PROTEOMIC SAMPLE PREPARATION

Two-dimensional electrophoresis

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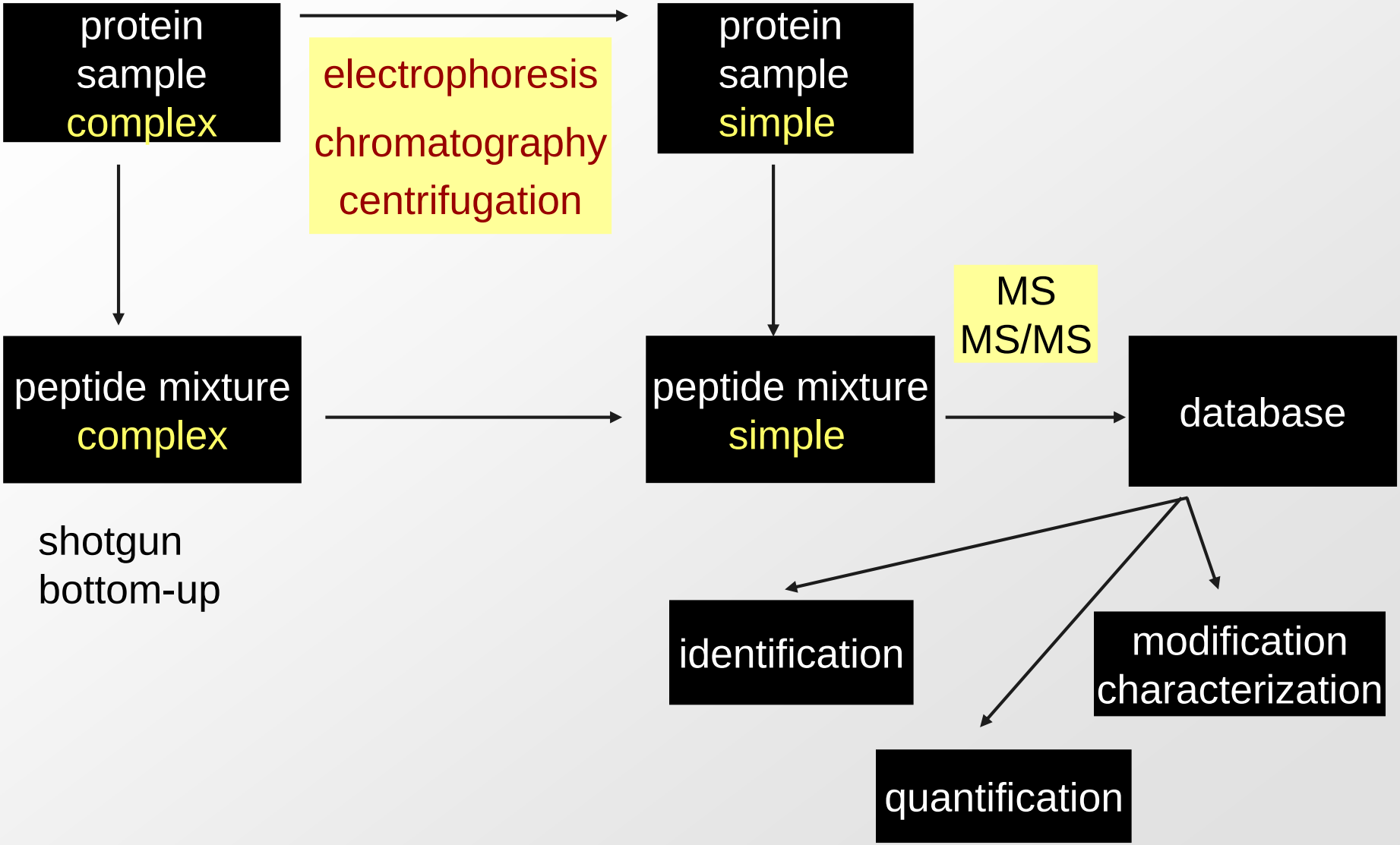
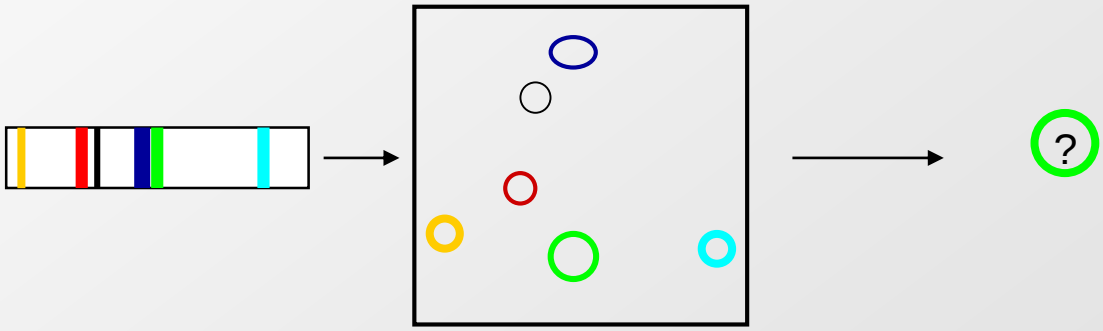
NCBR National Centre for Biomolecular Research

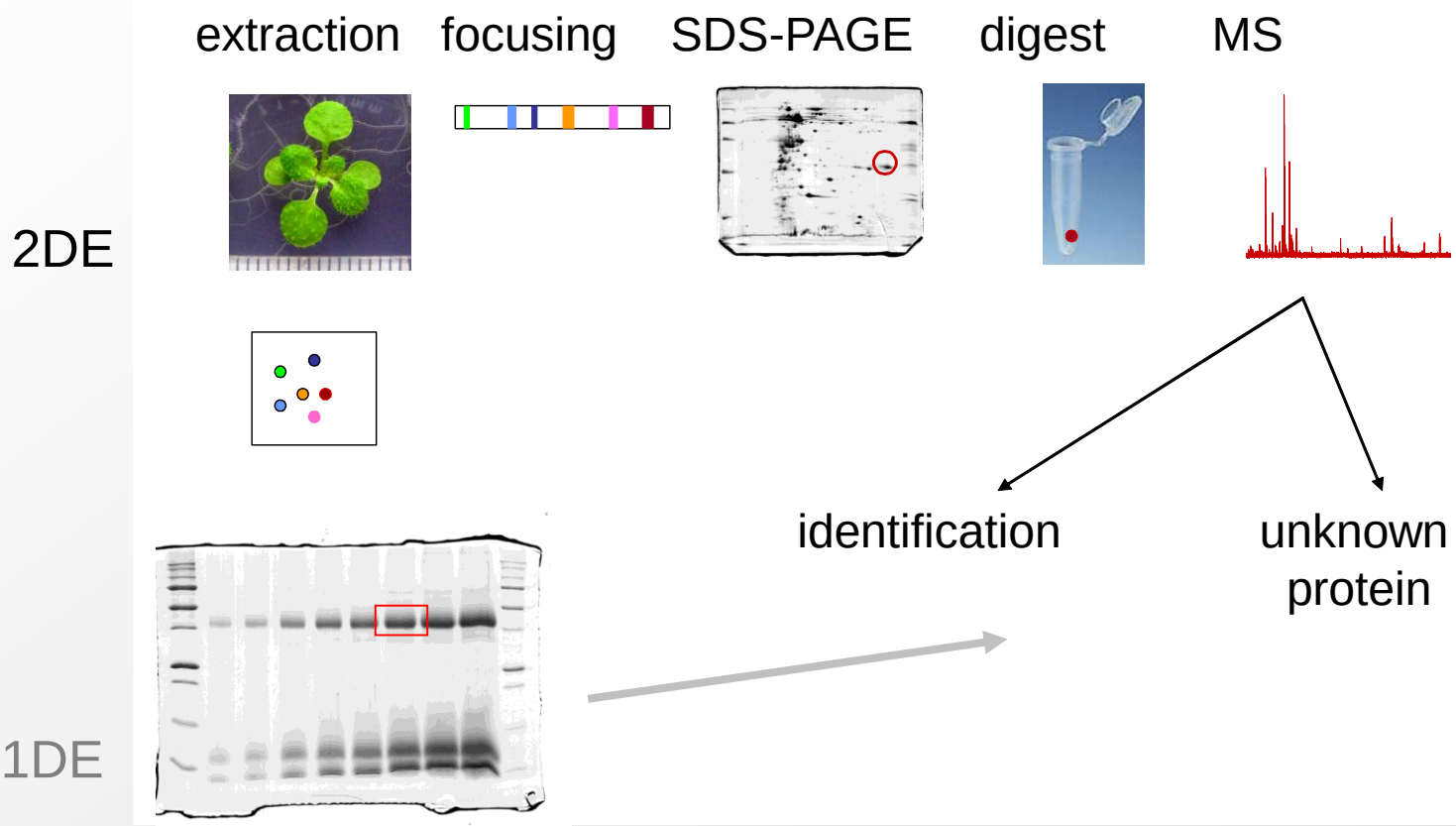

protein sample complex
electrophoresis
chromatography
centrifugation
protein sample simple
peptide mixture complex
peptide mixture simple
MS
MS/MS
database
shotgun
bottom-up
identification
modification characterization
quantification

I. SEPARATION
II. PREFRACTIONATION

Two-dimensonal electrophoresis 2-DE

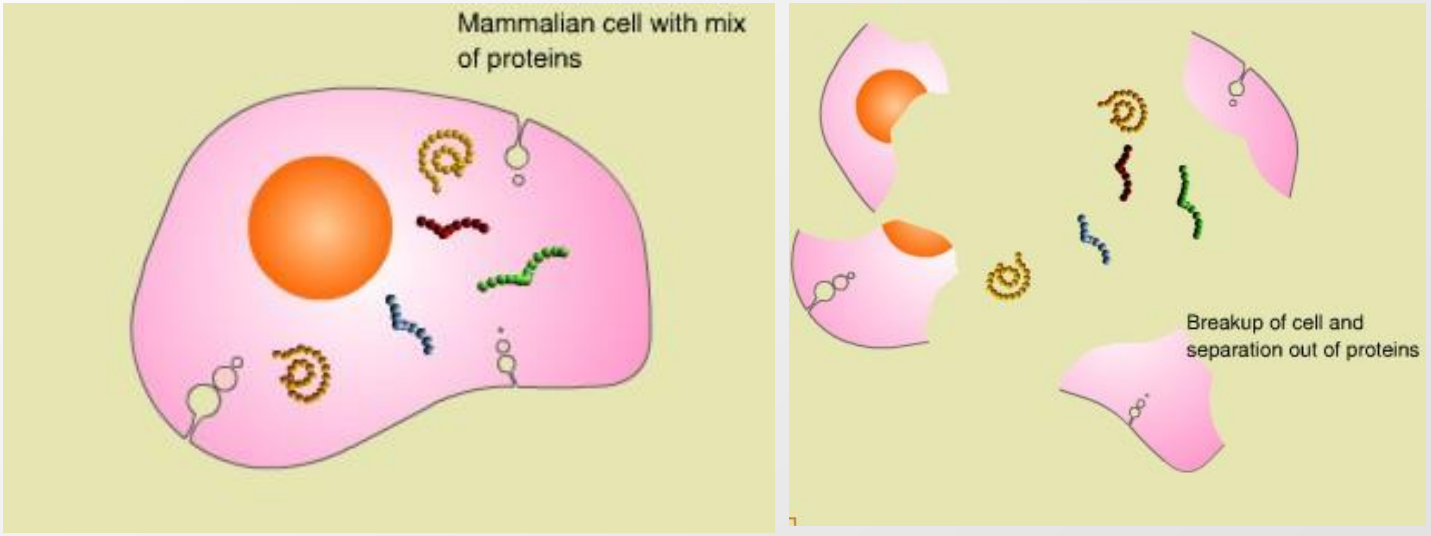


Proteomic experiment



HOMOGENIZATION

- mechanical
- ultrasound
- pressure
- freeze/thaw lysis
- detergent lysis



CryoMill

Liquid Nitrogene

Watch for keratins!

SAMPLE PREPARATION

Solubilization urea thiourea detergents

Reduction

DTT	TBP	TCEP
dithiotreitol	tributylphospine	Tris (2-carboxyethyl) phosphine hydrochloride

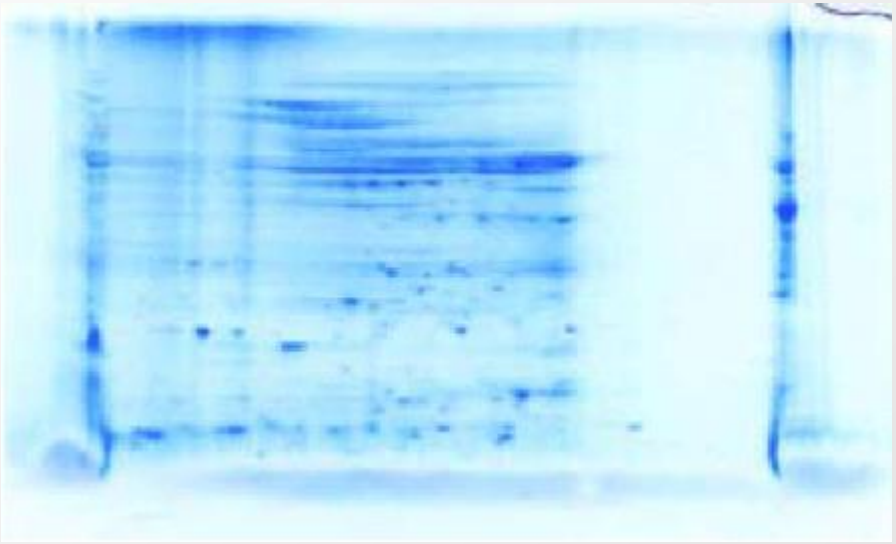
Inhibition of proteases, phosphatases glycosylases

