蛋白質體學技術簡介

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Common techniques in proteomics

- **Separation of proteins**
  - 1DE (convention)
  - 2DE (modern)
  - Multi-dimensional HPLC (modern)
- **Analysis of proteins**
  - Edman Sequencing (convention)
  - Mass Spectrometry (modern)
- **Database utilization**

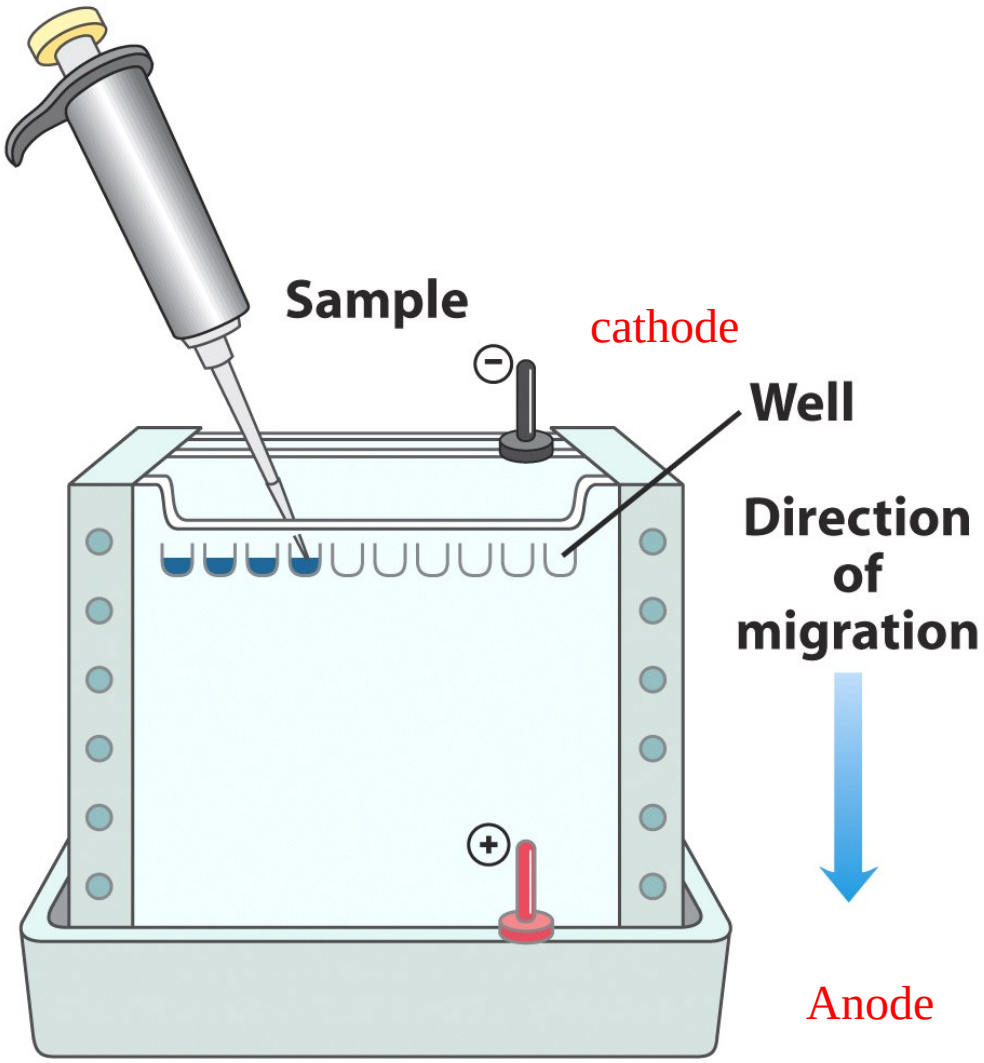
About the old techniques

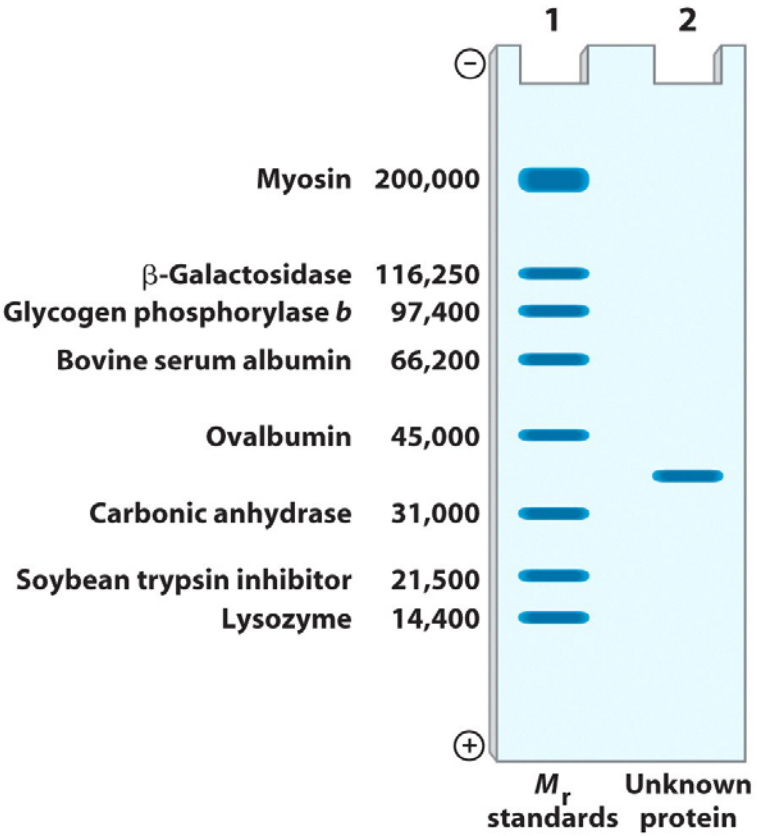
The pioneer of electrophoresis

The Nobel Prize in Chemistry 1948

Arne Wilhelm Kaurin Tiselius was born August 10,1902, in Stockholm. After the early loss of his father,the family moved to Gothenburg where he went to school, and after graduation at the local "Realgymnasium" in 1921, he studied at the University of Uppsala, specializing in chemistry. He became research assistant in The Svedberg's laboratory in 1925 and obtained his doctor's degree in 1930 on a thesis "The moving-boundary method of studying the electrophoresis of proteins" (published in *Nova Acta Regiae Societatis Scientiarum Upsaliensis*, Ser. IV, Vol. 7, No. 4) and was appointed Docent (Assistant Professor) in Chemistry from 1930 on.

Operation of SDS-PAGE





SDS-PAGE

- Poly-acrylamide gel electrophoresis
- Has not changed in the 32 years since its inception..the question answered is still the same
- Resolution depends on gel length
- Most convenient technique to separate protein

