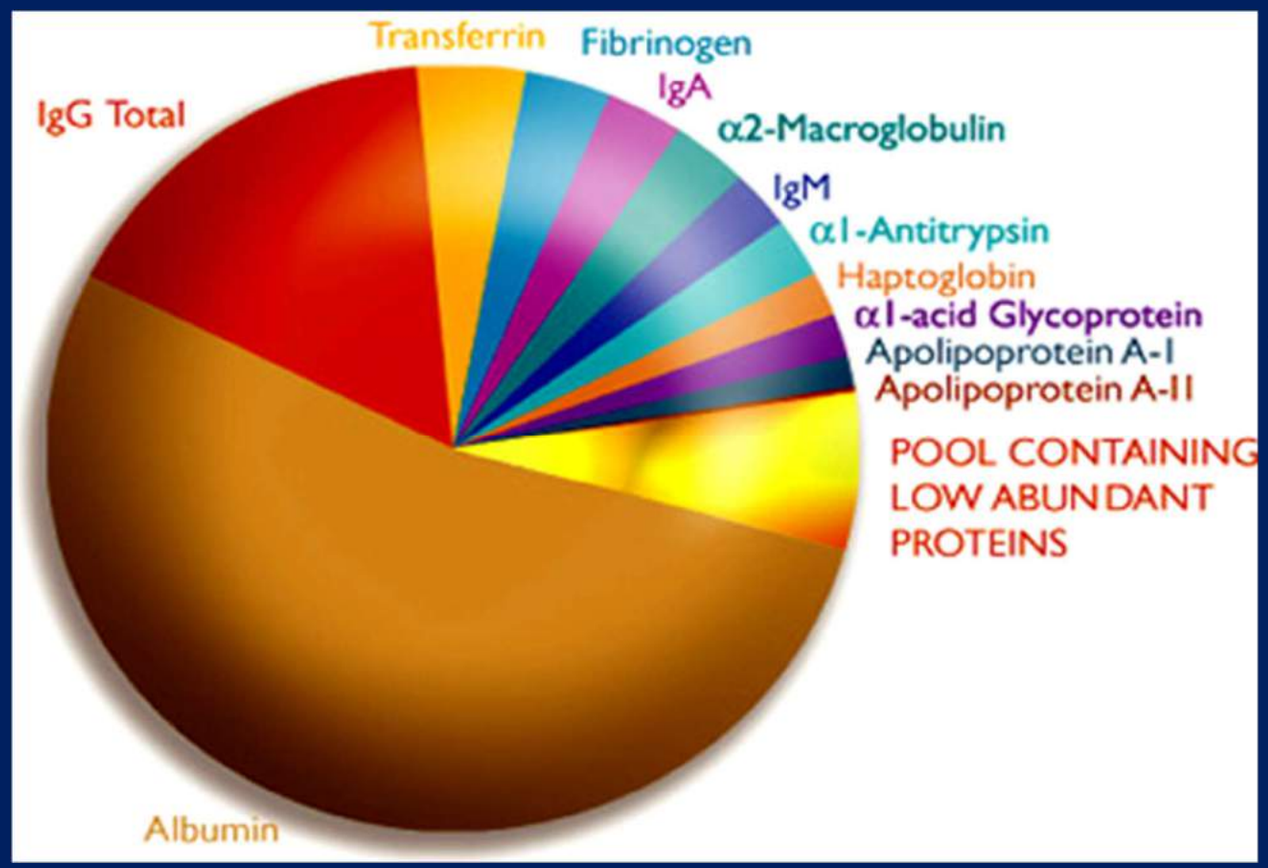
Targeting BIG3 –PHB2 complex – with ERAP / StERAP peptide in Breast Cancer