Contaminants removal

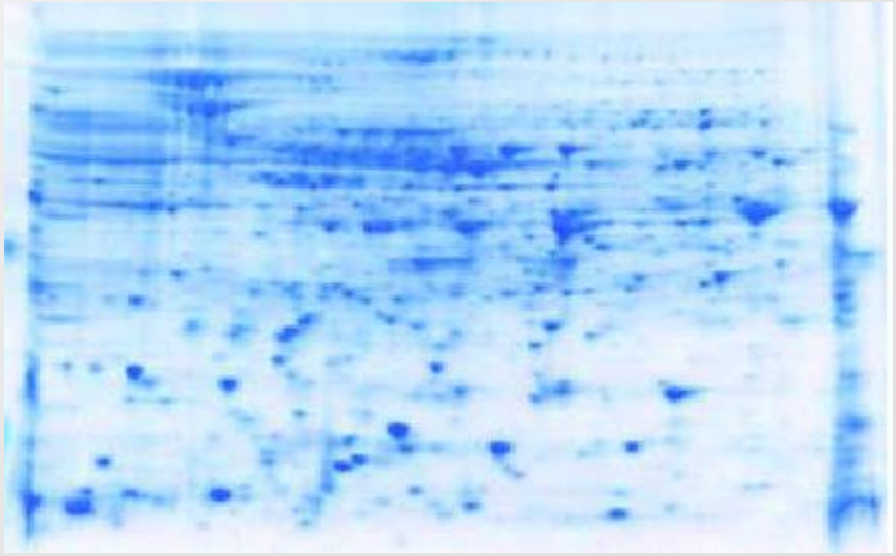
Protein assay

DETERGENTS

- no net charge
- 0.5 – 4%
- working in high urea



- non ionogenic
- zwitterion



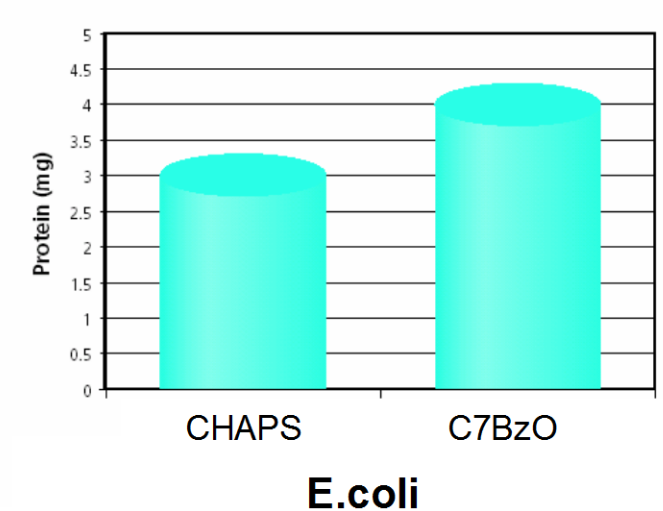
- SDS only up to 0.25%

C7BzO

3-(4-Heptyl)phenyl-3-hydroxypropyl)dimethylammoniopropanesulfonate

CHAPS

3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate



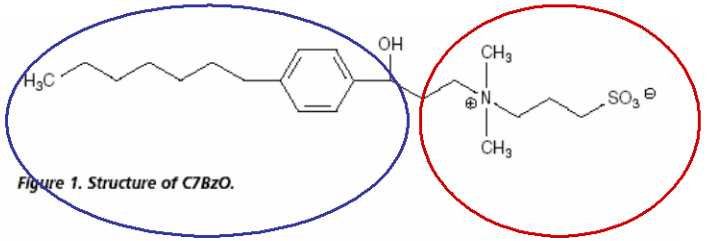
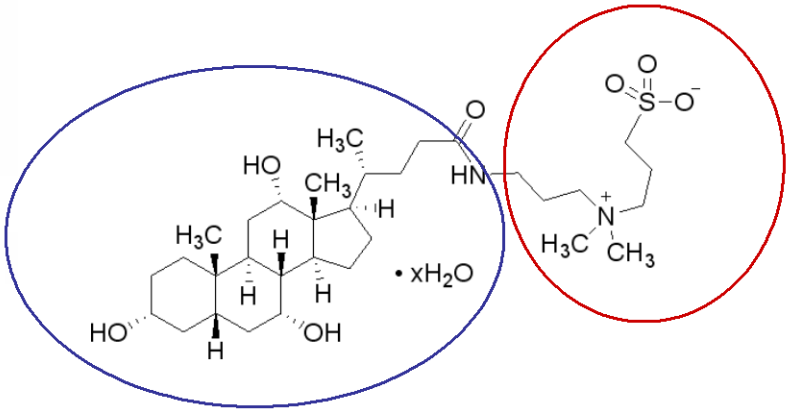


Figure 1. Structure of C7BzO.



RULE OF THUMB

- avoid proteolysis
- simple preparation
- fresh reagents
- fresh sample
- remove particles - spin
- remove contaminants

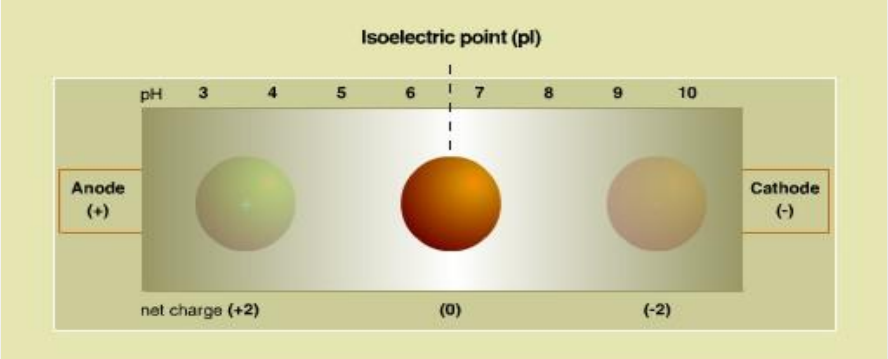
CONTAMINANTS

- salts, buffers
- small molecules
- ionic detergents
- nucleic acids
- polysaccharides
- lipids
- phenols

2-DE

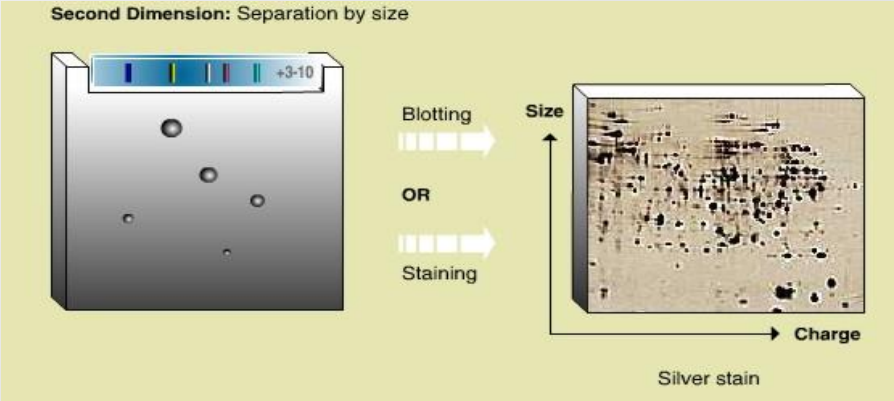
- first dimension

IEF



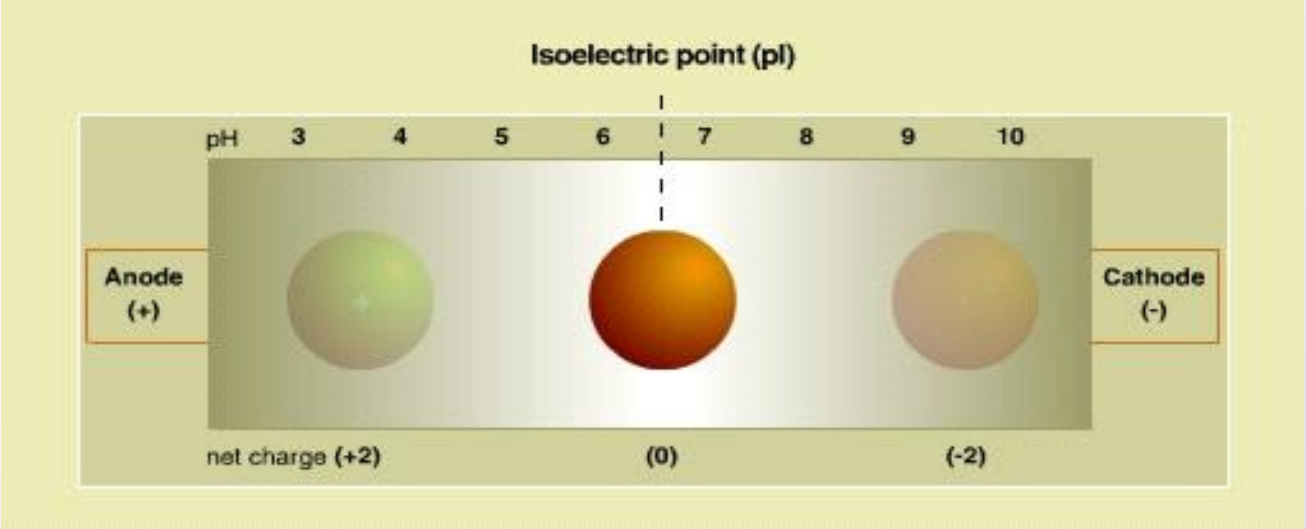
- second dimension

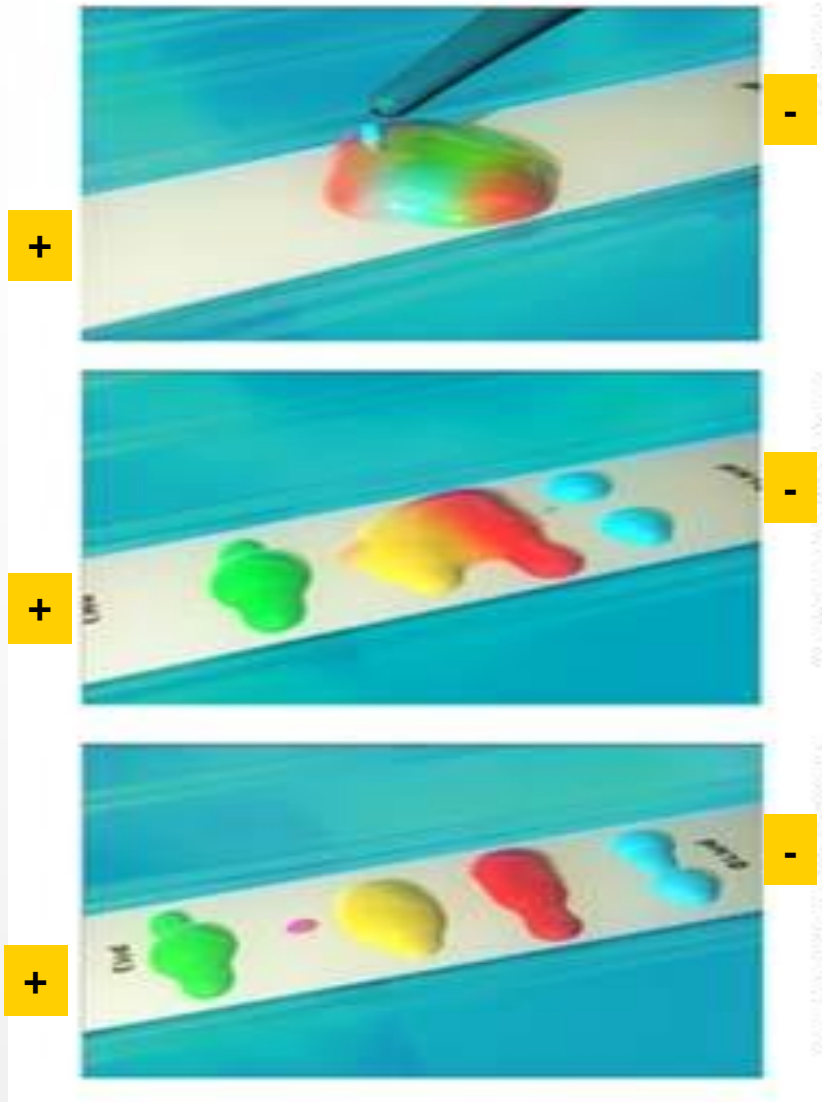
SDS-PAGE



1st dimension ISOELECTRIC FOCUSING

migration of charged molecules in pH gradient in electric field



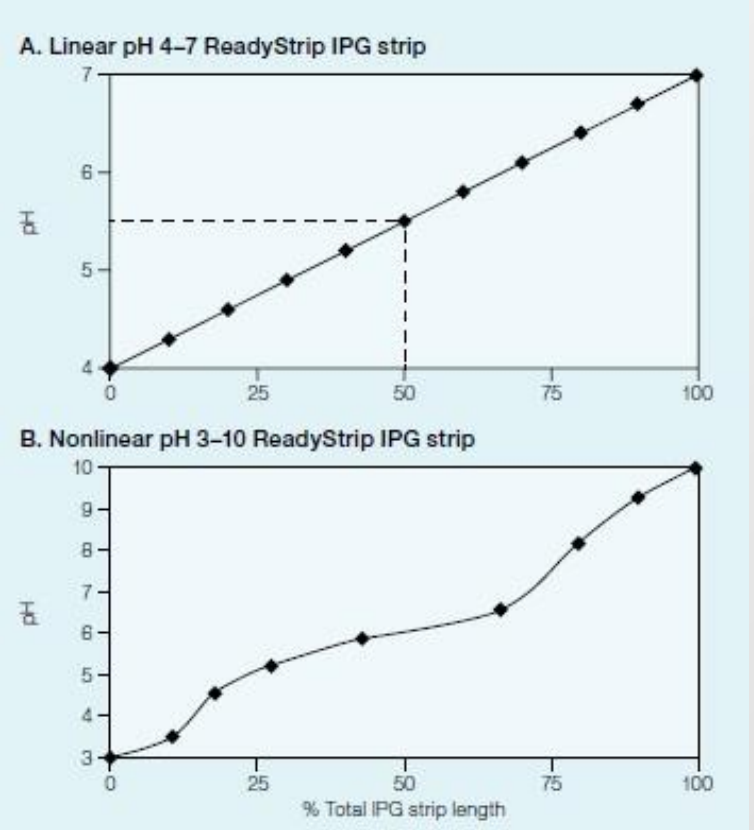


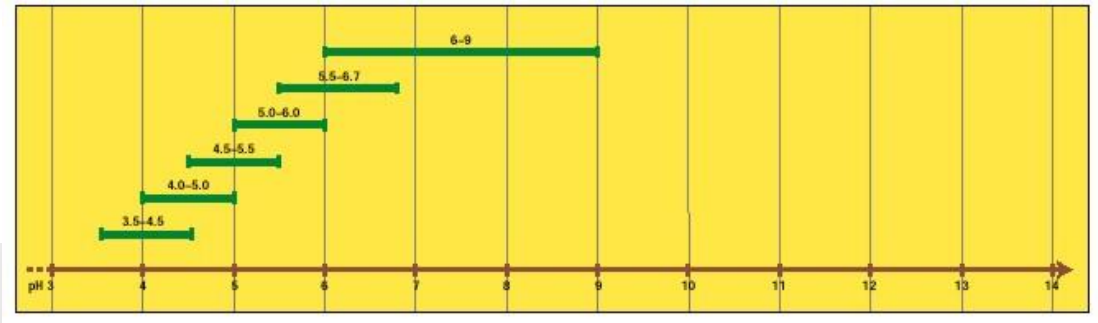
ISOELECTRIC FOCUSING

- immobilized pH gradient
- ampholytes

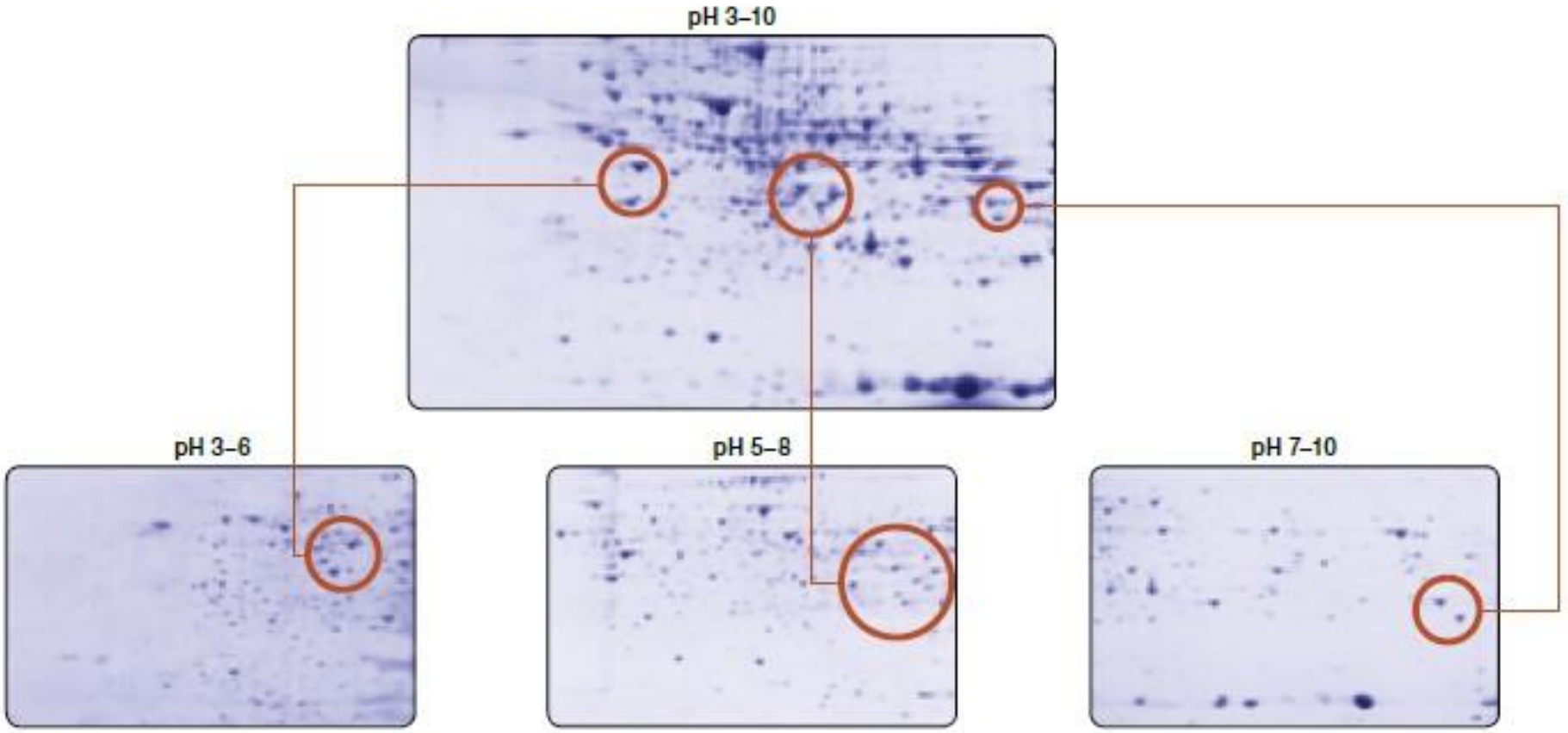
RANGE OF STRIP
SIZE OF STRIP

!