Edman degradation

- **Introduced in 1949**

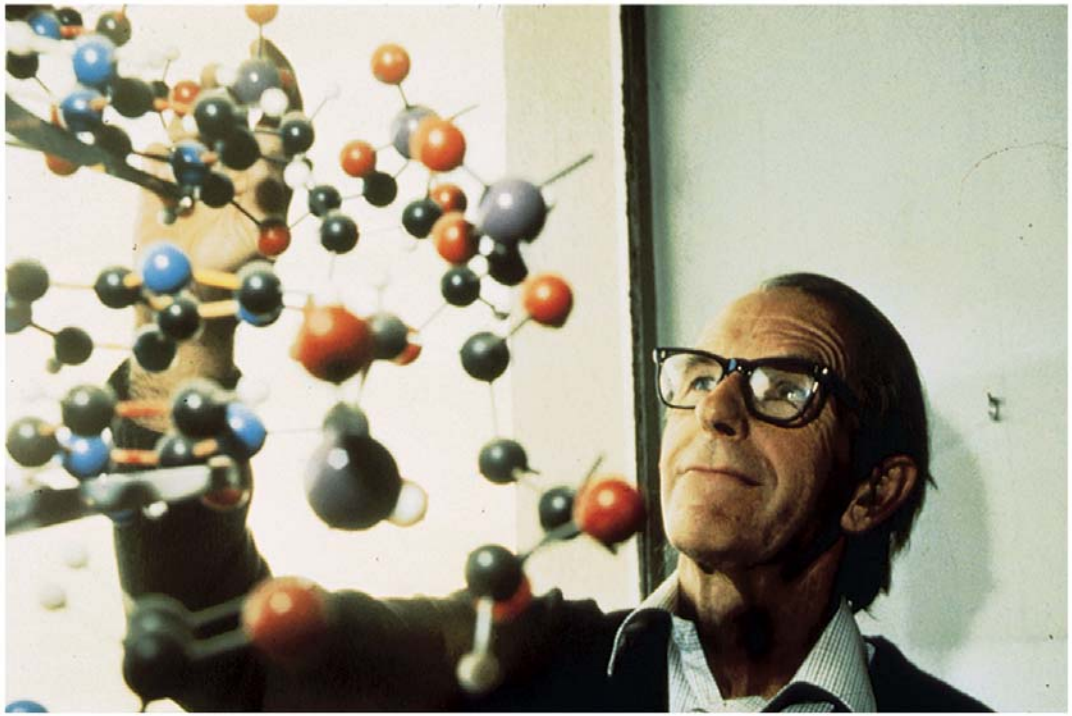
  1. Run PAGE
  2. Digest whole protein mixture and start sequencing

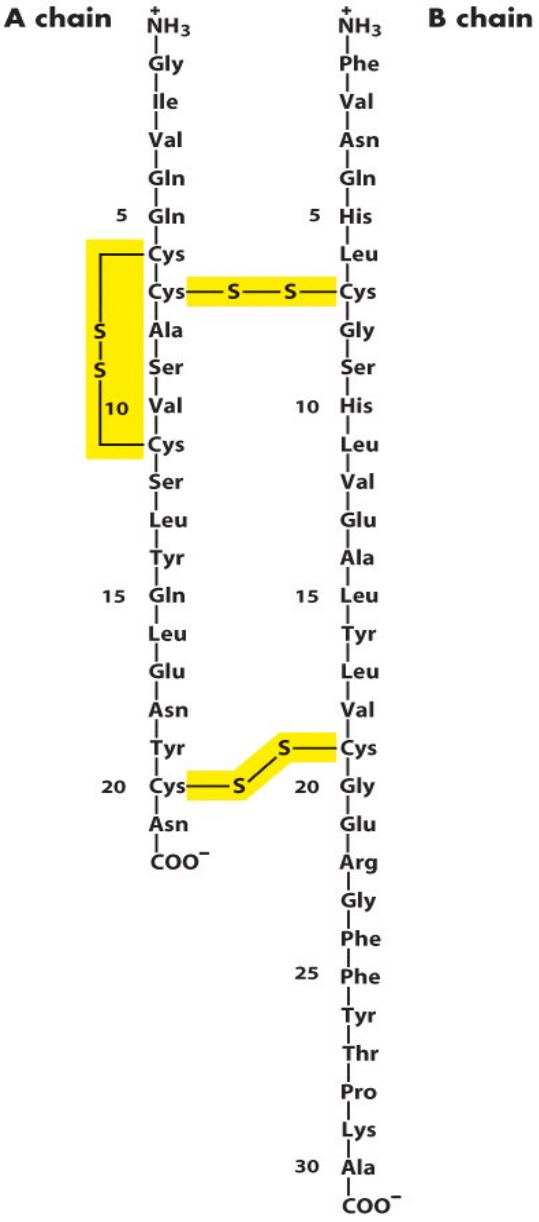
- **Only works with high abundance proteins**

Edman degradation

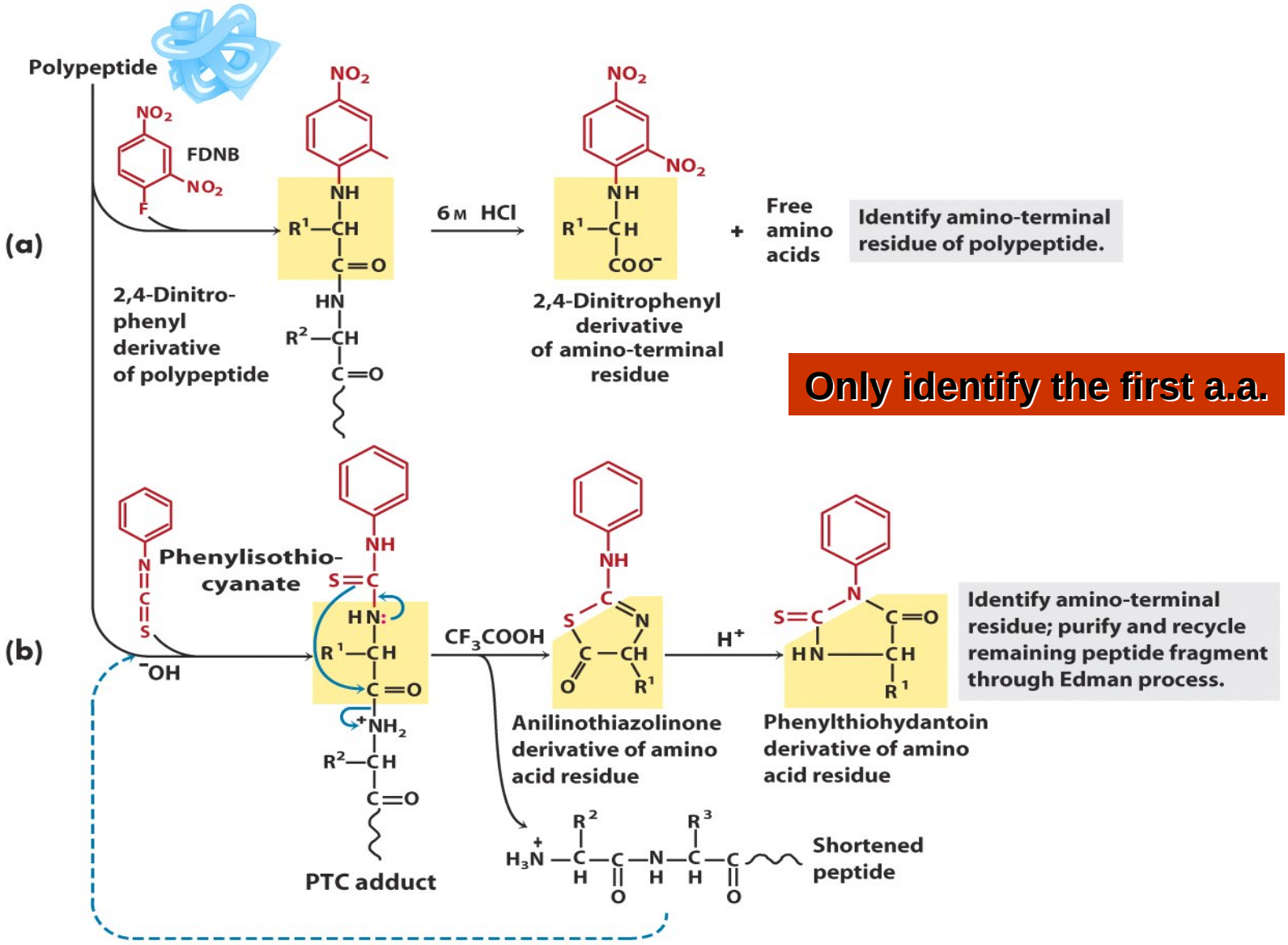
1953, Frederick Sange
sequence of insulin