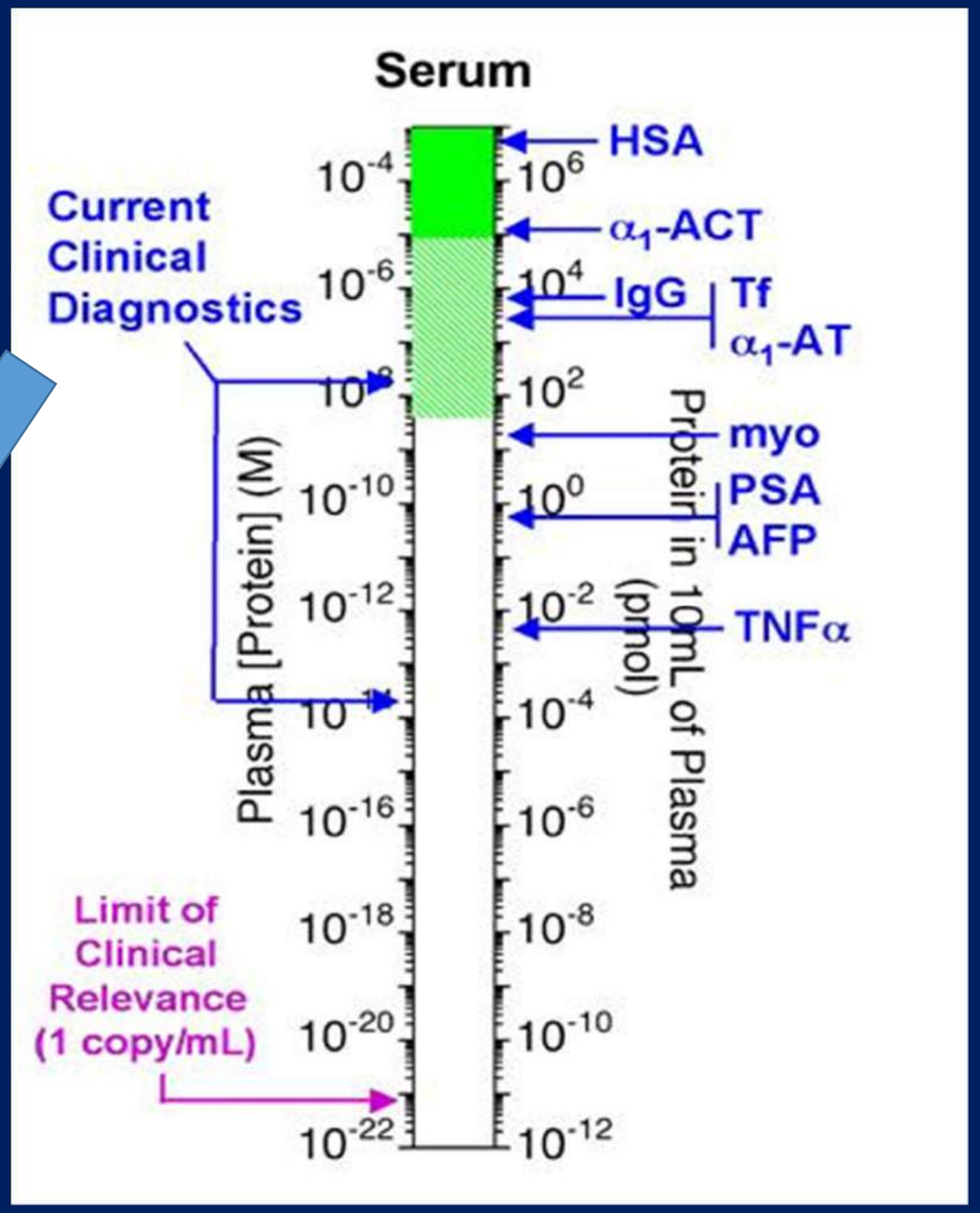


StERAP – A cell-permeable peptide inhibits the interaction of BIG3 & PHB2 (PHB2 released). And Repression of E2 signaling pathway and cell growth.

Nat. Commun., 4, 2443, 2013; **Sci Rep**., 7, 1821, 2017

SERUM PROTEOMICS & ITS CHALLENGES



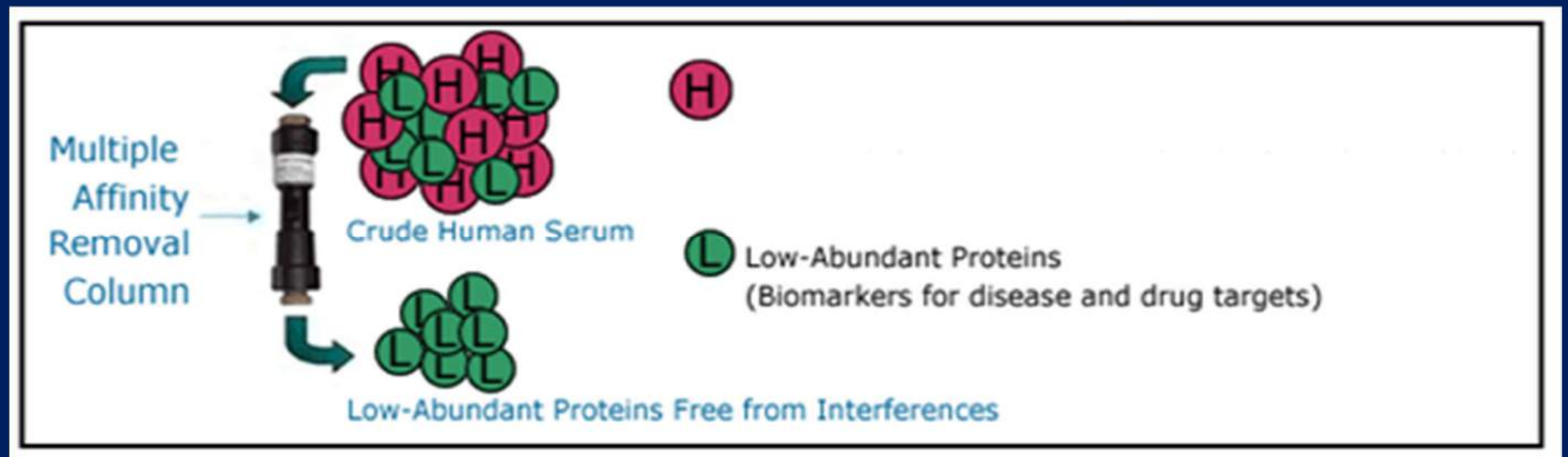


METHODOLOGY

Serum (40 µl)

Human Proteins Depleted: Albumin, IgG, Antitrypsin, IgA, Transferrin, Haptoglobin, Fibrinogen, Alpha2-Macroglobulin, Alpha1-Acid Glycoprotein, IgM, Apolipoprotein AI, Apolipoprotein AII, Complement C3, Transthyretin