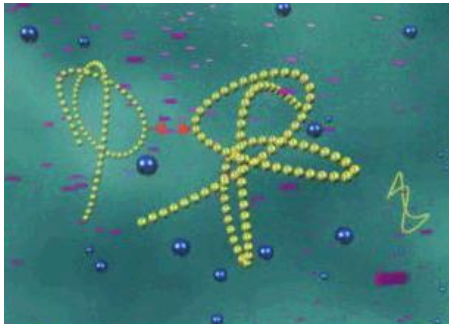


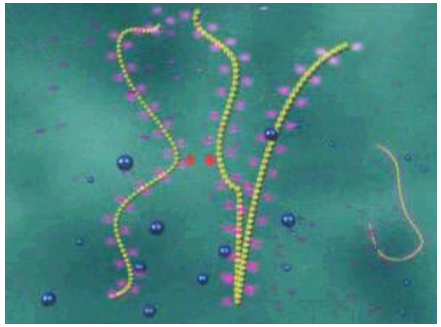
RANGE OF STRIP

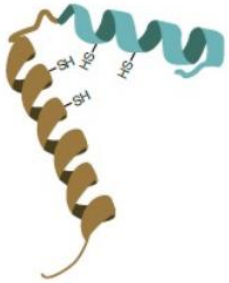


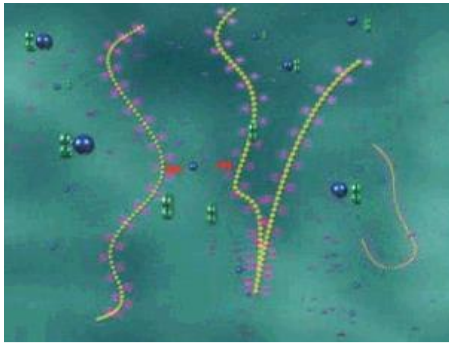
EQUILIBRATION OF STRIP !

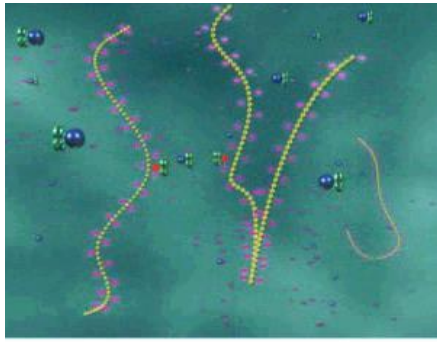
denaturation **SDS** •

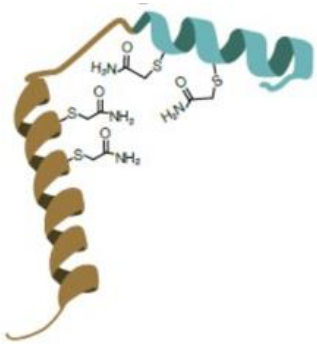








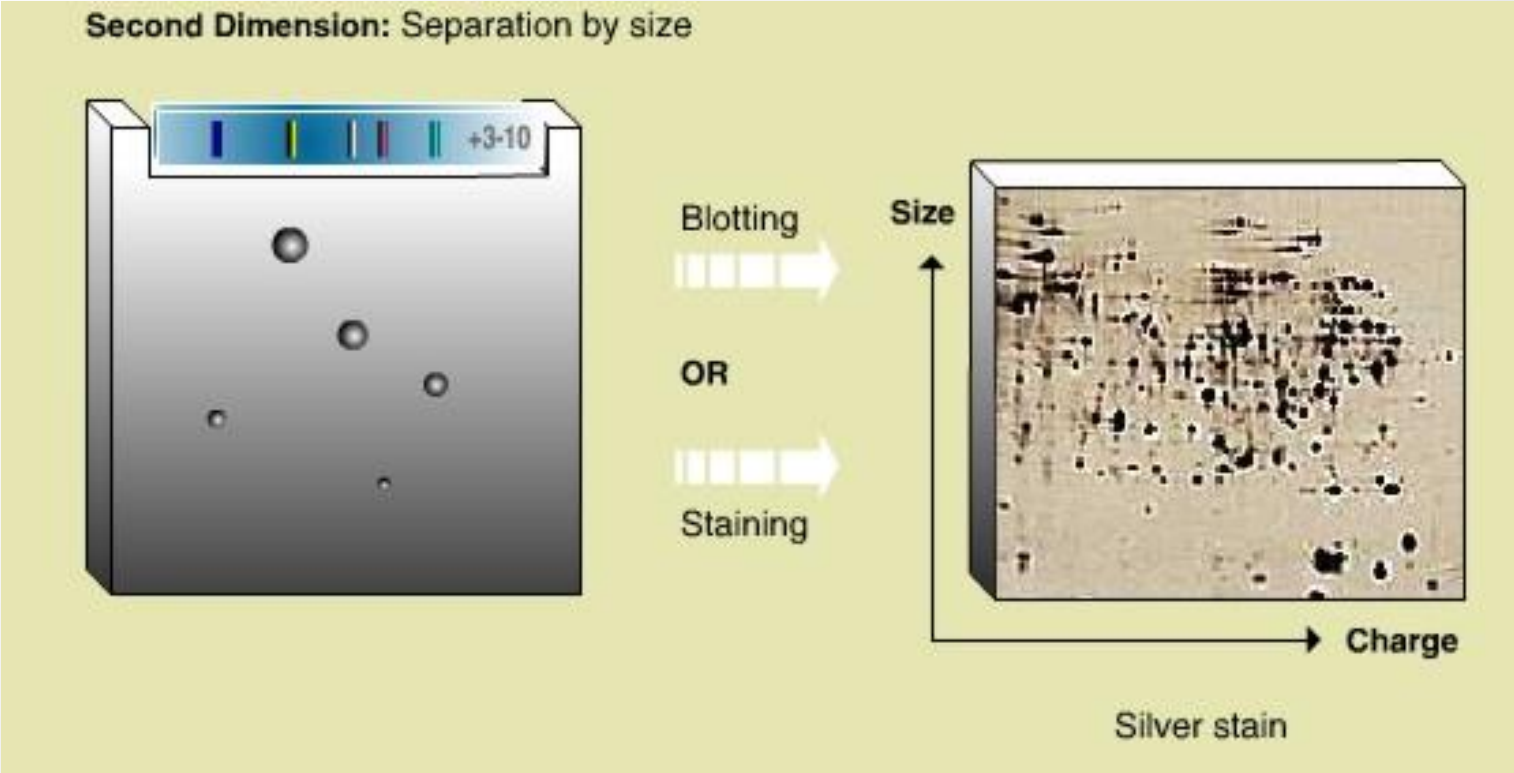




reduction **DTT** ● alkylation **IAA** •

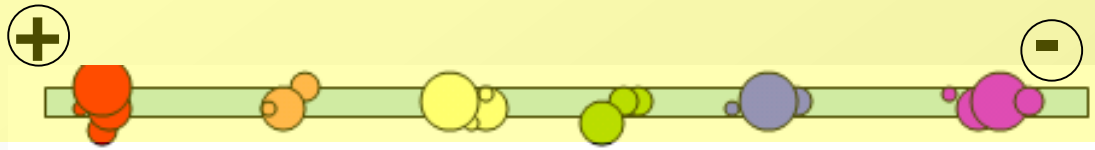
2nd dimension SDS-PAGE !

Migration of anions in electric field according to MW



FOCUSING

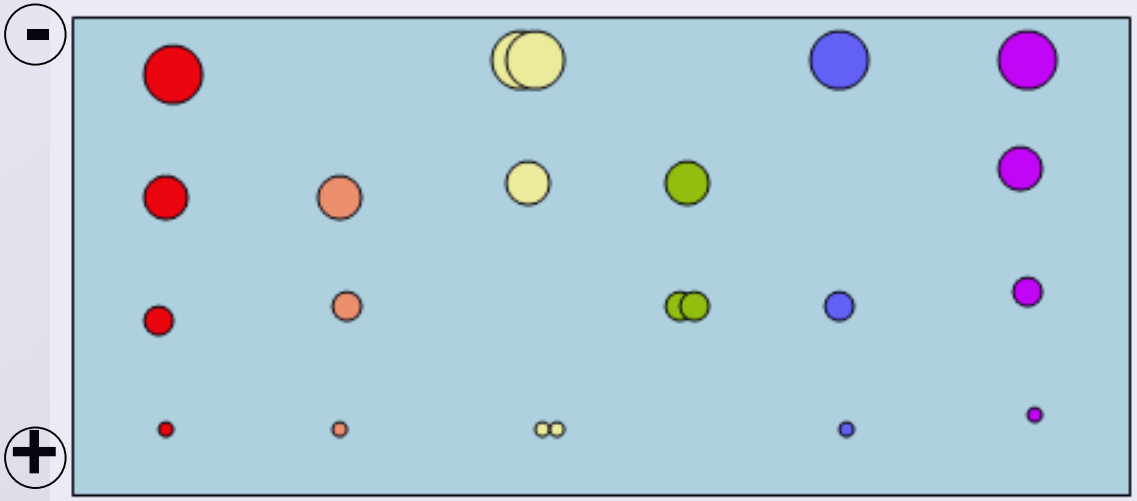
!



STRIP

equilibration

SDS-PAGE



GEL

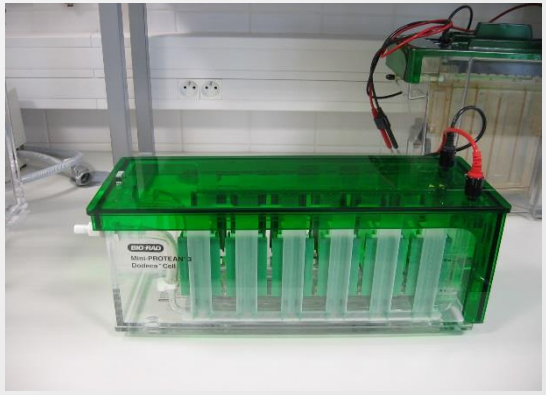
Gel orientation

2-DE INSTRUMENTATION

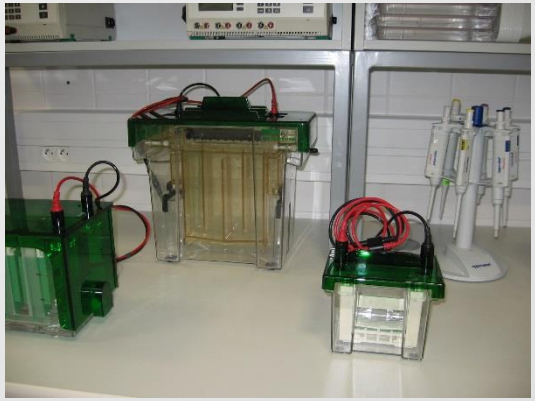
- Protean IEF
- Protean Dodeca Cell
- Densitometer GS-800
- FLA-7000, STORM

PDQuest, Quantity One

Protean Plus Dodeca Cell



Mini-Protean 3 Dodeca Cell



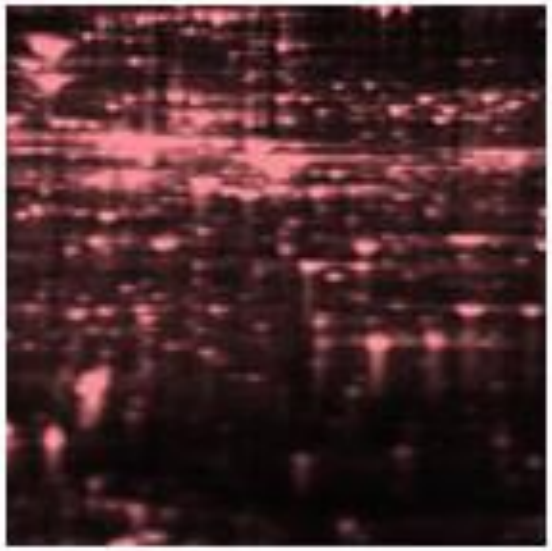
Protean II xi Cell

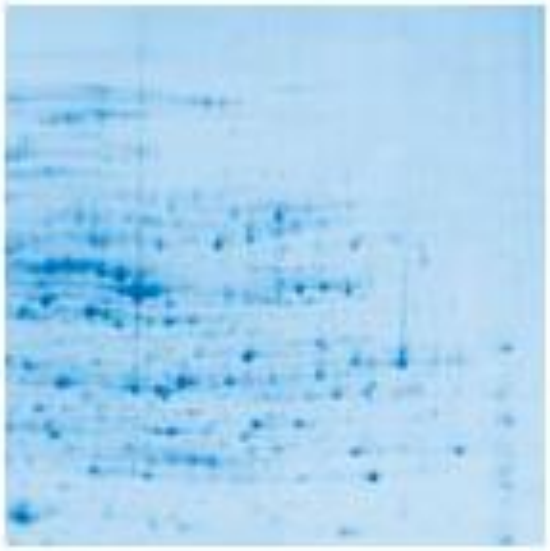
PROTEIN DETECTION

- gel x blot
- visualisation → staining
  → radioactivity assay
  → immunodetection

- staining in gel
  - post-electrophoretic
  - pre-electrophoretic
  - protein specific
  - PTM specific
  - visible spectrum
  - fluorescence