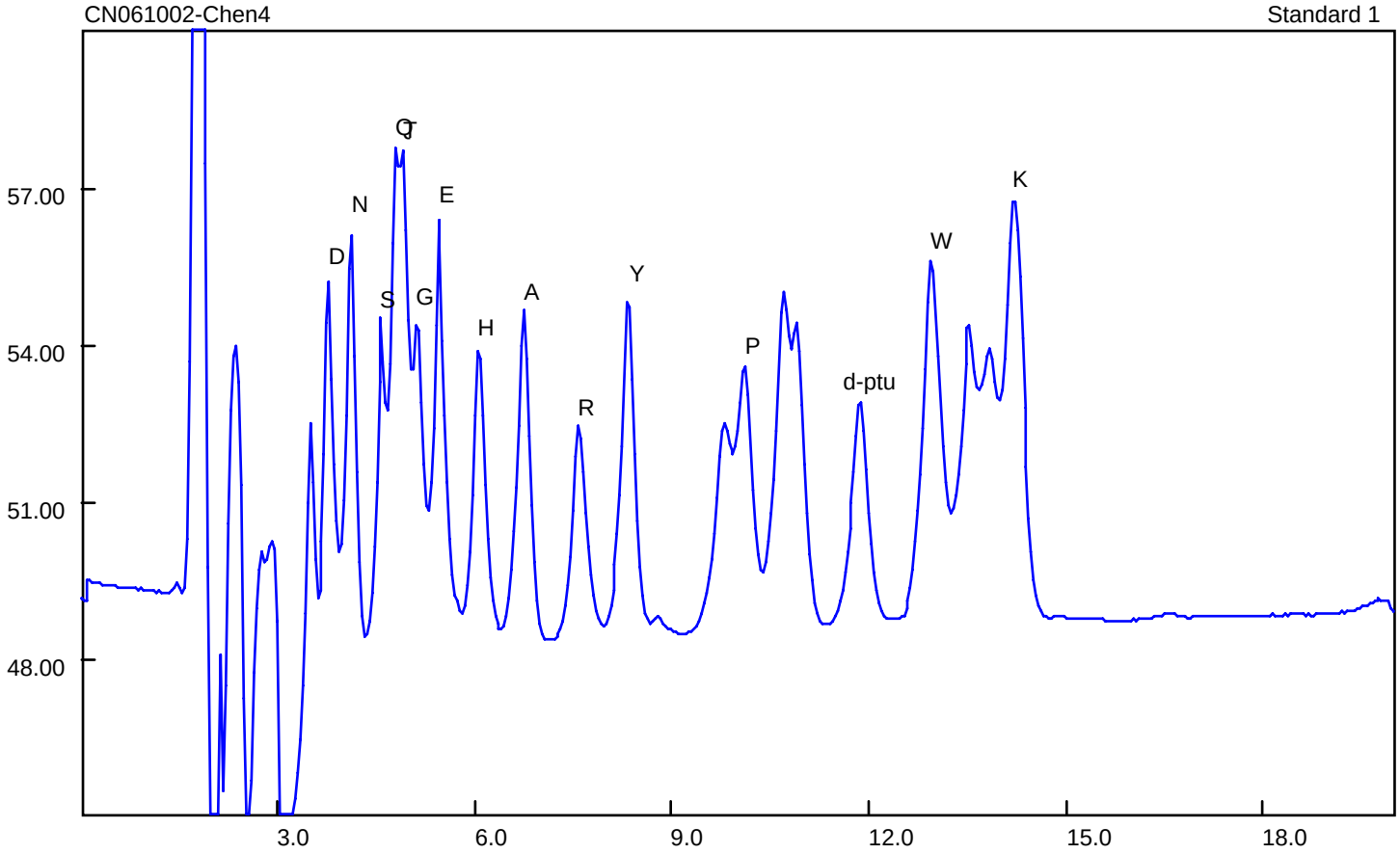
1958 Nobel Prize in Chemistry
1980 Nobel Prize in Chemistry:
ing