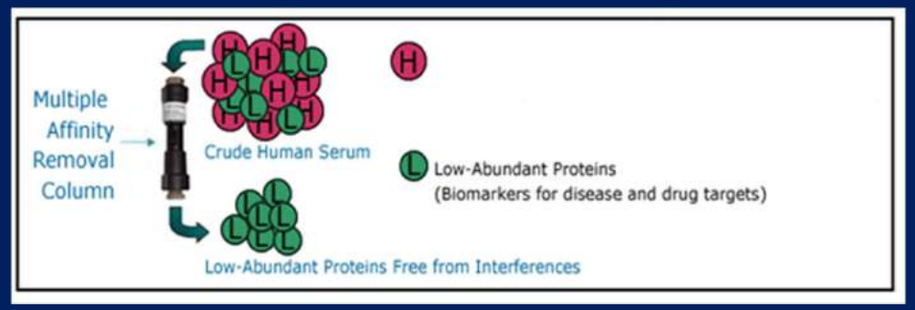
HIGH-ABUNDANT PROTEIN REMOVAL



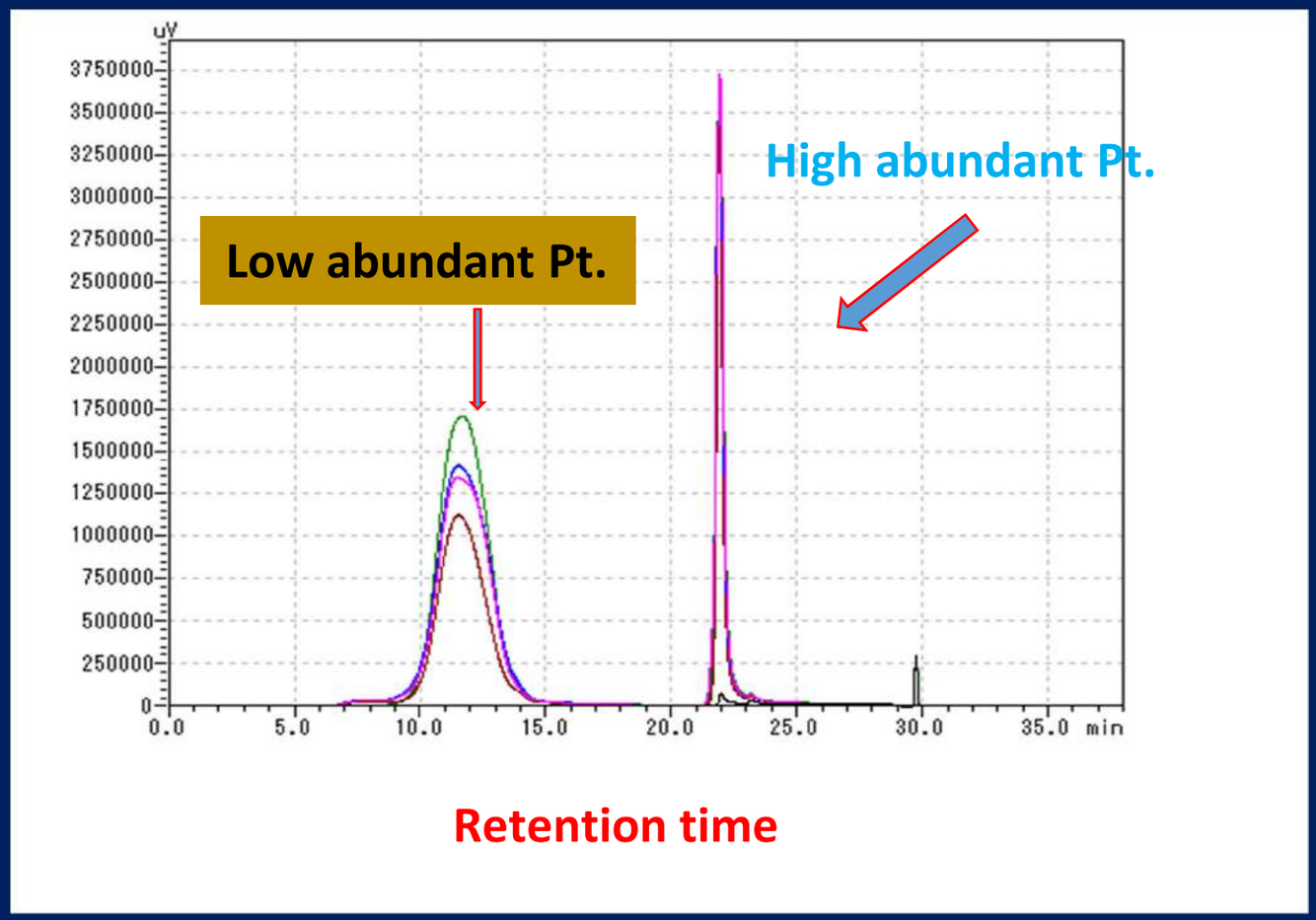
High-Recovery Protein Desalting LC Column

Agilent's Macroporous Reversed-phase C18 (mRP-C18)

MARS COLUMN CHROMATOGRAM



Human Proteins Depleted: Albumin, IgG, Antitrypsin, IgA, Transferrin, Haptoglobin, Fibrinogen, Alpha2-Macroglobulin, Alpha1-Acid Glycoprotein, IgM, Apolipoprotein AI, Apolipoprotein AII, Complement C3, Transthyretin