PROTEIN DETECTION IN GEL



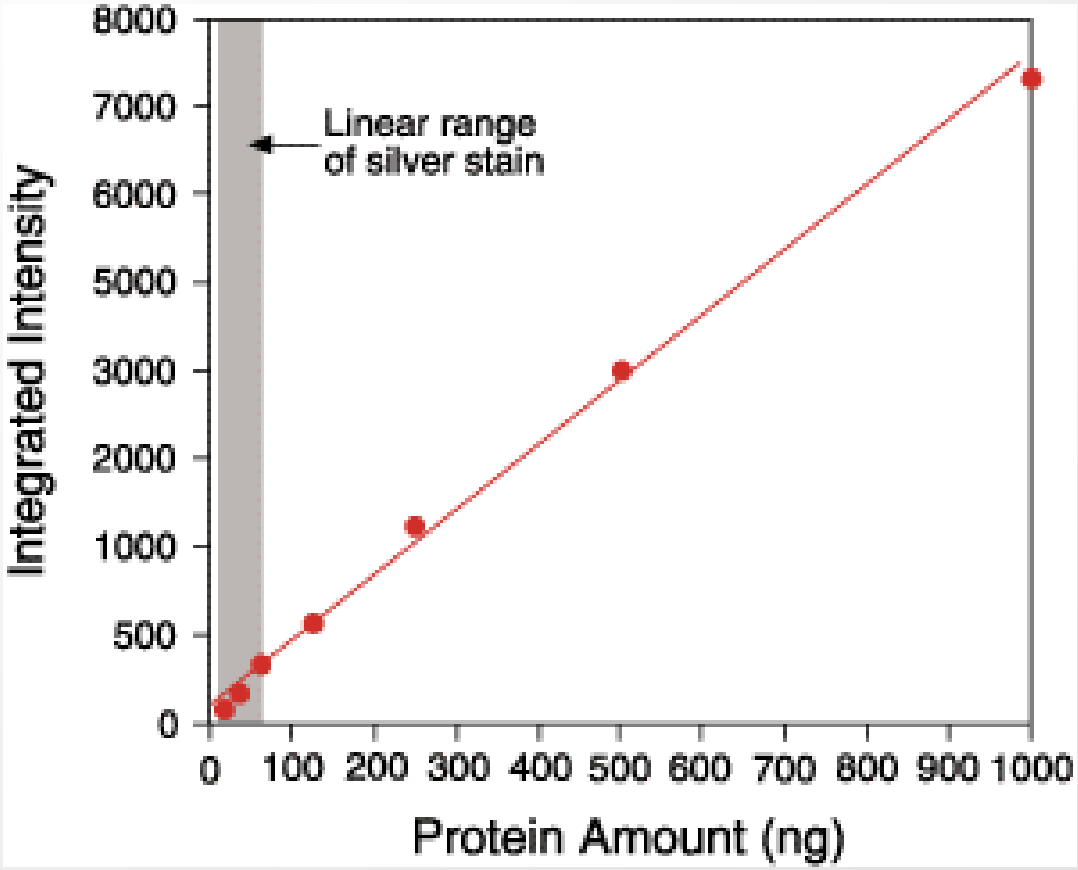


Sypro Ruby	Coomassie	silver
1.4 ng	36 ng	0.6 ng

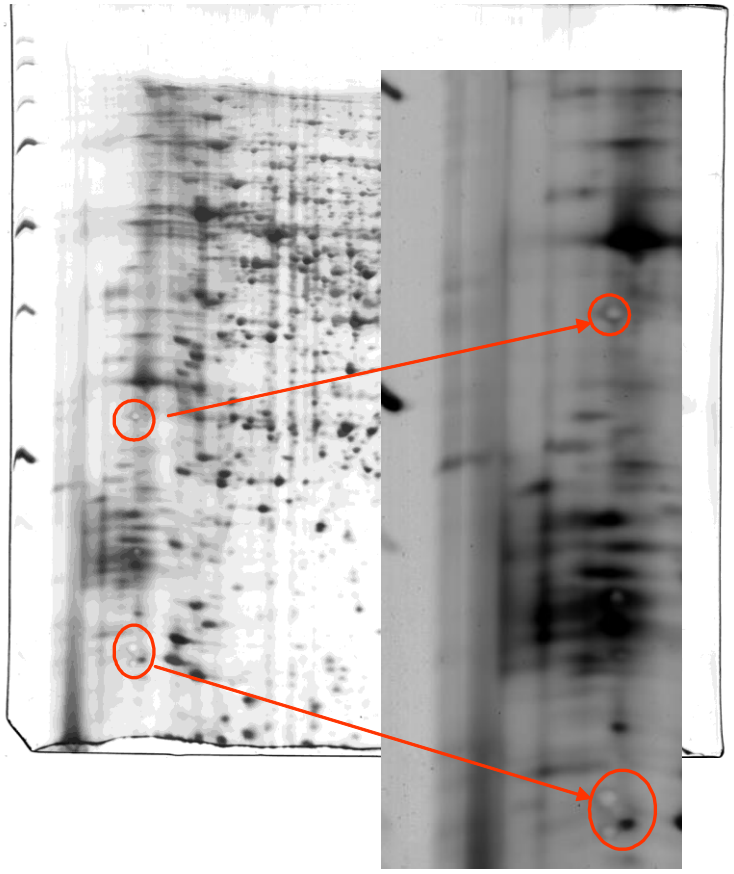
PTM specific staining *Pro-Q Diamond* *Pro-Q Emerald*

PROTEIN STAINING – LINEARITY

Sypro Ruby



Ag



Sypro Ruby

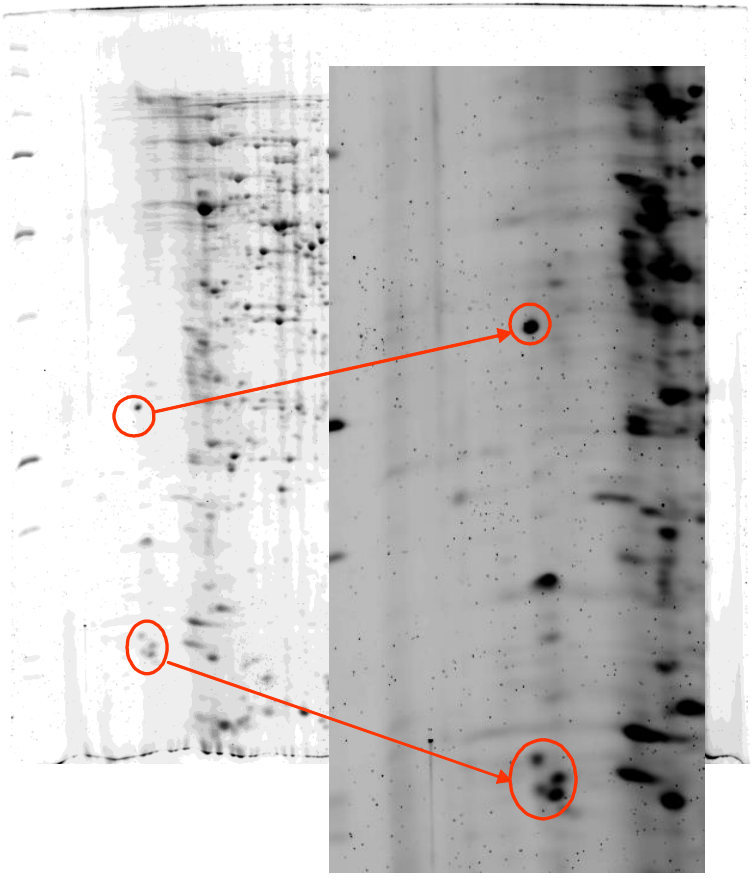
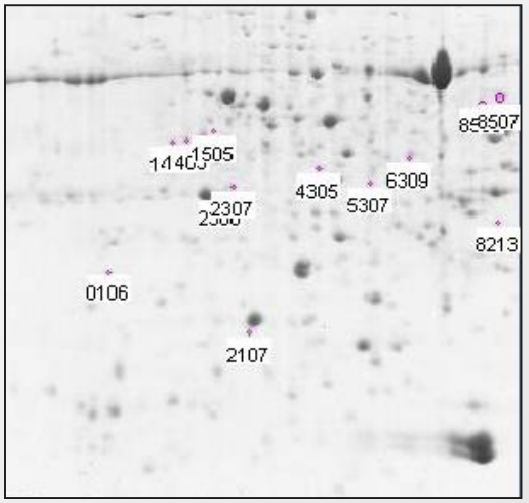
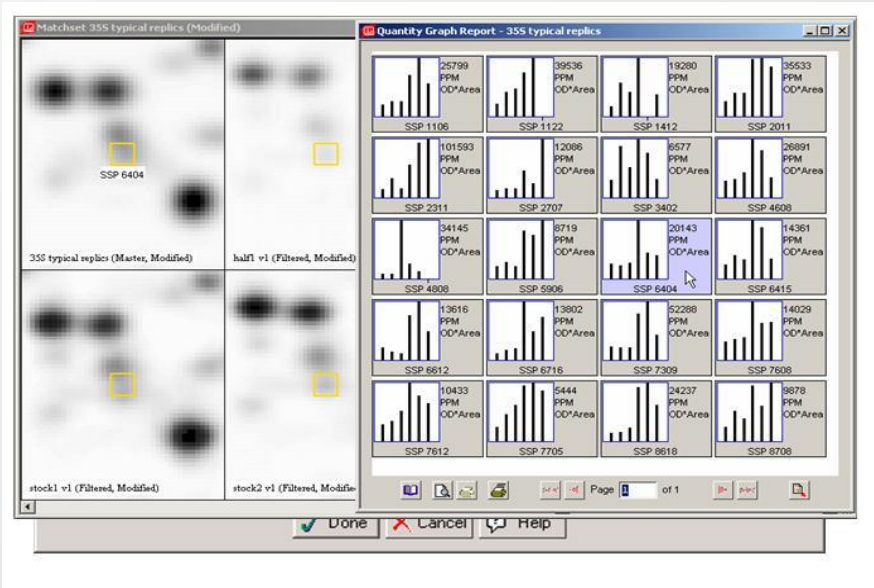


IMAGE ANALYSIS

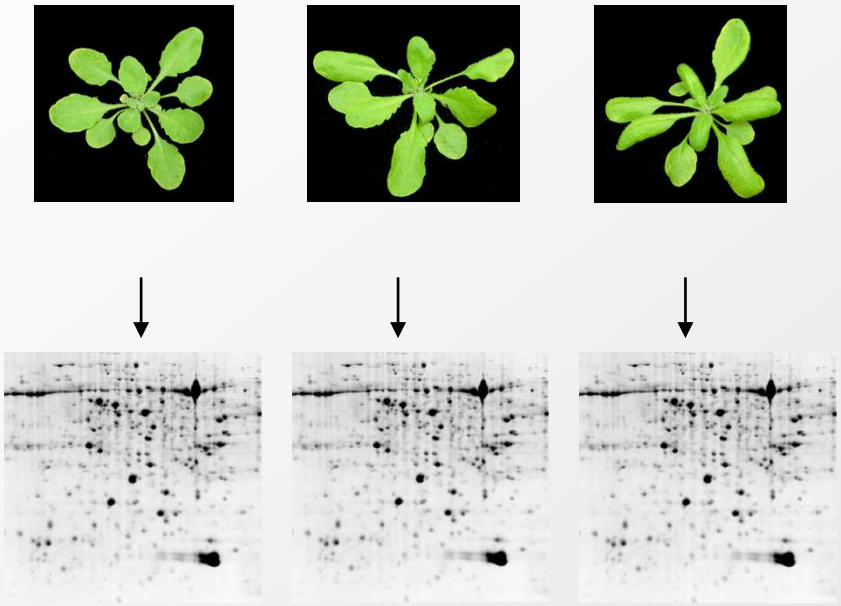
- quality
- quantity





biological variability

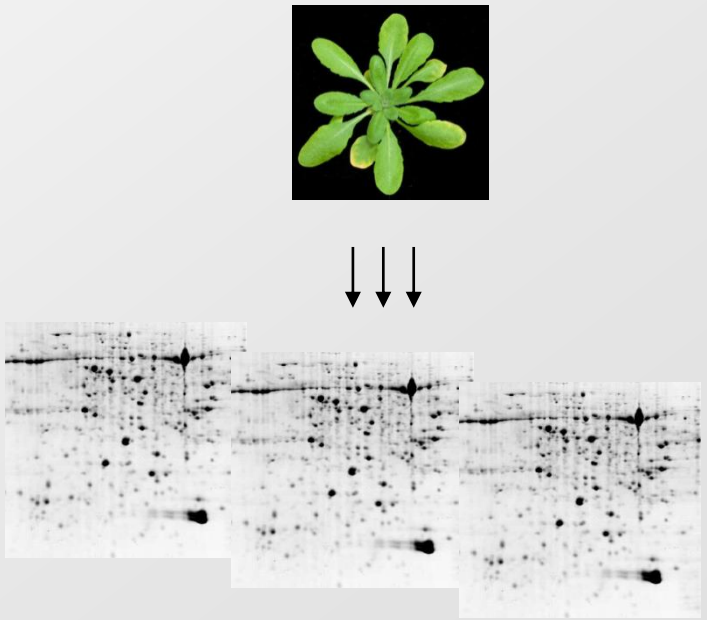
three plantelets analysed
separately under same conditions



biological replicates

technical variability

same plantelet analysed
three times under same conditions



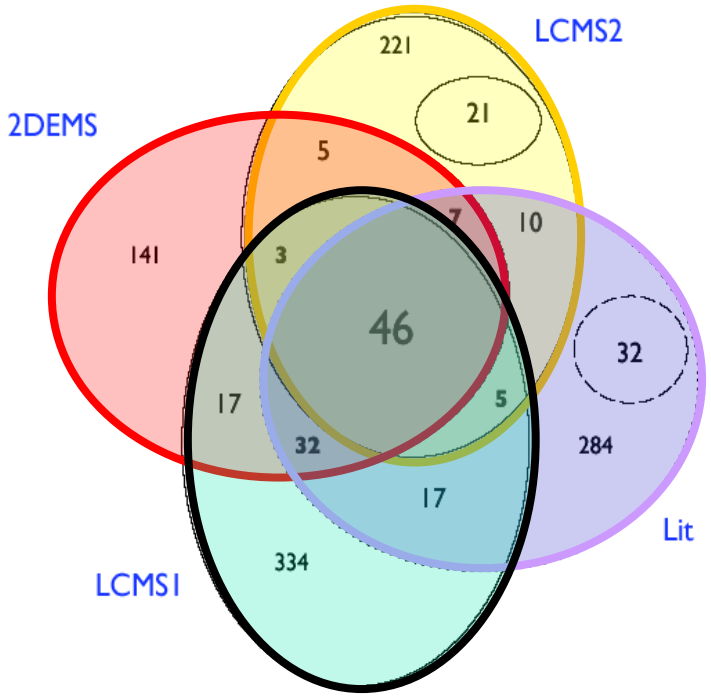
technical replicates

2D or not 2D ?

- visual aspects
- reproducibility
- dynamic range
- extreme proteins (membrane, basic...)
- difficult automatization
- postdigestion extraction

Different Platforms See Different Plasma Proteomes: Small Overlap of Four Plasma Proteome Datasets

(Number of NR proteins)



- 46 proteins in all four lists
- 195 proteins in 2 or more lists
- 1175 NR proteins total

From: The Human Plasma Proteome: A Non-Redundant List Developed by Combination of Four Separate Sources, N. L. Anderson et al, Molec. Cell Proteomics, 3: 311-326 (2004).