Edman degradation

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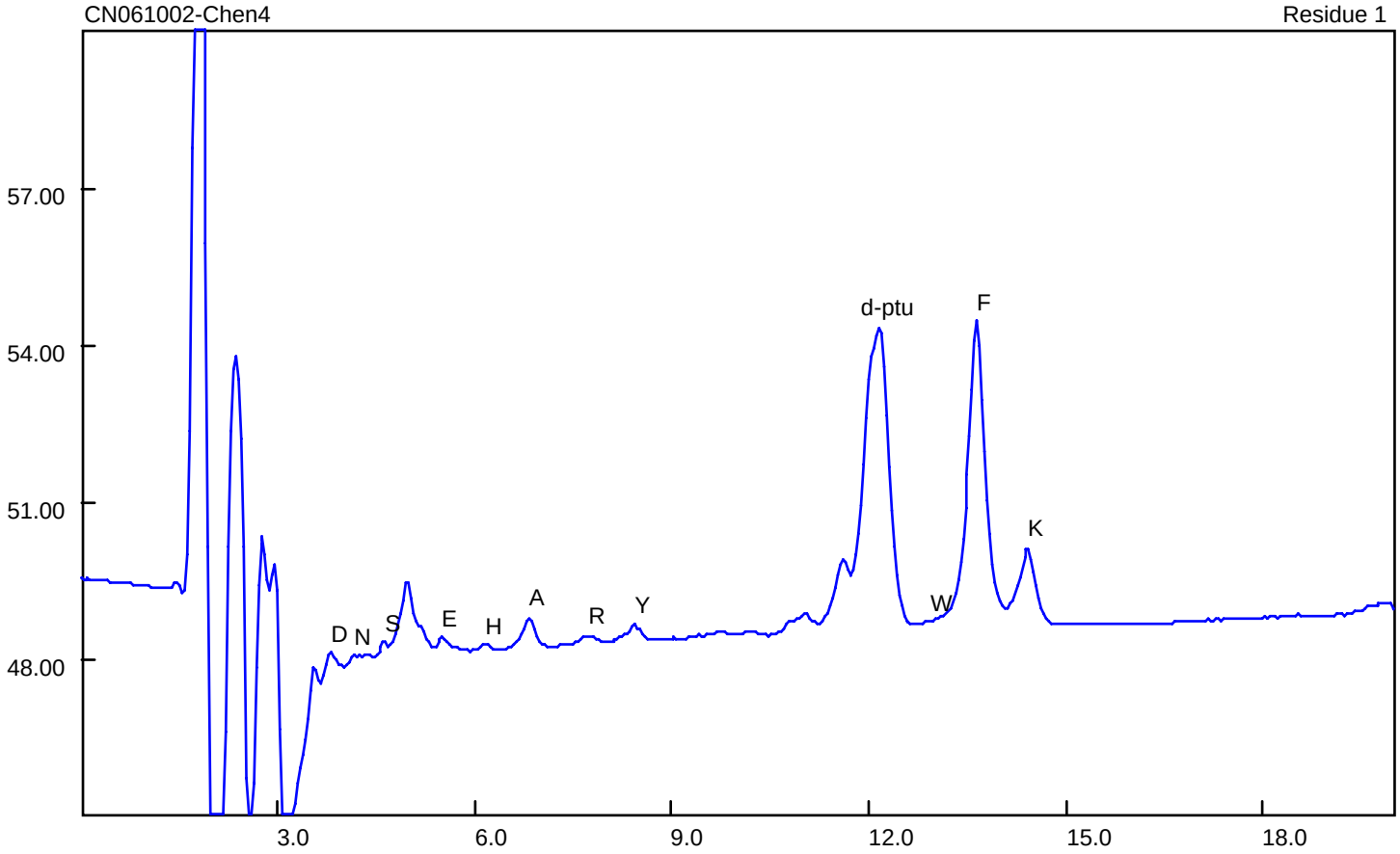
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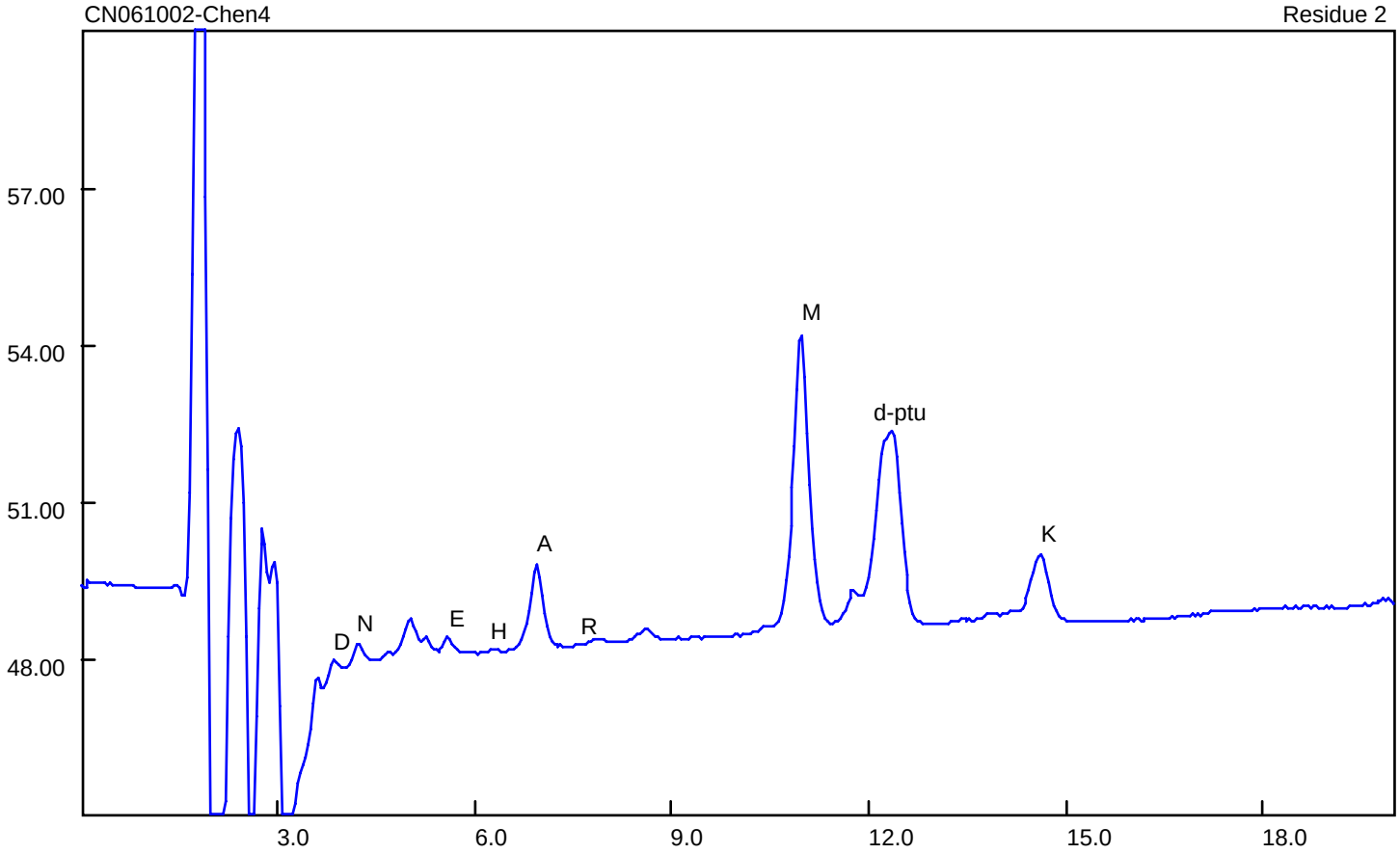
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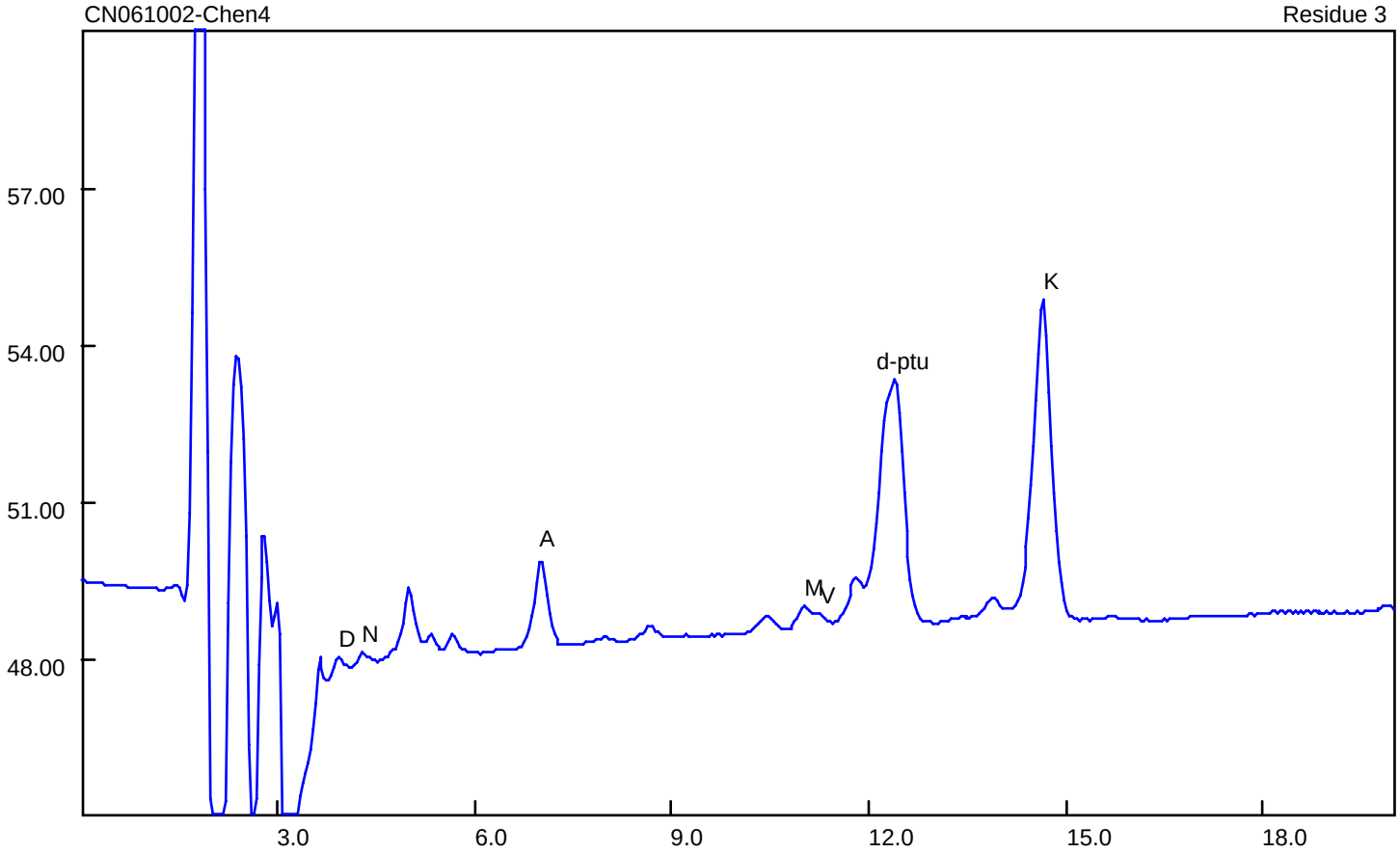