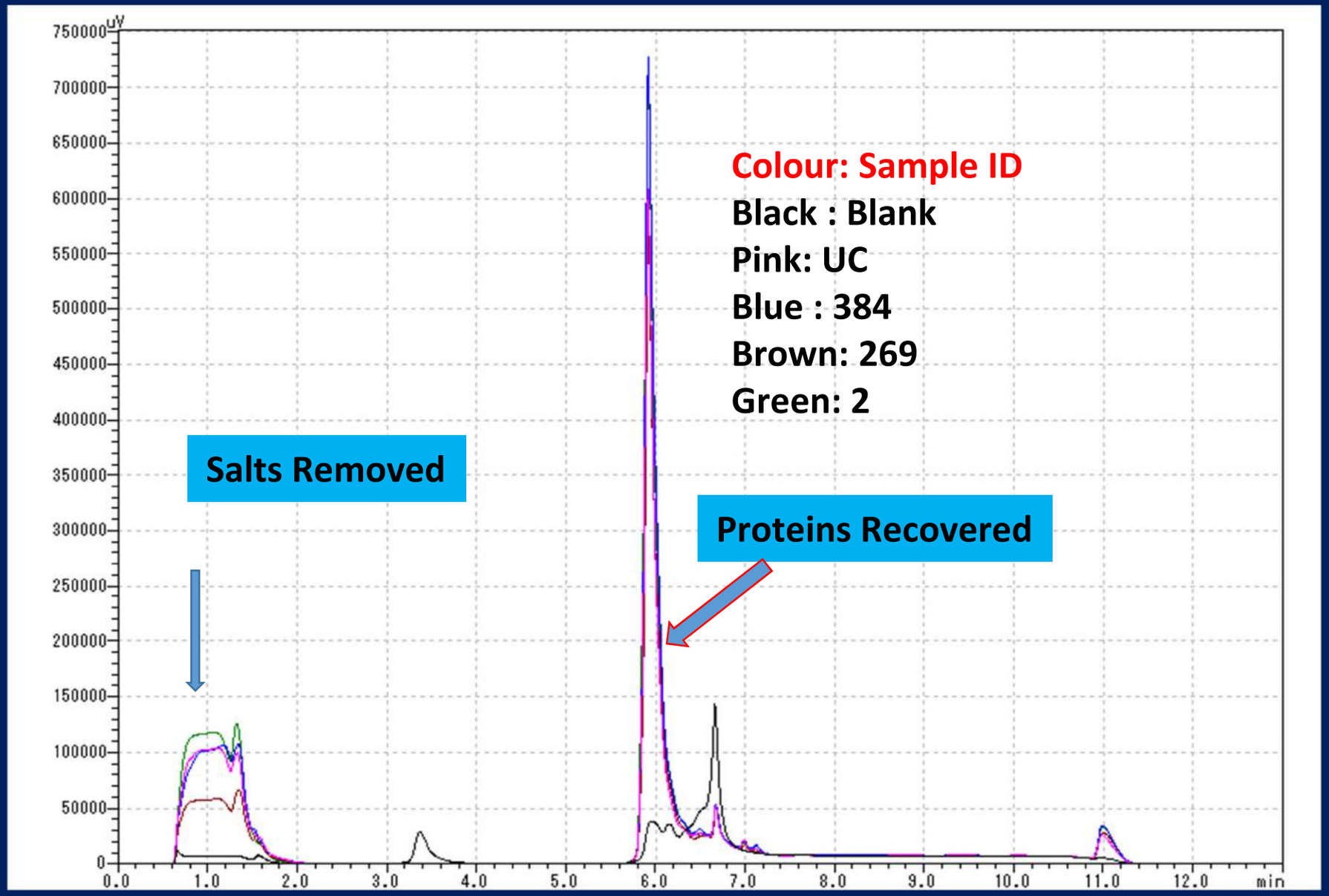


Color: Sample ID

Black : Blank
Pink: UC - 114
Blue : 115
Brown: 116
Green: 117

4.6 x 100 mm
Mobile
Phase(elution): Low pH Urea sol.