PPI

MULTIDIMENSIONAL CHROMATOGRAPHY

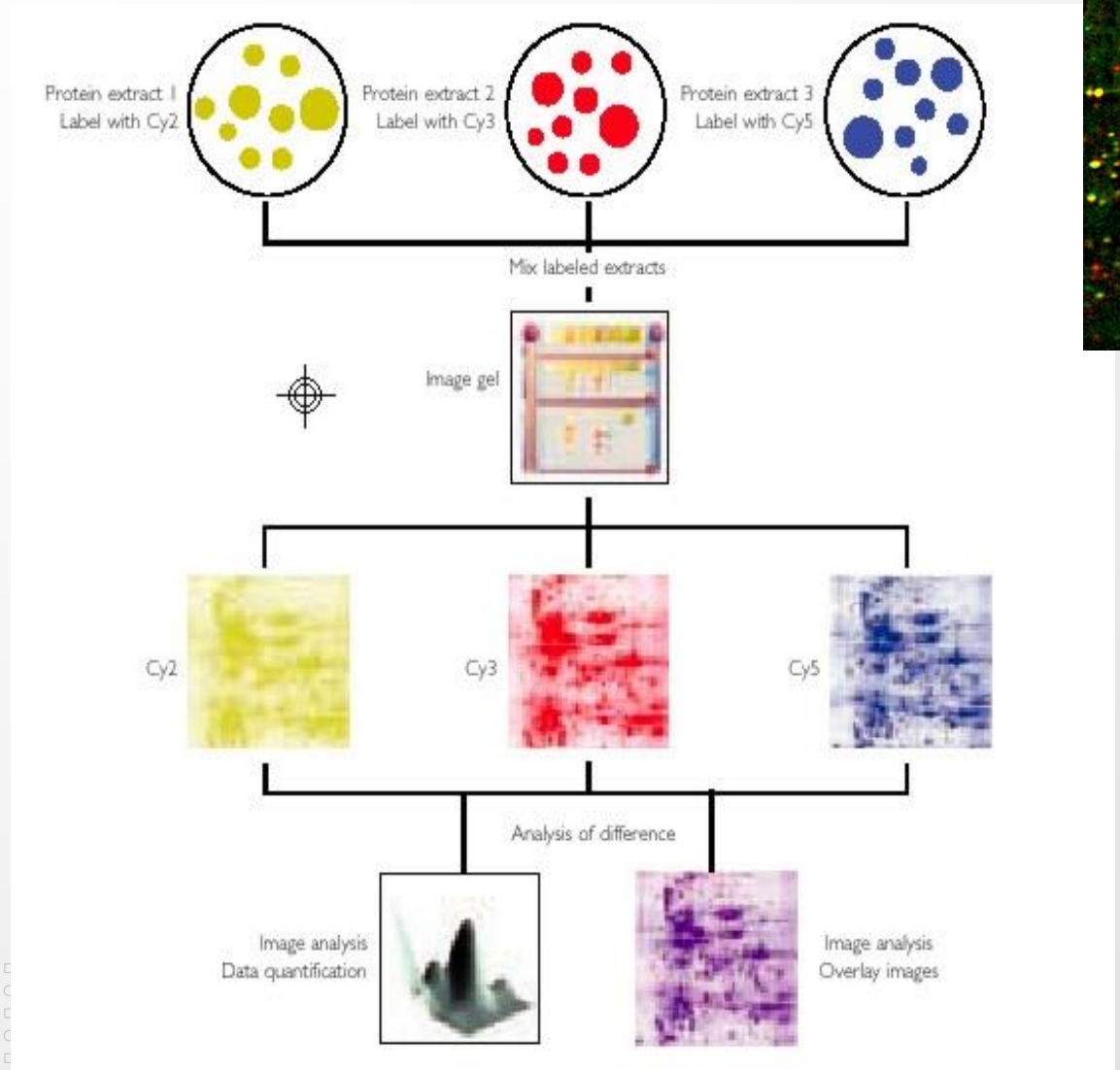
FOR

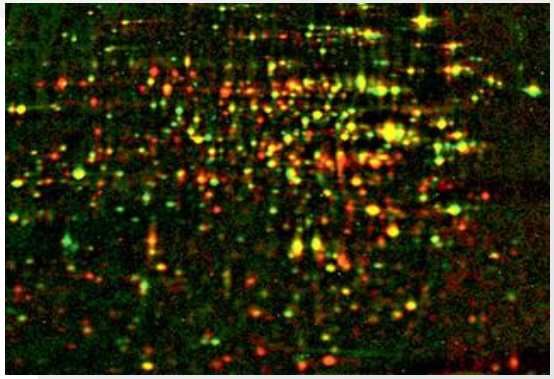
- large sample volumes
- on-column concentration
- membrane proteins, basic proteins
- no staining
- peptides – going directly to MS
- automatization

AGAINST

- vizual aspects lost: pI a Mr
- LC - serial analysis
- GE - more samples in paralel

Difference Gel Electrophoresis DIGE





BIOMARKERS

… NEEDLE IN HAYSTACKS

prefractionation ▪ separation ▪ identification ▪ control vs. sample

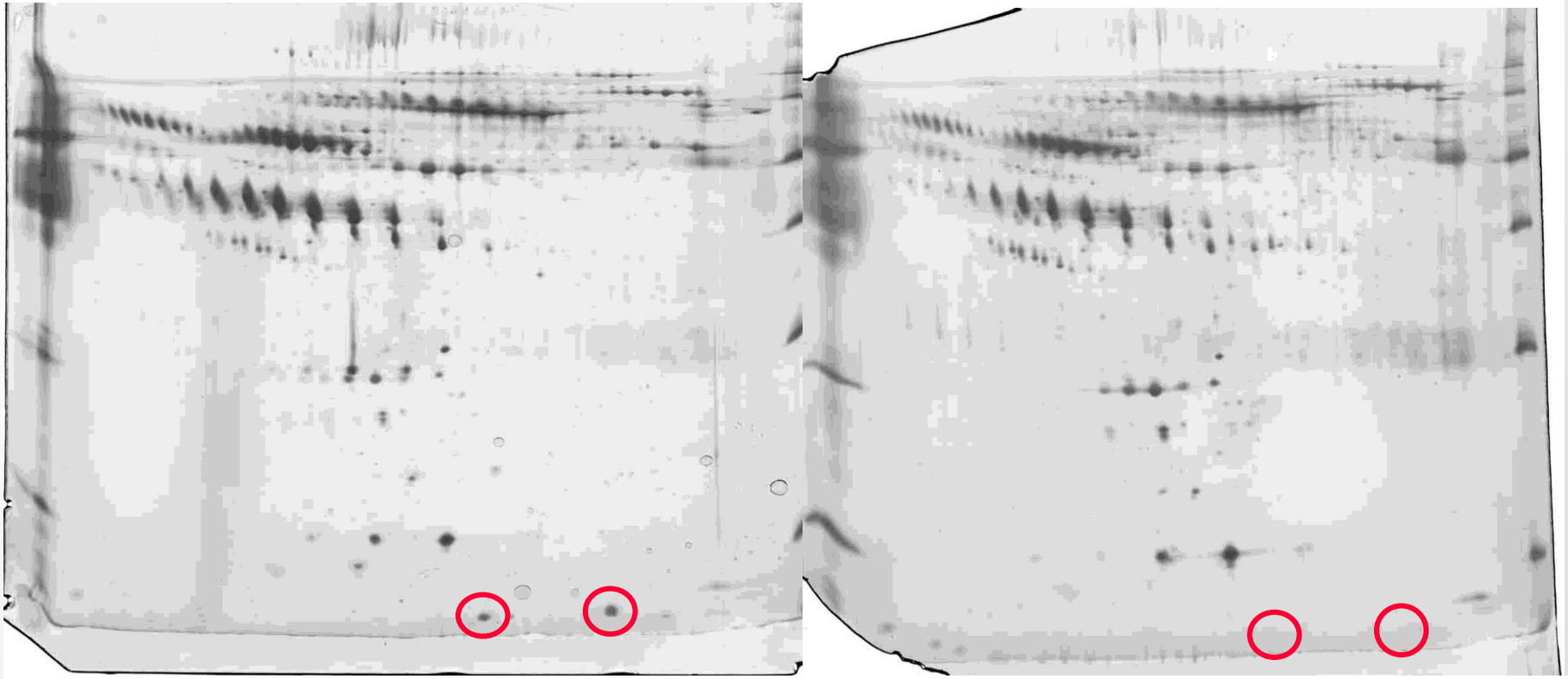
- **haystack** - proteins without relation to disease
- **neeedles** – disease specific proteins
- potencial needles **difficult to validate**
- are needles worth further examination?
- often contain **PTM,** difficult to be identified by MS

biological variability!

Biomarkers in human plasma

Day 21 – before clinical manifestation

Day 44 – after clinical manifestation



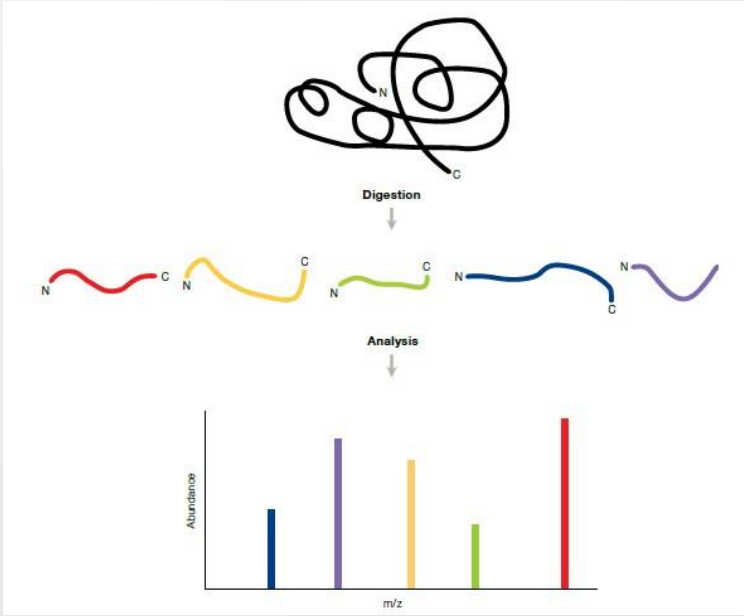
separation → identification

DIGEST

trypsin Glu-C Asp-N thermolysin

MAVEPFPRRPITRPHASIEVDTSGTGGSAGSSEKVF
CLIGQAEGGEPNTVYELRNYAQAKRLFRSGELLDAI
ELAWGSNPNYTAGRILAMRIEDAKPASAEIGGLKIT
SKIYGNVANNIQVGLEKNTLSDSLRLRVIFQDDRFN
EVYDNIGNIFTIKYKGEEANATFSVEHDEETQKASR
LVLKVGDQEVKSYDLTGGAYDYTNAIITDINQLPDF
EAKLSPFGDKNLESSKLDKIENANIKDKAVYVKAVF
GDLEKQTAYNGIVSFEQLNAEGEVPSNVEVEAGEE
SATVTATSPIKTIEPFELTKLKGGTNGEPPATWADKL
DKFAHEGGYYIVPLSSKQSVHAEVASFVKERSDAGE
PMRAIVGGGFNESKEQLFGRQASLSNPRVSLVANS
GTFVMDDGRKNHVPAYMVAVALGGLASGLEIGES
ITFKPLRVSSLDQIYESIDLDELNENGIISIEFVRNRTN
TFFRIVDDVTTFNDKSDPVKAEMAVGEANDFLVSE
LKVQLEDQFIGTRTINTSASIIKDFIQSYLGRKKRDN
EIQDFPAEDVQVIVEGNEARISMTVYPIRSFKKISVS
LVYKQQTLQA

- IN-GEL
- IN-SOLUTION



MS

G I G O

G I G O

GARBAGE IN - GARBAGE OUT

LITERATURE

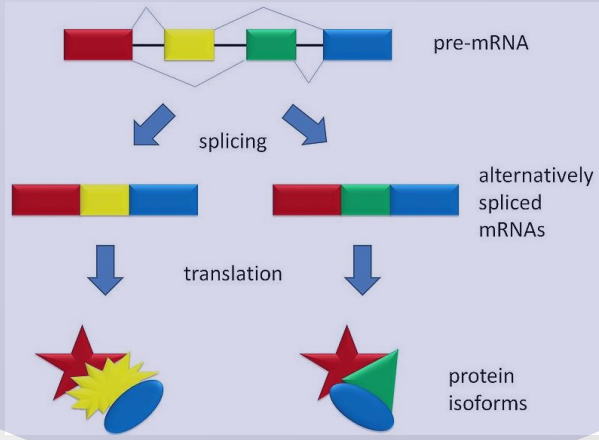
- R.M. Twyman: Principles of Proteomics
- R.Westermeier, T.Naven, H-R Höpker: Proteomics in Practice
- A.J.Link: 2D Proteome Analysis Protocols
- Current Protocols in Protein Science
- R.J.Simpson: Proteins and Proteomics
- T.Rabilloud: Proteome Research: Two-dimensional Gel
- Electrophoresis and Identification Methods
- A. Görg, W. Weiss, M.J.Dunn: Proteomics 2004, 4, 3665, rev.
- I. Miller, J. Crawford, E. Gianazza: Proteomics 2006, 6, rev.
- F.Chevalier: Proteome Science 2010, 8:23, review
- R. Burgess, M. Deutscher: Guide to Protein Purification

I. SEPARATION

II. PREFRACTIONATION

GENOME →

PROTEOME



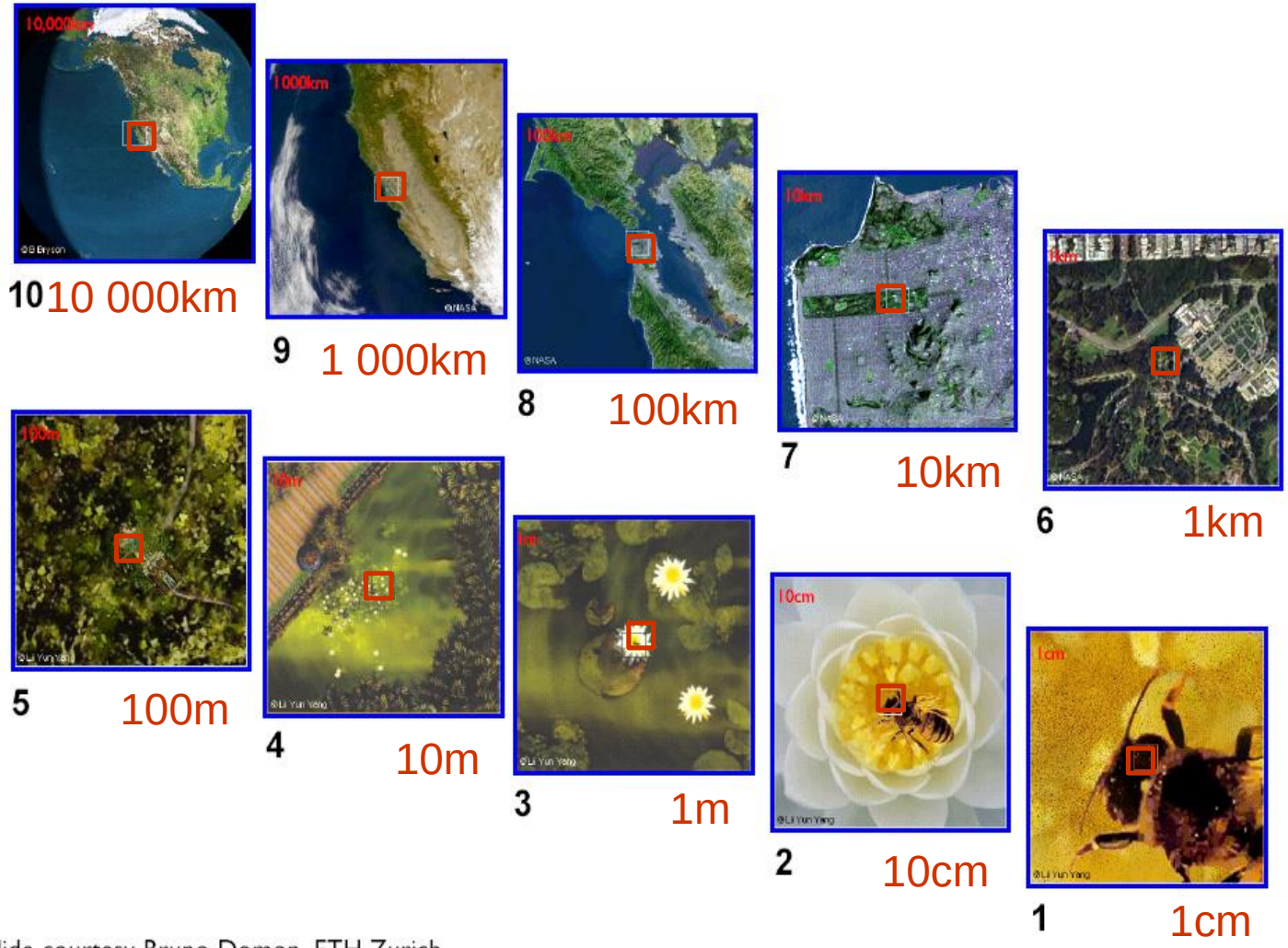
ISOFORMS

PTM ~200 variants (fosforylation, glykosylation, acylation, methylation…)