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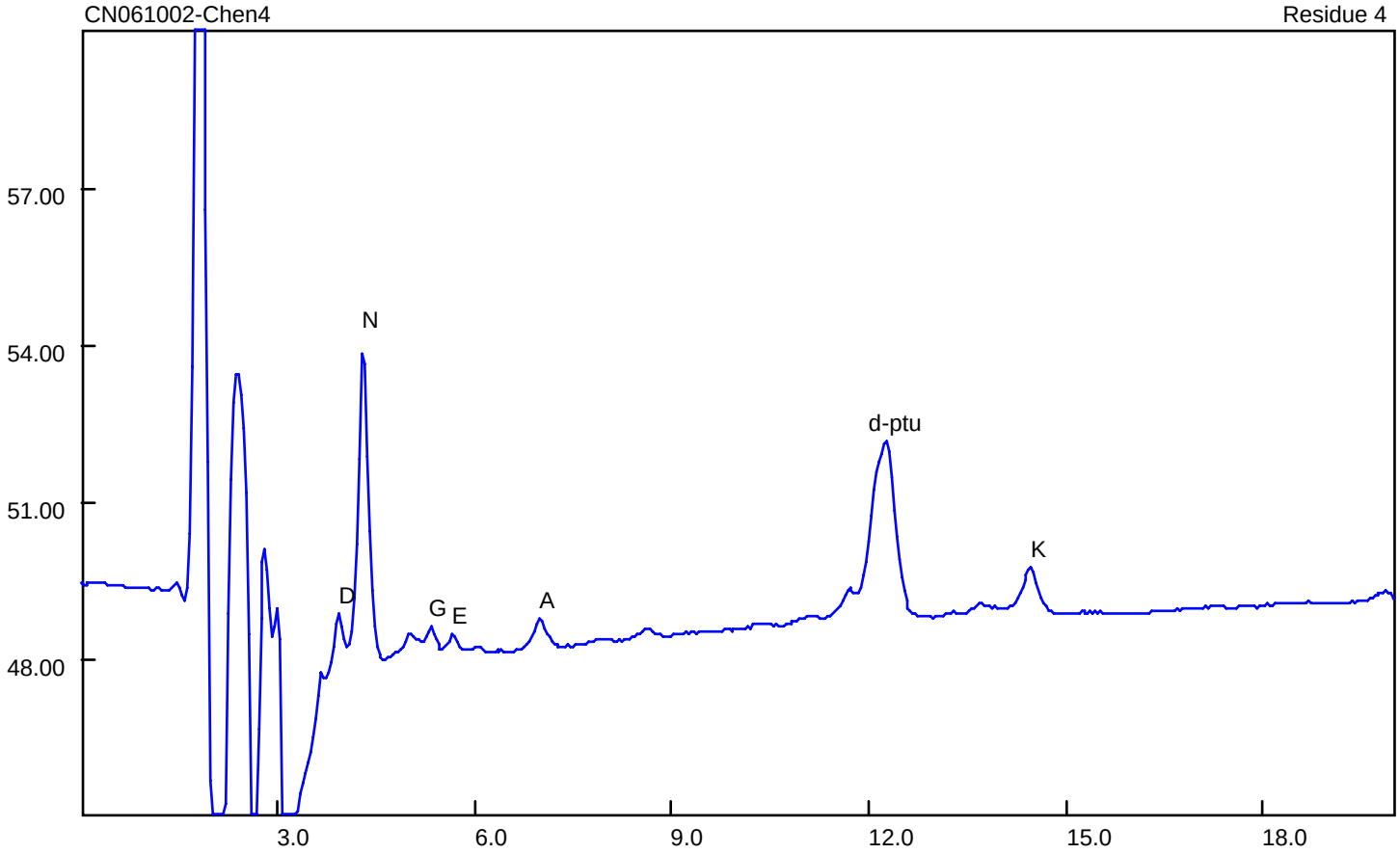
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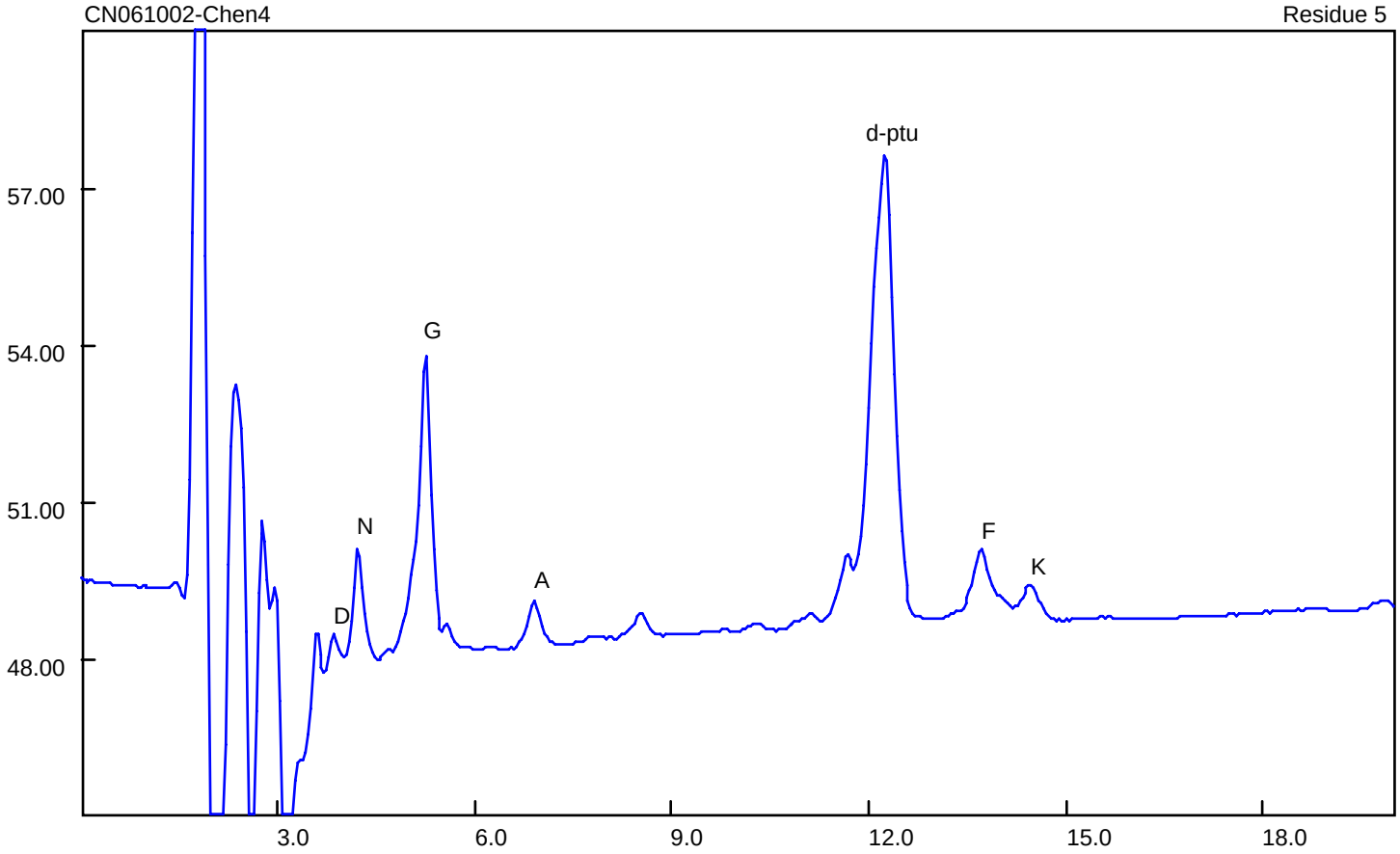
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