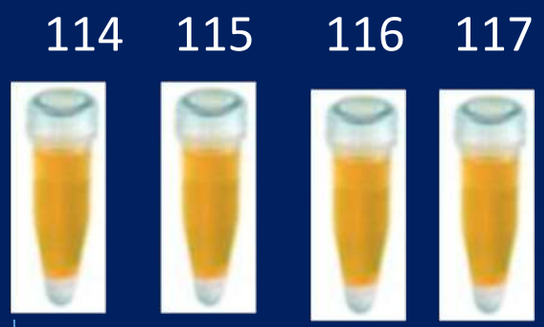
mRP COLUMN CHROMATOGRAM



4.6 x 50 mm
Mobile
Phase (elution): ACN,0.1 % TFA

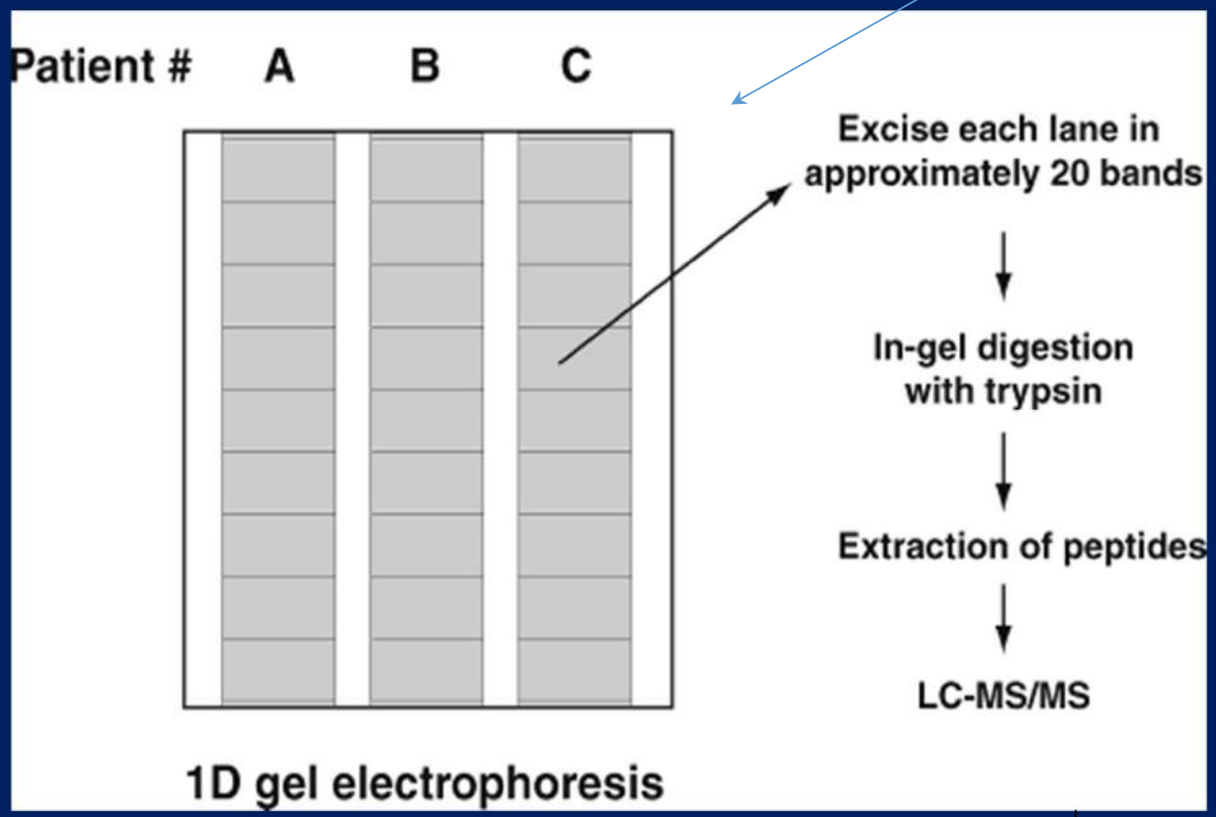
METHODOLOGY / Strategy

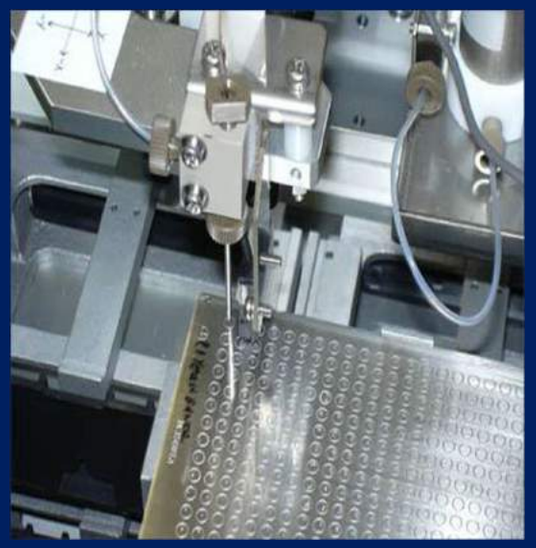
iTRAQ –



Protein iTRAQ Labeling -

iTRAQ labeled samples <u>combined</u> and 20 ug of labeled sample subjected to SDS-PAGE





4800 MALDI **TOF/TOF** (Applied Biosystems)

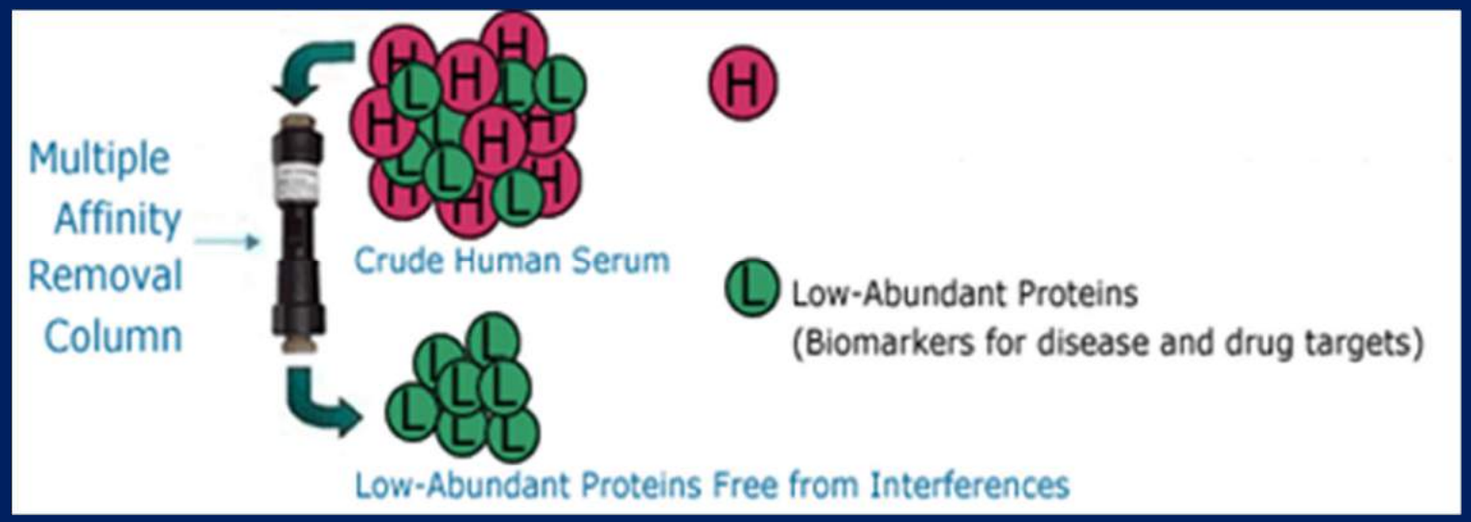
KYA Tech. Nano-HPLC 100 um x 15 cm

Mobile Phase : 70 %ACN,0.1 % TFA (elution)