CONCENTRATION RANGE ~ 10 orders of magnitude ↓

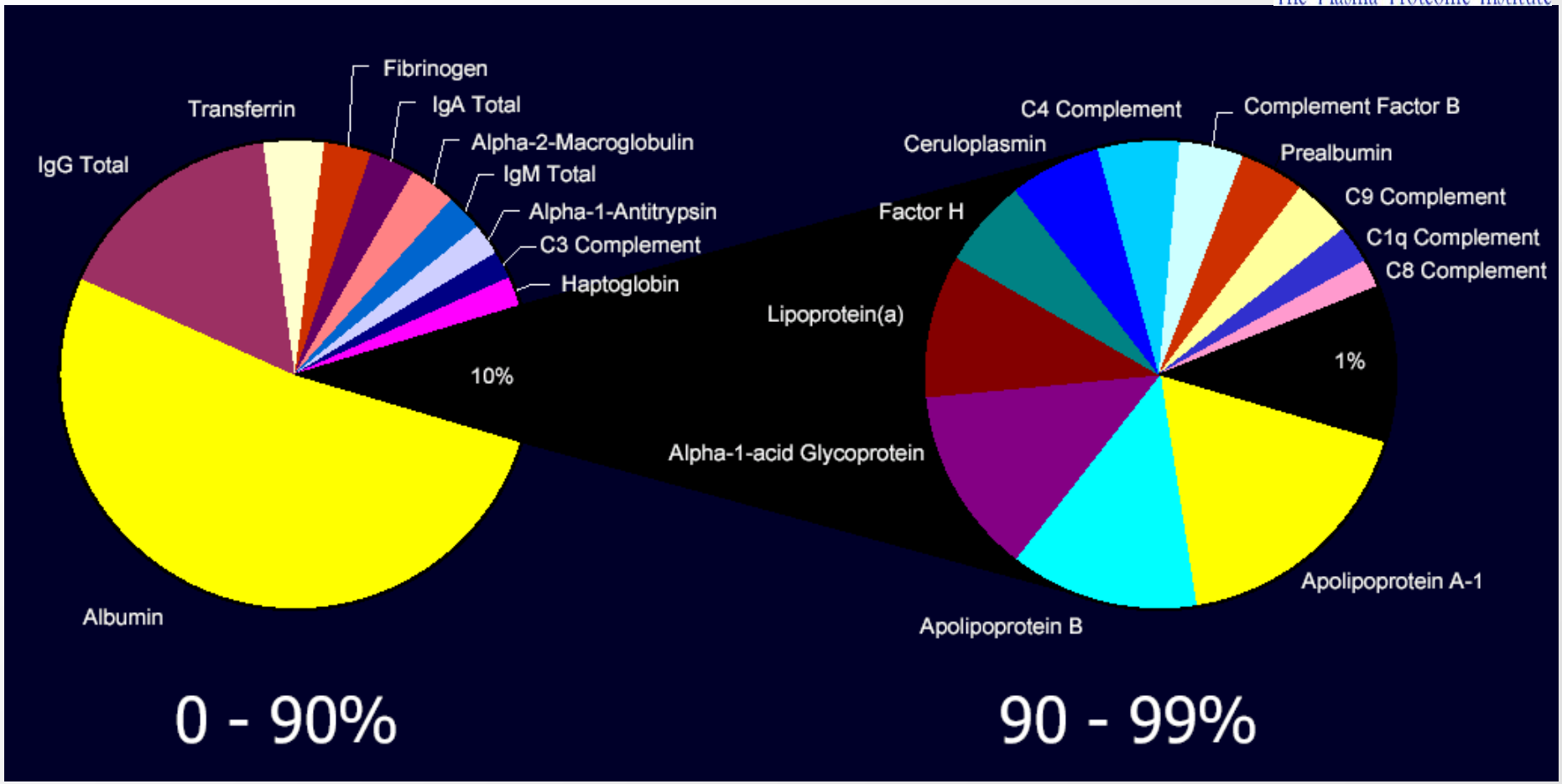
PREFRACTIONATION → MS

10^{10} Really Is Wide Dynamic Range



Slide courtesy Bruno Domon, ETH Zurich

Abundant proteins in human plasma

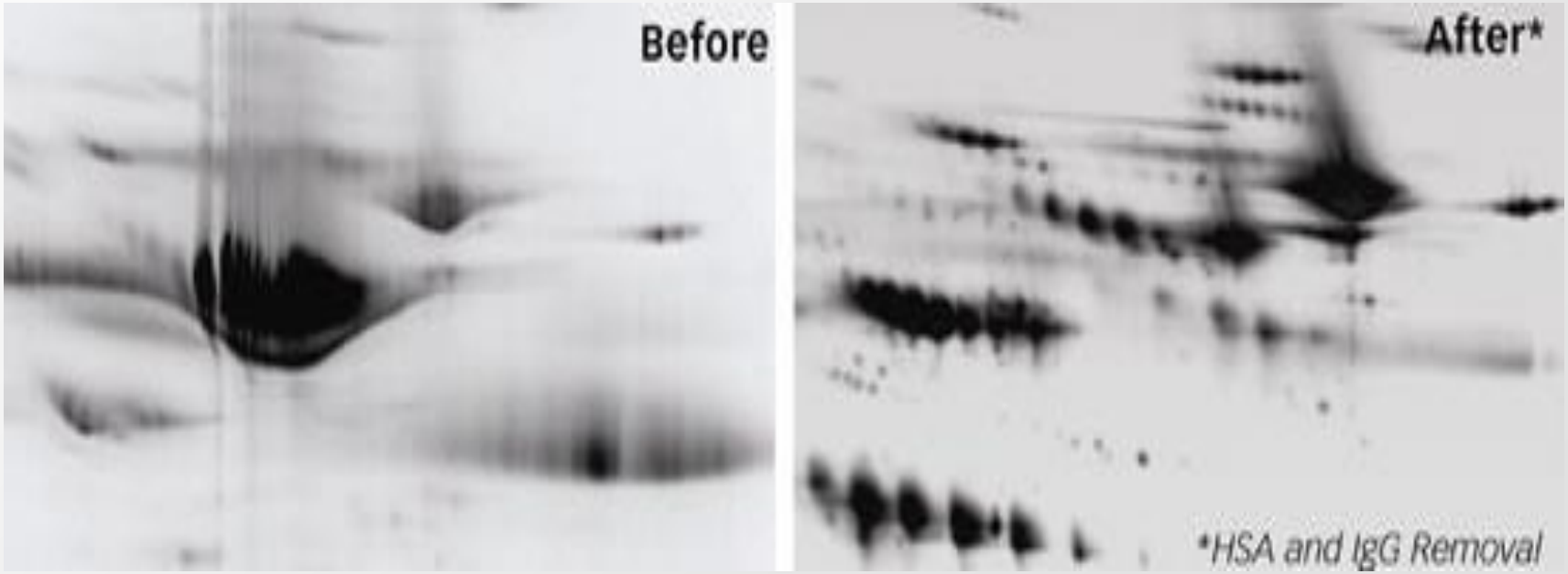


AFFINITY DEPLETION

Removal of abundant proteins by affinity chromatography

HSA

IgG

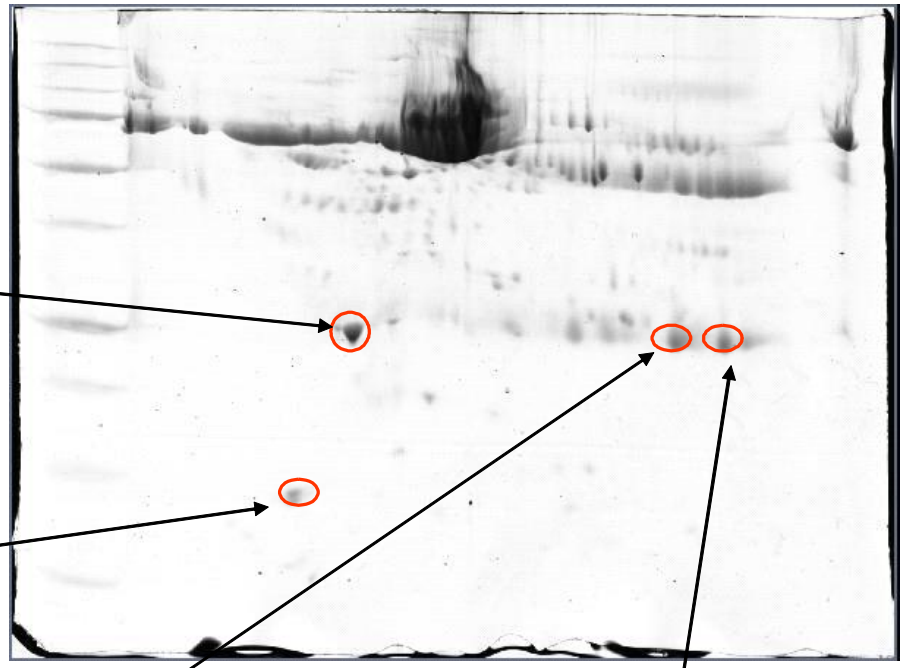


Human plasma – bound fractions after affinity depletion

ALBUMIN

IgG

Staining CBB G-250



Apolipoprotein

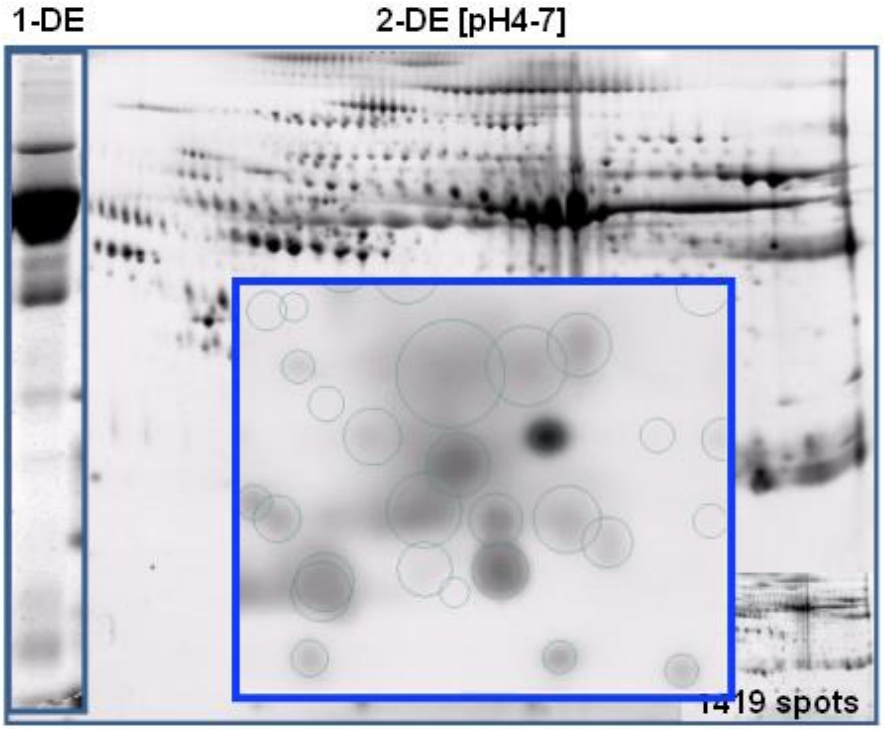
albumin

Immunoglobulin kappa light chain

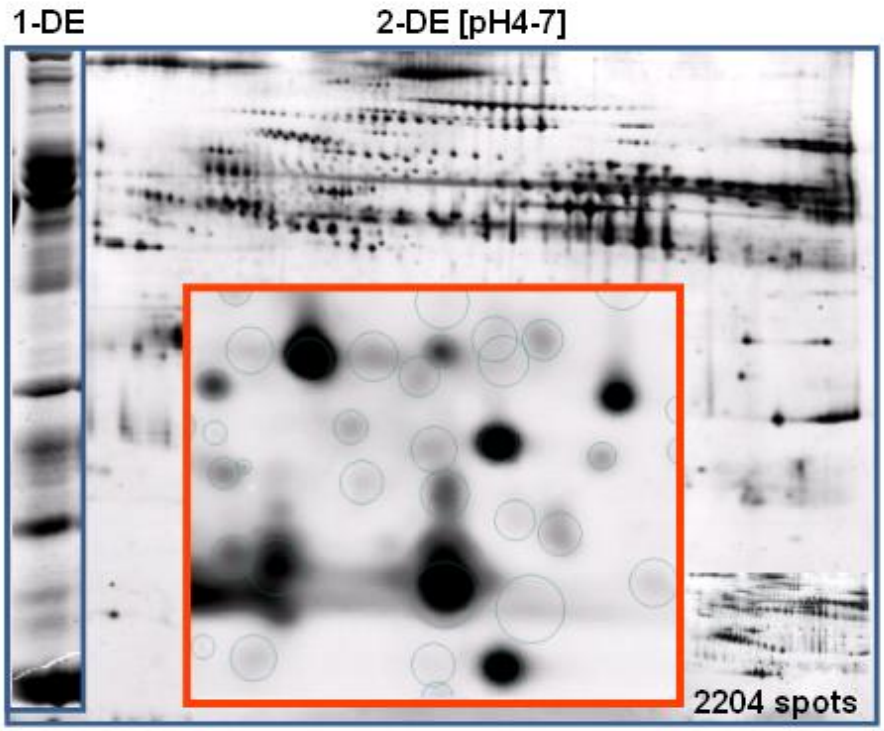
Immunoglobulin light chain

CPPL
Combinatorial Peptide Ligand Library