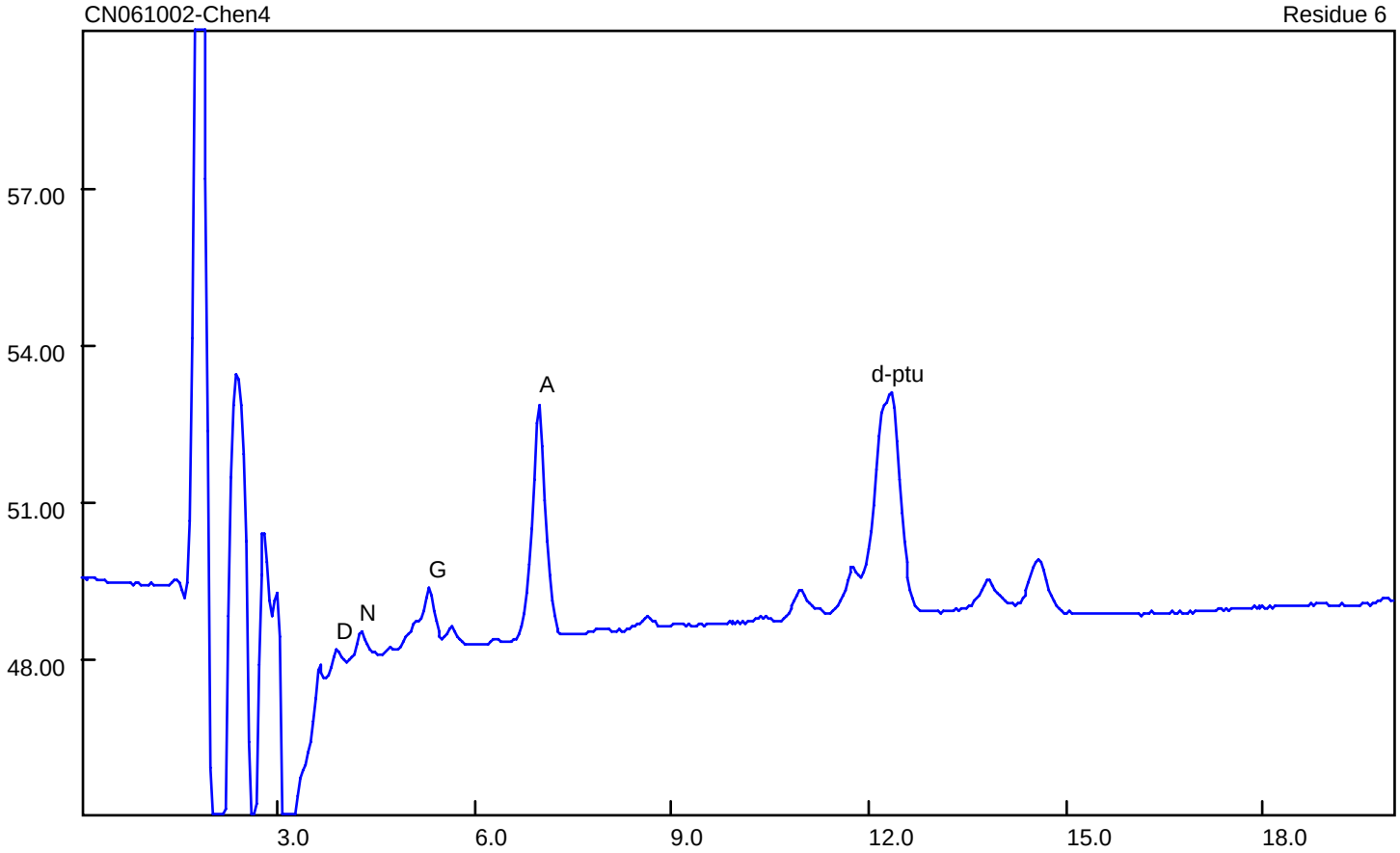
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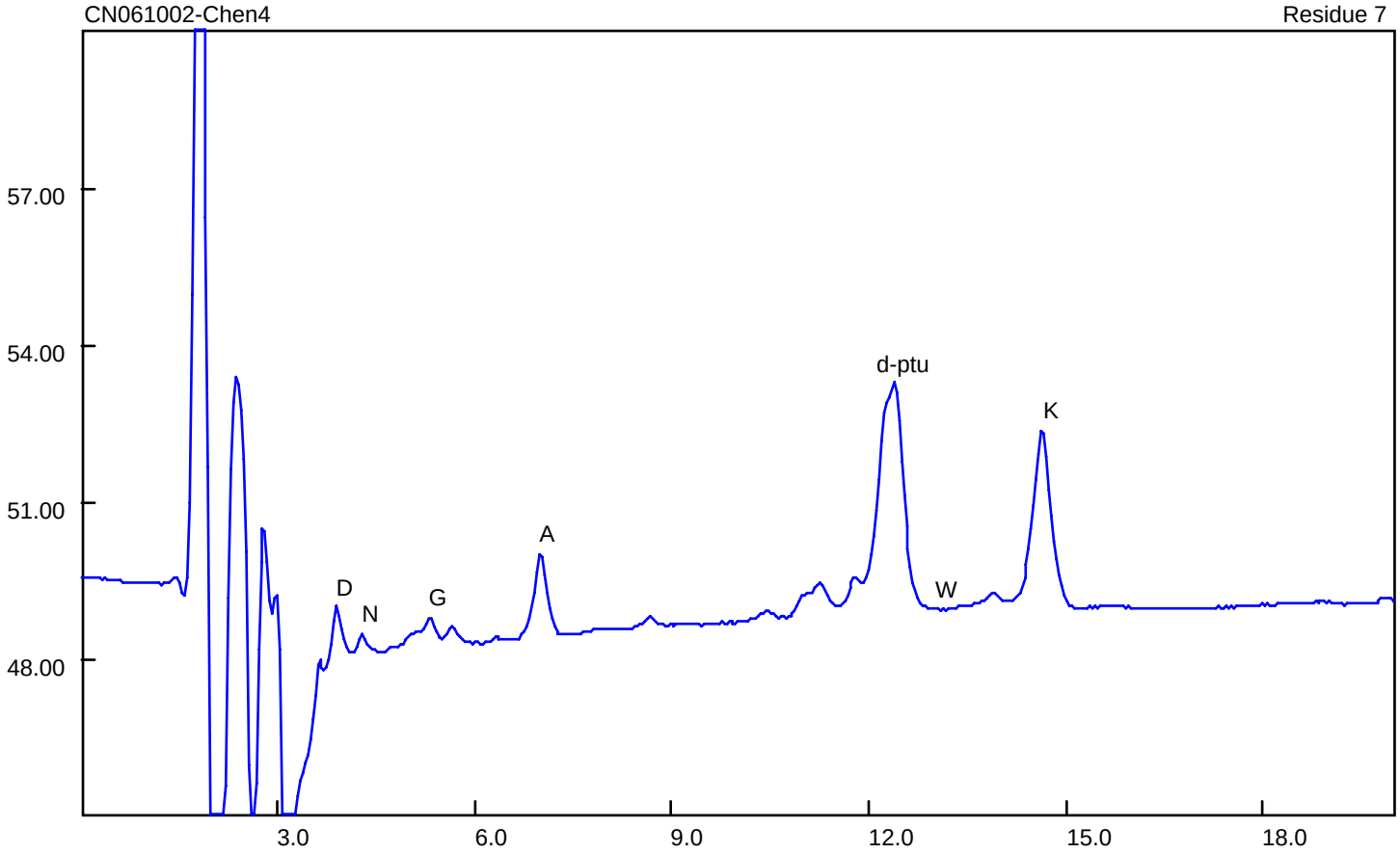
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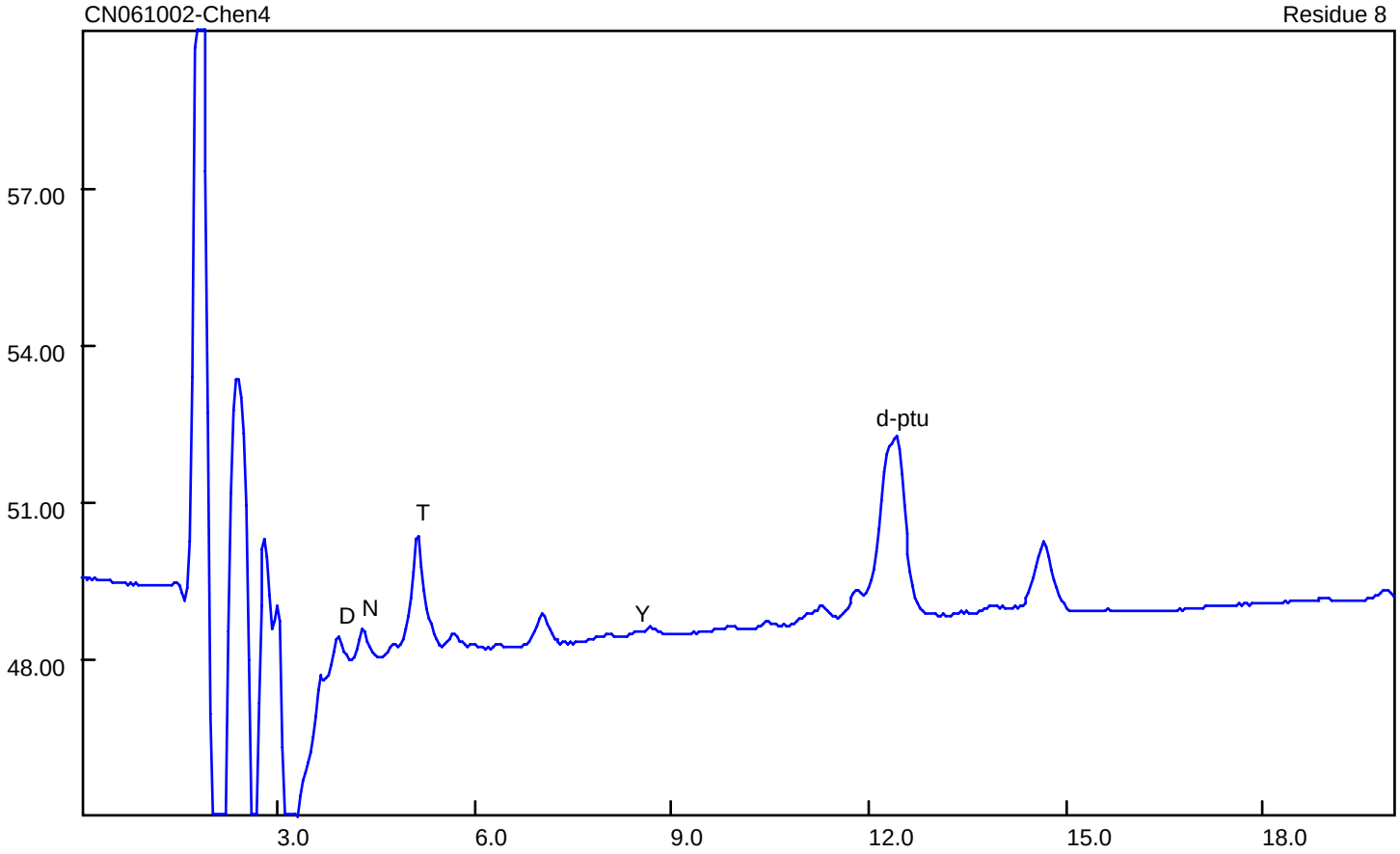