Protein Pilot Software 2.0

~ 450 Proteins Identified

PROTEIN AND PEPTIDE iTRAQ LABELING



Protein iTRAQ

i TRAQ Labeling -

Peptide iTRAQ

Pro iTRAQ –

114 115 116 117

combined

Protein Fractionation – SDS-PAGE

In-gel trypsin digestion

KYA Tech.
Nano-HPLC
100 um x 15 cm

LC-MS/MS

~ 450 Proteins Identified

In-solution trypsin digestion

Pep iTRAQ –

114 115 116 117

combined

SCX Chromatography

LC-MS/MS

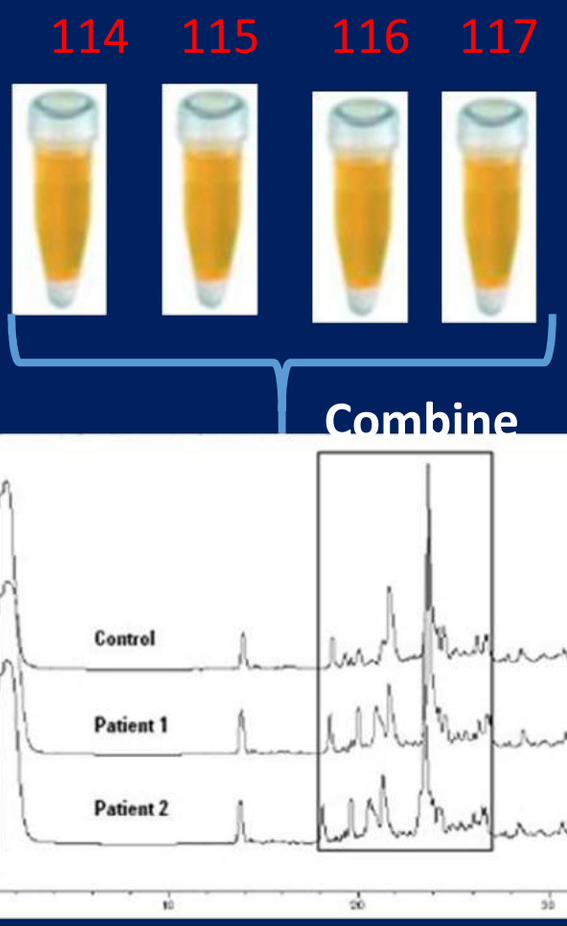
~ 700 Proteins Identified

NEWER METHOD / TECHNOLOGY EXPLORATION I mRP PROTEIN RACTIONATION

Protein iTRAQ Labeling -

iTRAQ –

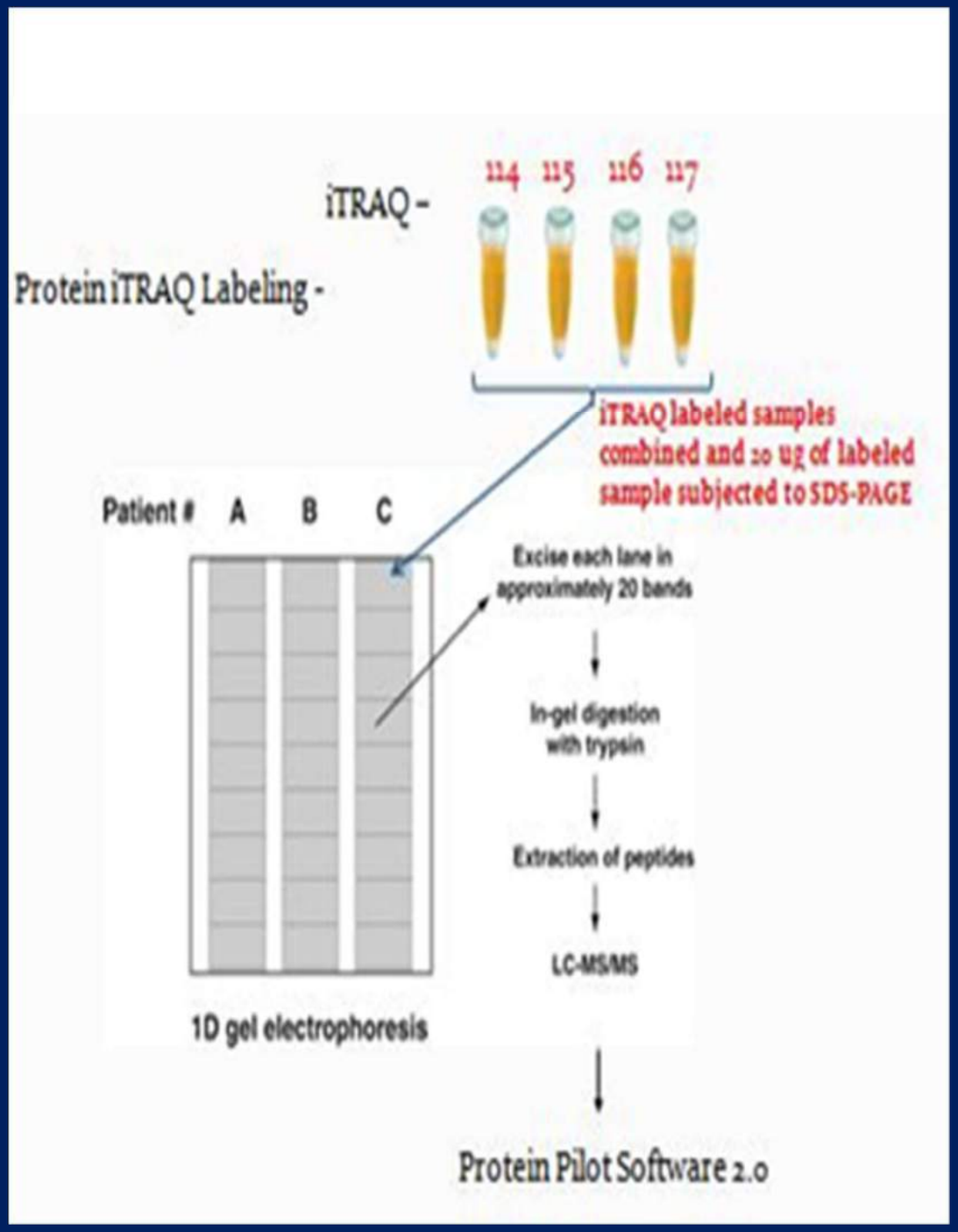
100 ug - labeled protein mRP PROTEIN FRACTIONATION