Native Human Serum

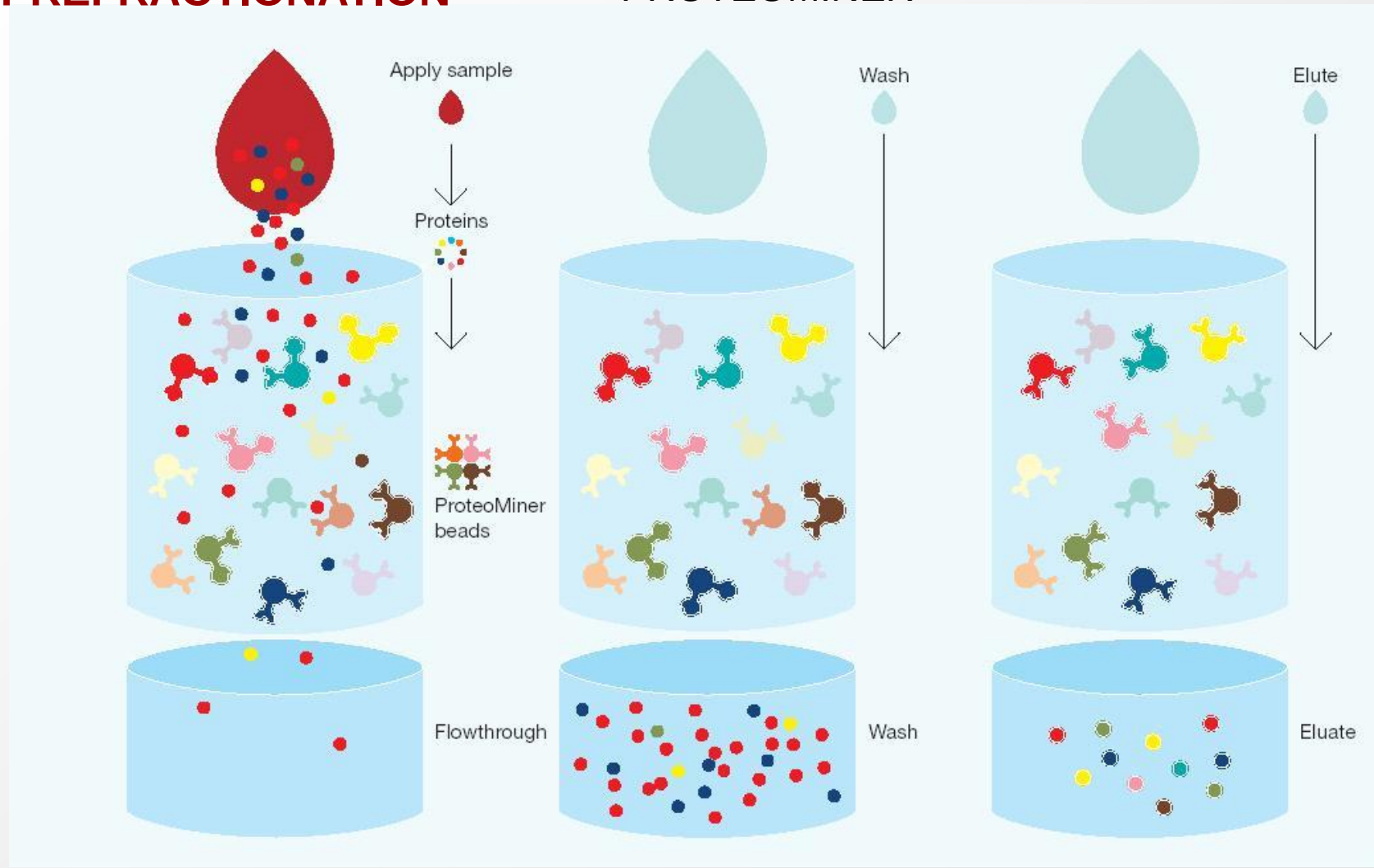


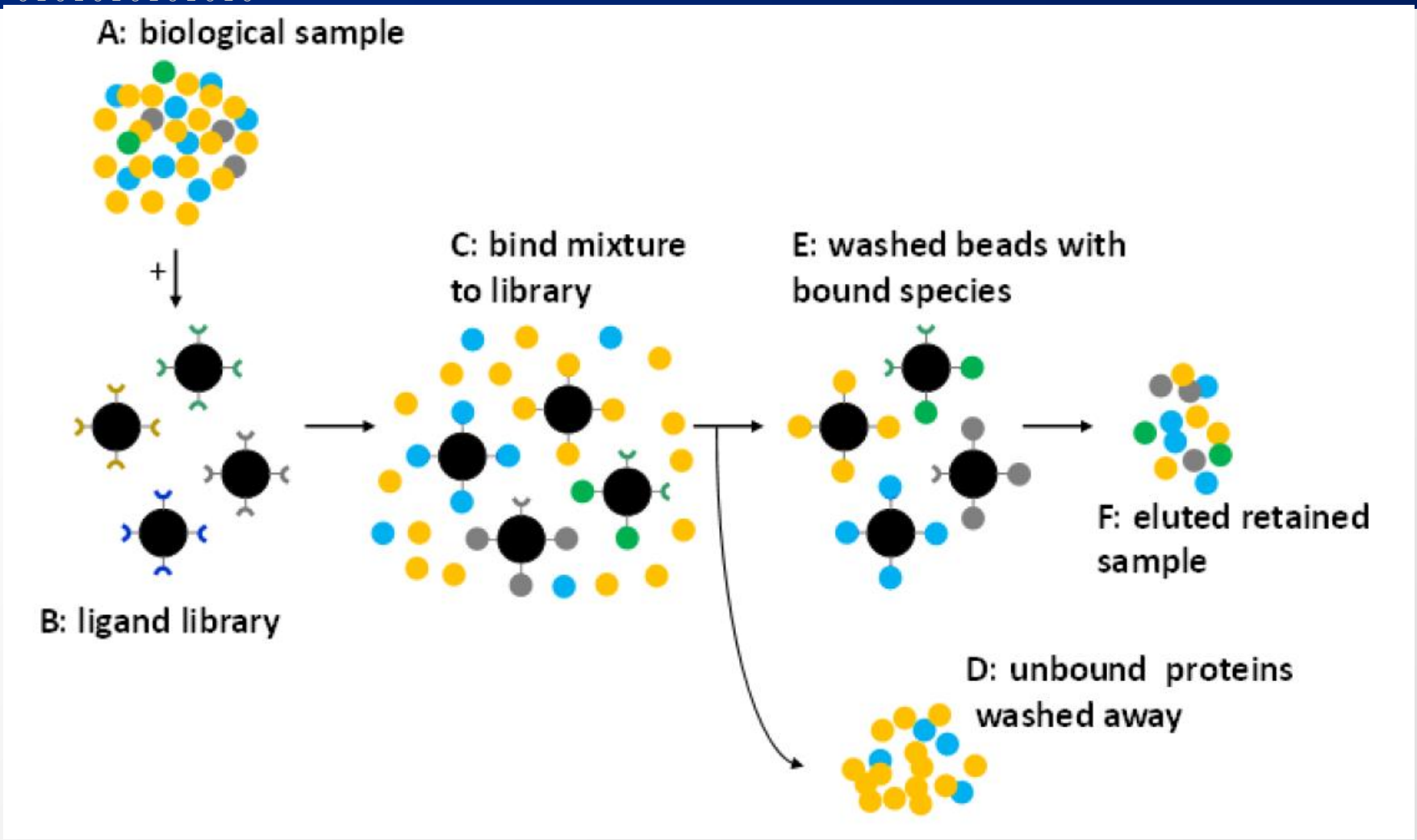
Human Serum Fractionated by ProteoMiner



PREFRACTIONATION

PROTEOMINER




A: biological sample
+
B: ligand library
C: bind mixture to library
D: unbound proteins washed away
E: washed beads with bound species
F: eluted retained sample

IEF prefractionation

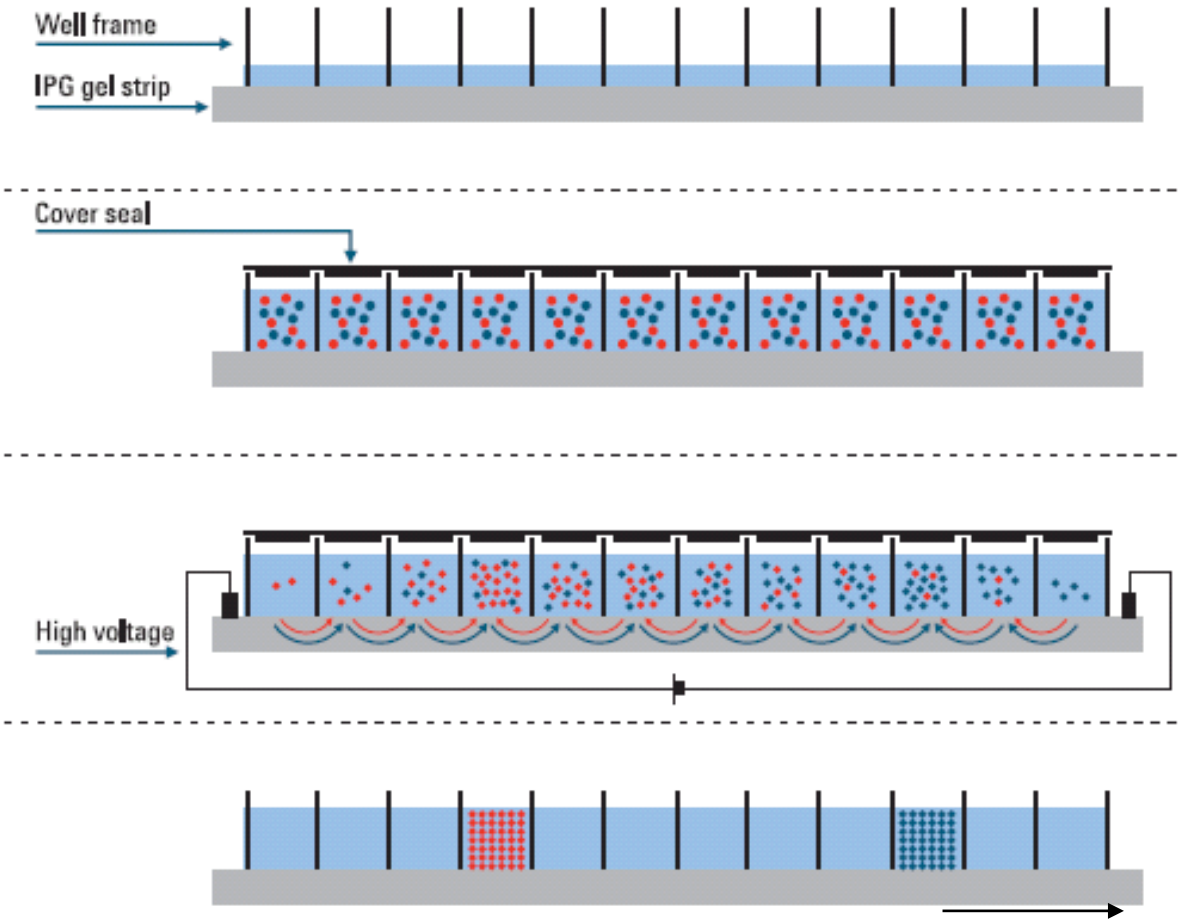
MicroRotofor

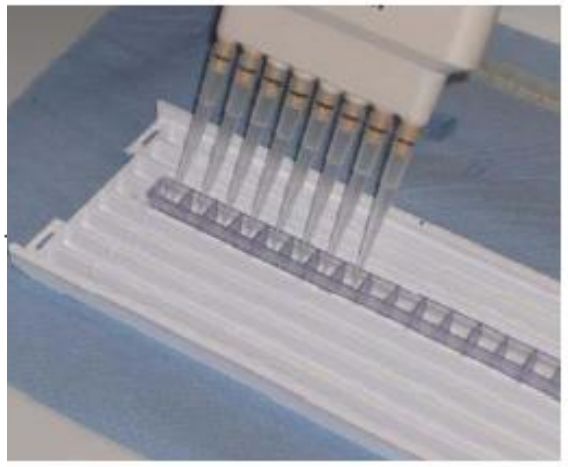
- prefractionation in solution

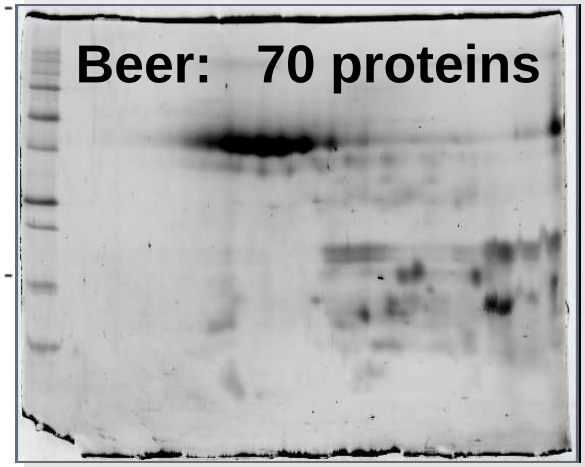
OffGel Fractionator

- prefractionation in solution using IPG strip

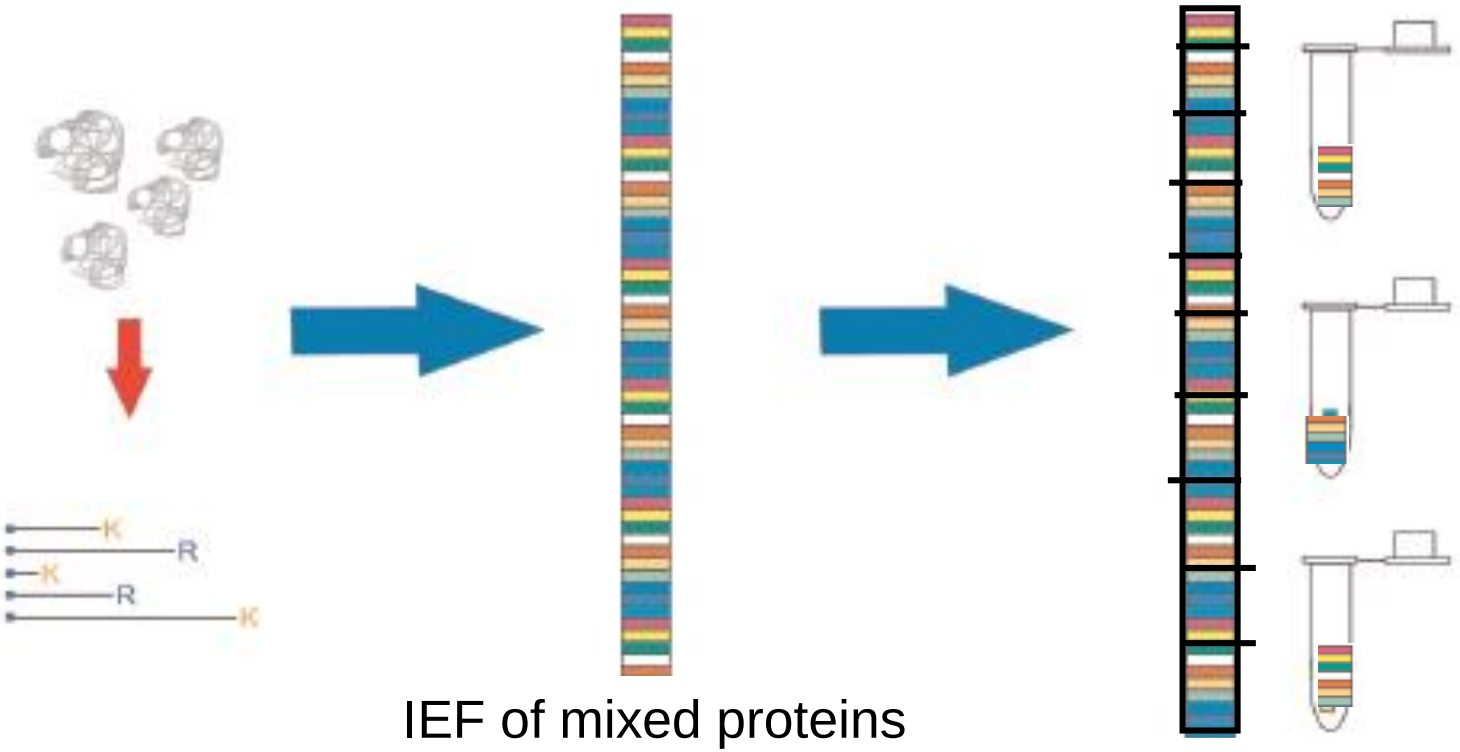
OFFGEL IEF prefractionation of proteins or peptides