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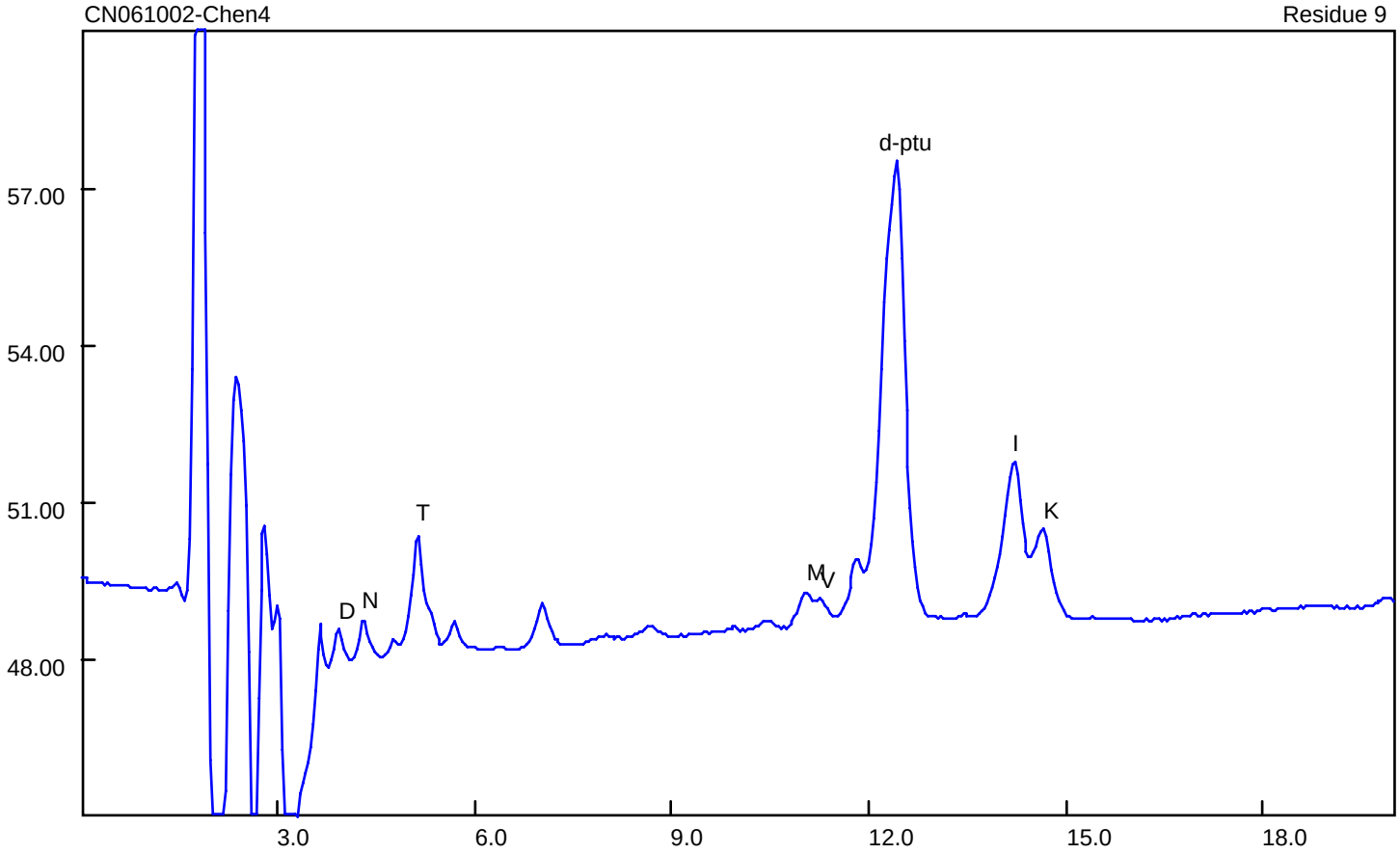
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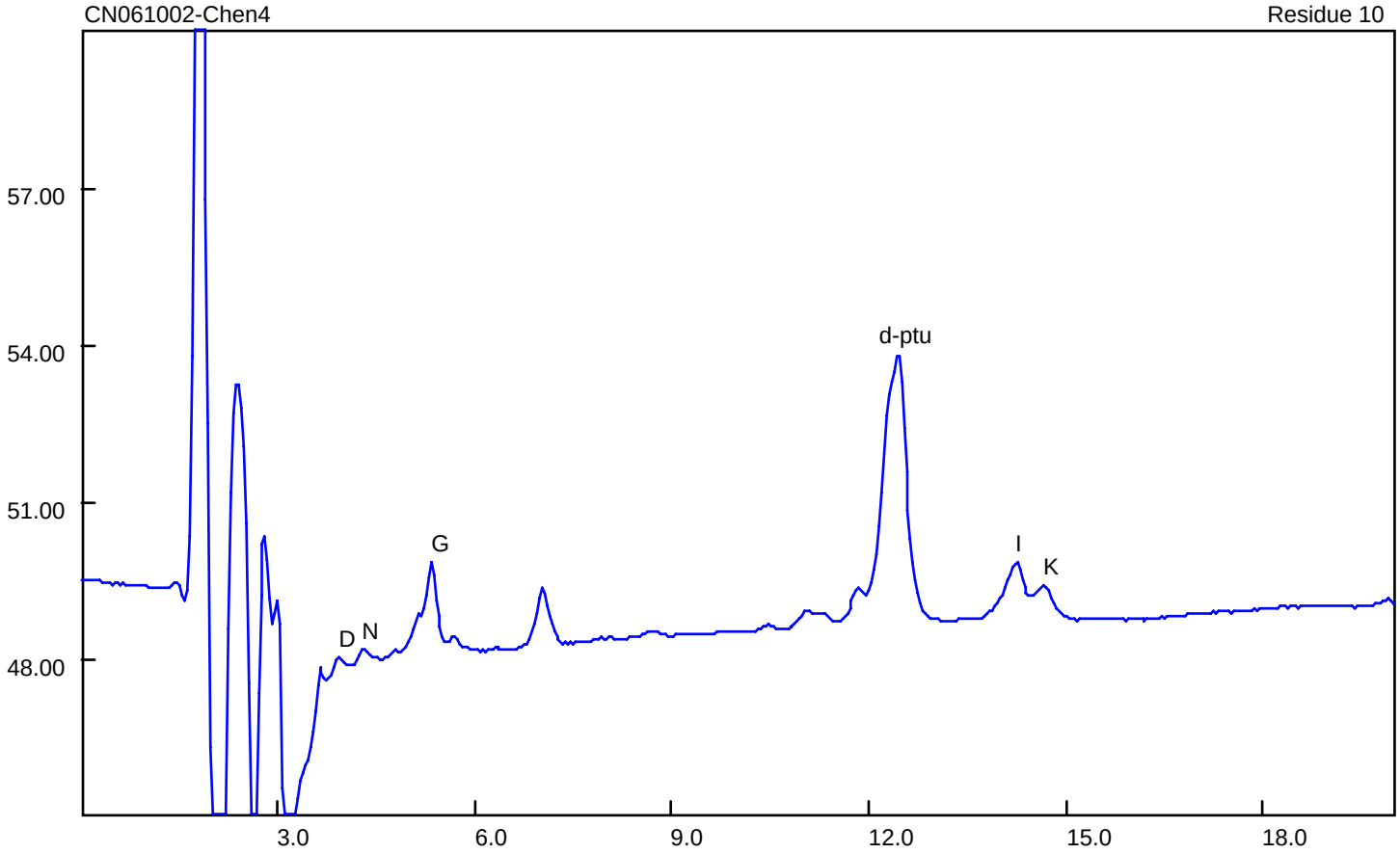
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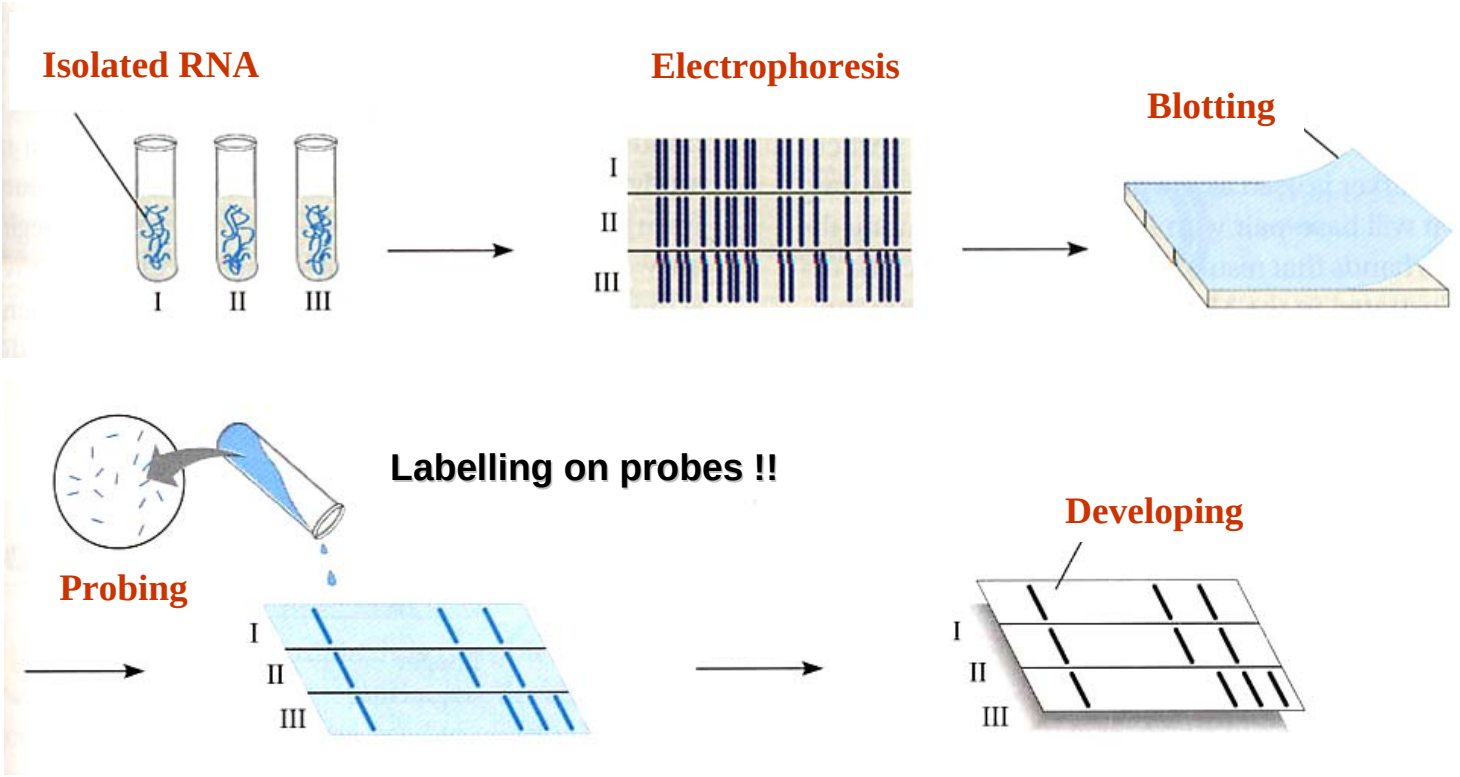
Technology, Now and then