In-solution trypsin digestion

LC-MS/MS

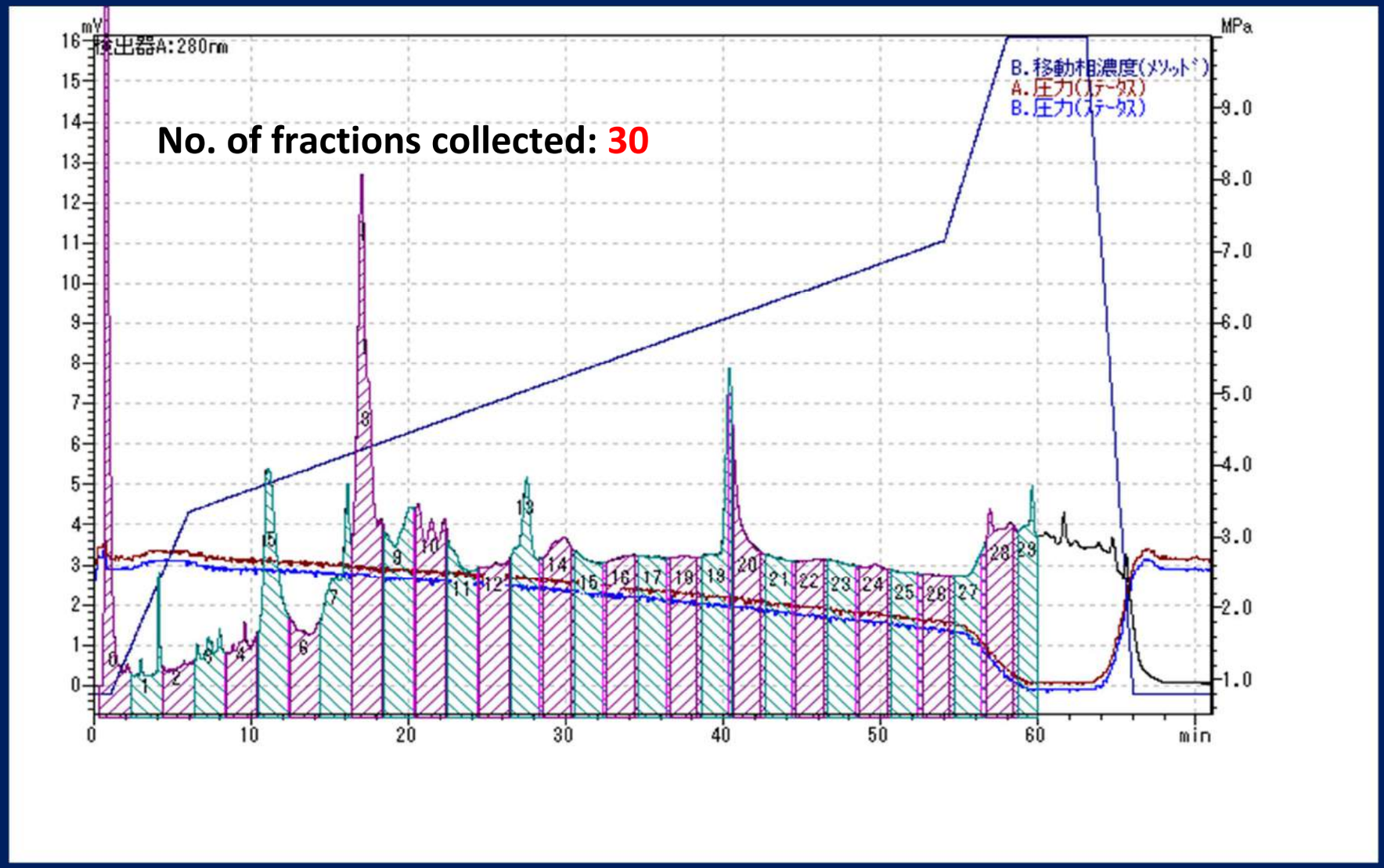
~ 650 Proteins Identified



~ 450 Proteins Identified

LABELED PROTEIN SAMPLE (100 µg) - mRP PROTEIN FRACTIONATION

UV Absorbance at 280 nm

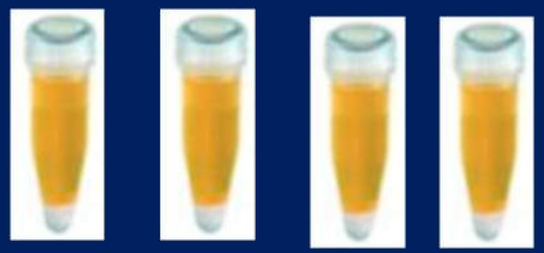


Retention time (min)

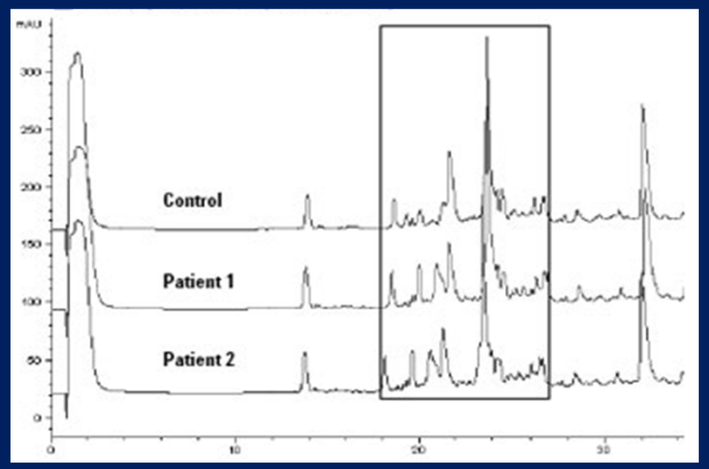
4.6 x 50 mm
Buffer ‘A’: 0.1 % TFA
Buffer ‘B’: 0.1 % TFA in ACN

EXPLORATION II

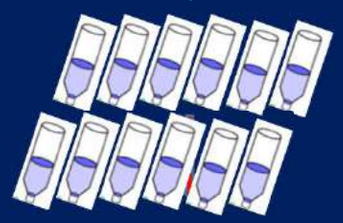
114 115 116 117



Combine



Present Method



~ 650 Proteins Identified

In-solution trypsin digestion

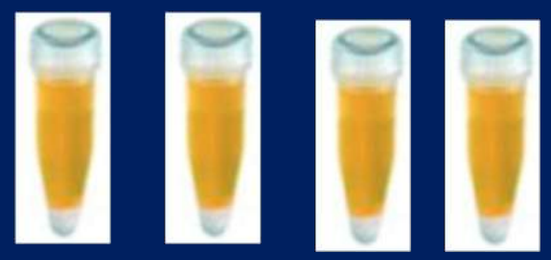
LC-MS/MS

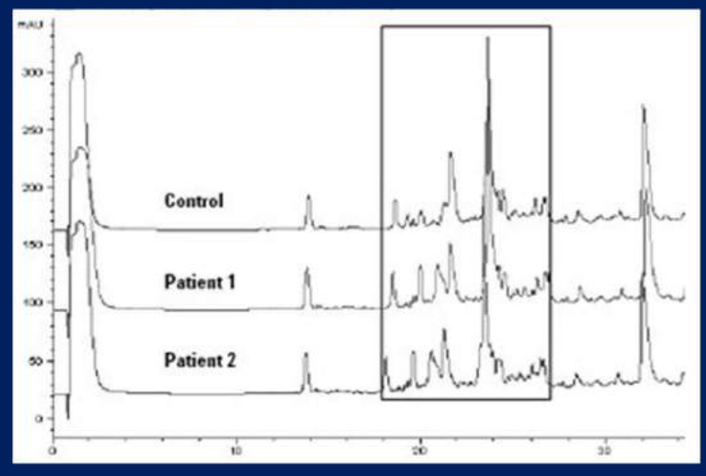
i TRAQ Labeling -

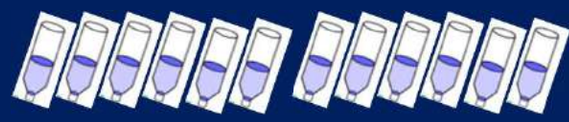
100 ug - labeled protein
mRP PROTEIN FRACTIONATION