IPG-IEF



Protein digest

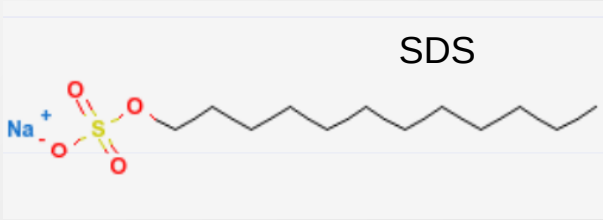
IEF of mixed proteins on the strip

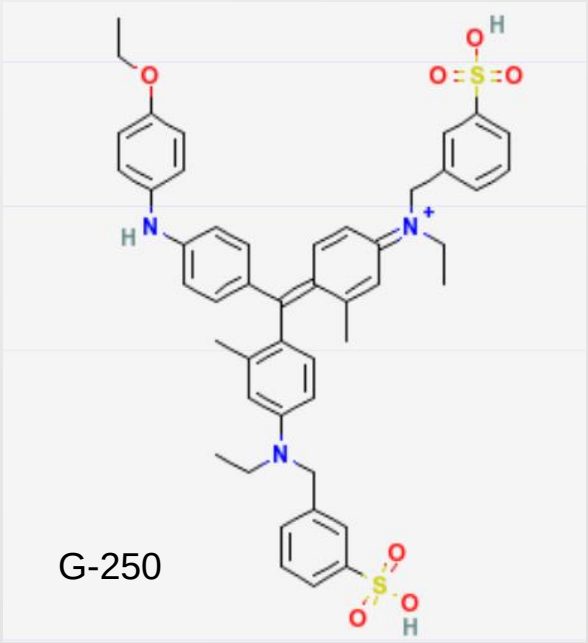
fractions of strip

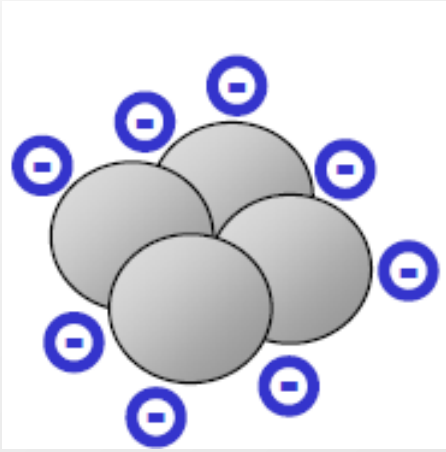
JOURNAL OF BIOMOLECULAR TECHNIQUES, VOLUME 16, ISSUE 3, SEPTEMBER 2005

Blue Native Electrophoresis BNE

- Separation of native proteins
- Separation of membrane complexes
- Solubilization by non-ionic detergents
- Charged by Coomassie G-250
- BN PAGE gel (strip/band) as 2D

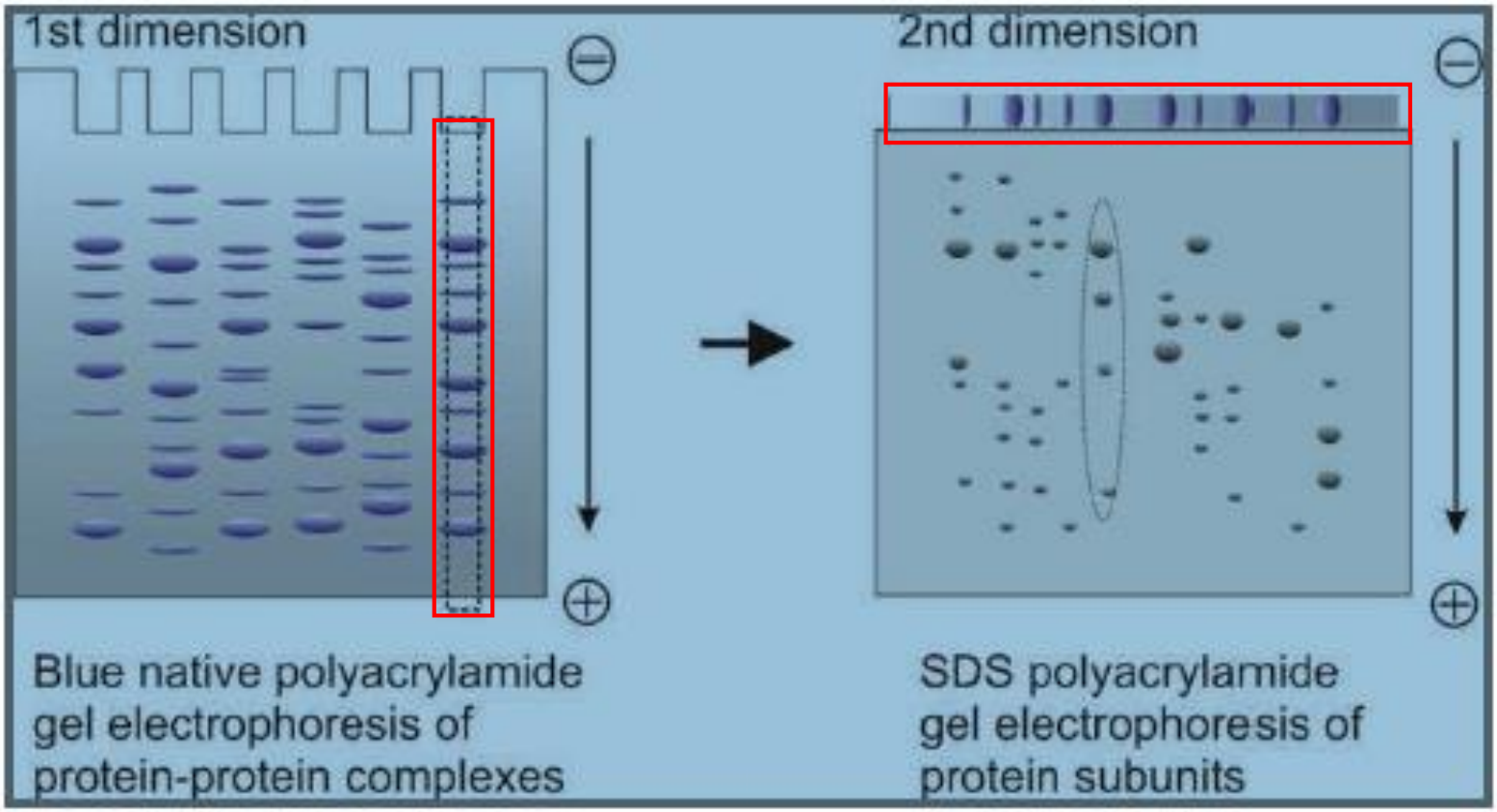






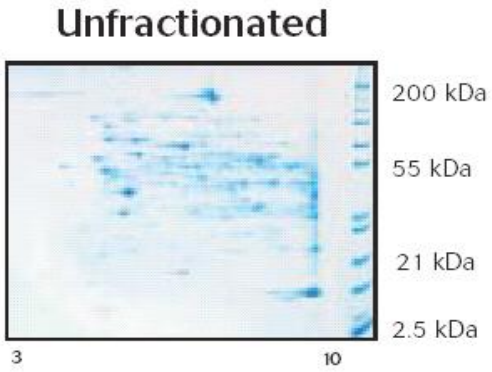
2DE

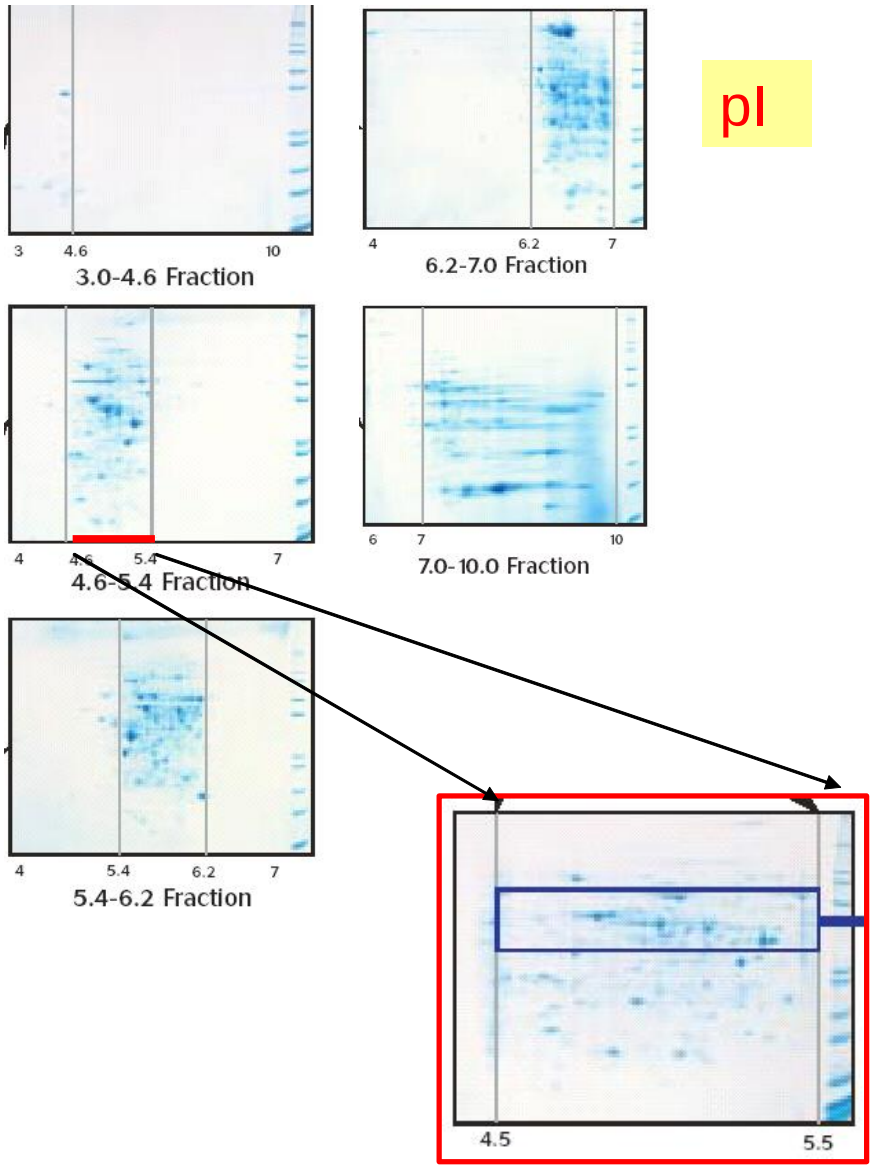
BNE SDS-PAGE



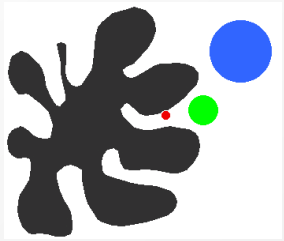
PREFRACTIONATION

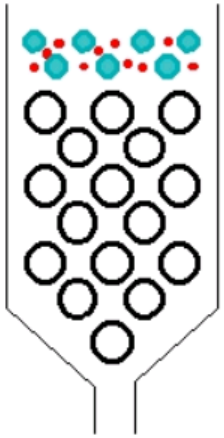
MICRO RANGES

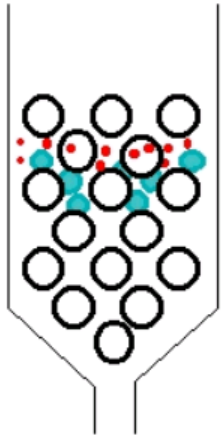


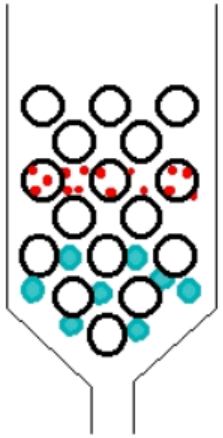


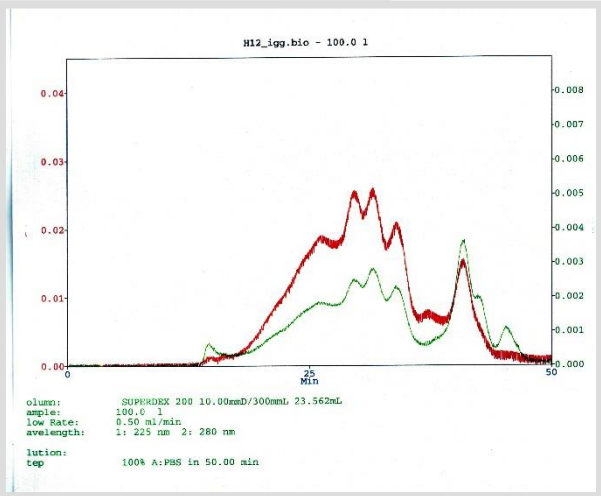
GEL CHROMATOGRAPHY











FASP Filter aided sample preparation

PROTEINS

Lysate
SDS DTT

spin

8M Urea
IAA

spin

SDS
DTT

Ammonium
bicarbonate

spin

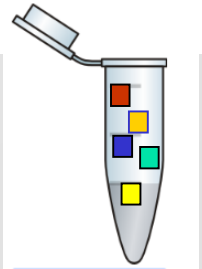
8M Urea
IAA

Trypsin

incubation
spin

proteins
nucleic
acids

PEPTIDES

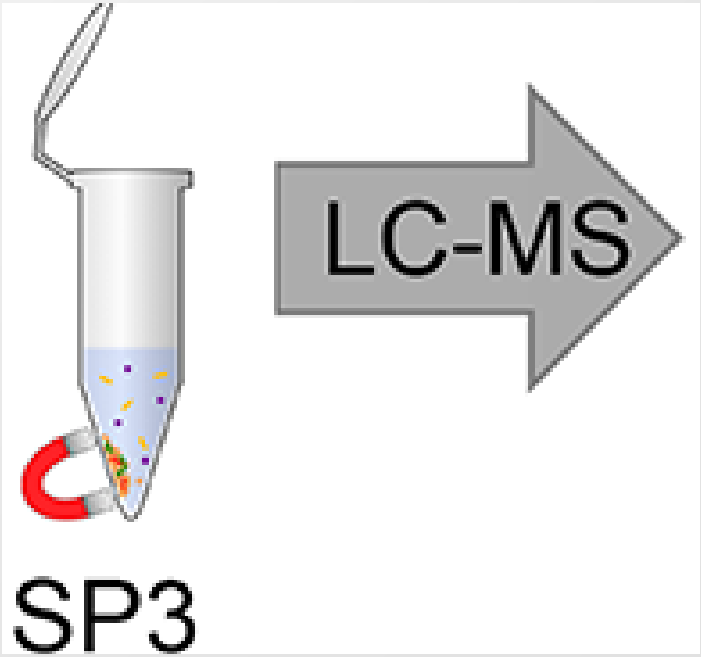


Nature Methods 6, 359 - 362 (2009)

“single vessel” approach:

SP3 single-pot solid-phase-enhanced sample preparation

- surface-functionalized (i.e. carboxylate-coated) paramagnetic beads trap proteins and peptides in hydrophilic layers when the organic composition of the buffer is increased and the pH adjusted.
- the beads can be immobilized within a magnetic field
- efficient removal of contaminating agents including chaotropes and detergents by washingwith different organic solvents (i.e., ethanol and acetonitrile
- after rinsing, bound proteins or peptides can be eluted from the beads using an aqueous solution.
- **protein cleanup, enzymatic digestion, desalting, and peptide recovery in a single tube**.



“single vessel” approach

iST in-StageTip method

- complete sample preparation in a single reactor
- resembles an in-solution digestion with the advantages of a single FASP-like reaction vessel that avoids the use of a filter membrane.
- the C18 disk serves as a physical barrier for insoluble material and macromolecules.
- additionally, it enables final peptide cleanup using solid-phase extraction (SPE).
- One drawback of iST as compared to FASP is the limitation regarding the use of certain reagents (i.e., iST cannot remove SDS).

MOTIVATING LITERATURE FOR ADVANCED READERS

Two-dimensional gel electrophoresis in proteomics: A tutorial
Thierry Rabilloud et al. *Journal of Proteomics* **2011**

Two-dimensional gel electrophoresis in proteomics: past, present and future
Thierry Rabilloud et al. *Journal of Proteomics* **2010**

Proteomic biomarker discovery: It's more than just mass spectrometry
Josip Blonder et al. *Electrophoresis* **2011**

Basics and recent advances of two dimensional – polyacrylamide gel electrophoresis
Sameh Magdeldin et al. *Clinical Proteomics* **2014**

Evaluation of FASP, SP3, and iST Protocols for Proteomic Sample Preparation in the Low Microgram Range
Malte Sielaff et al. *J. Proteome Res.* **2017**

For all the complex problems and difficult questions there is always one simple, easily comprehensible **w r o n g** answer.

INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