Future Plan

114 115 116 117

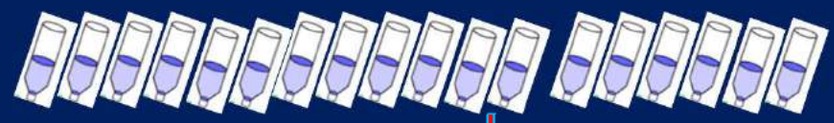






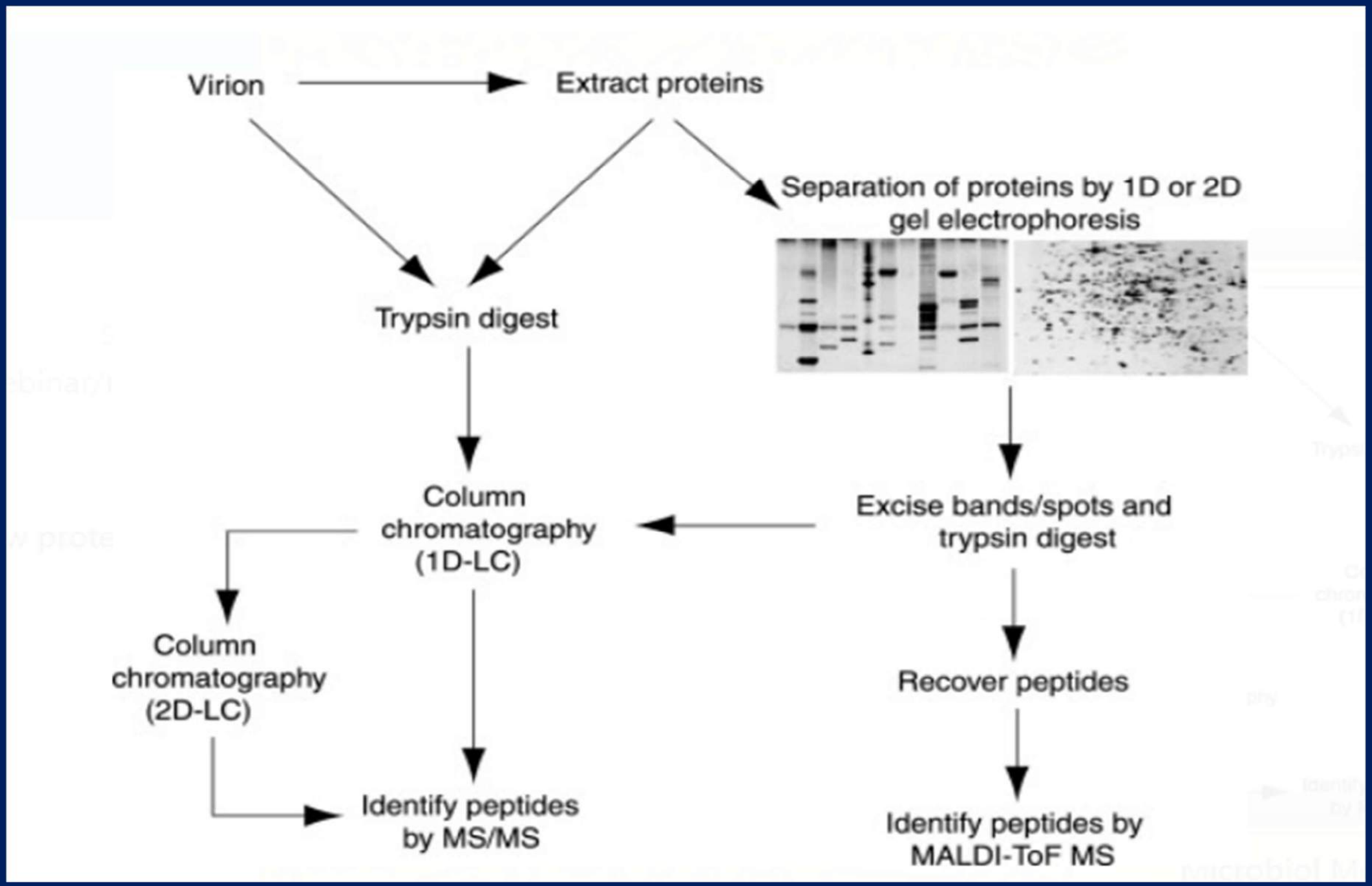
In-solution trypsin digestion

SCX column Fractionation



LC-MS/MS

Viral Proteomics



Microbiol Mol Biol Rev. 2007 Jun; 71(2): PMC1899879; PMID: 17554050 Viral Proteomics

MASS SPECTROMETRY BASED PROTEOMICS
&
ITS APPLICATION IN MEDICINE

Summary

- Proteomics & Mass Specs
- Life Cycle of a Proteomics Project
- Proteomics Core Facility -> Management - **(Balance -> Vision, Requirements / Capital)**
- Different technologies to deal with different challenges
- Clinical Proteomics Project (Examples) **Identify of Biomarker to Testing if it Drug-able and Development of Molecular Targeted Therapy.**
- **Future of MS-based Proteomics (PTM / Protein-Protein interaction)**

Thank you