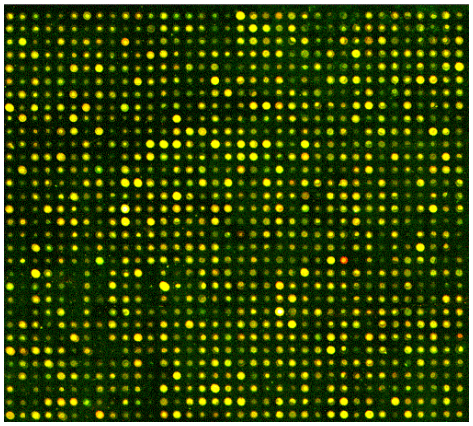
Traditional RNA technique : Northern blotting

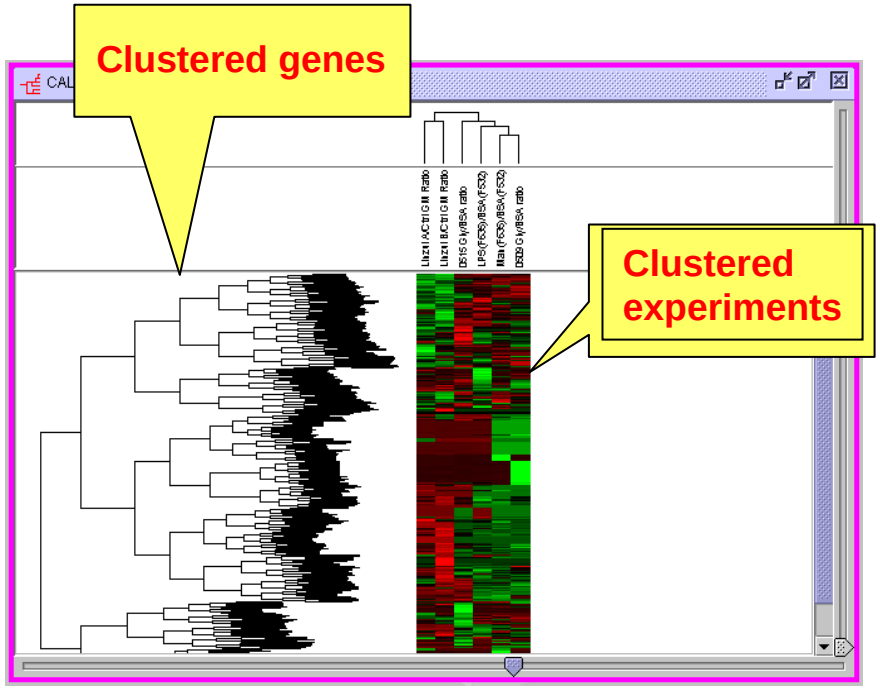


1. Estimated time to get results: 2-3days
2. Expressed Gene (mRNA) checked: 1-8 species
3. Accuracy: Low to moderate

High-throughput method: Microarray

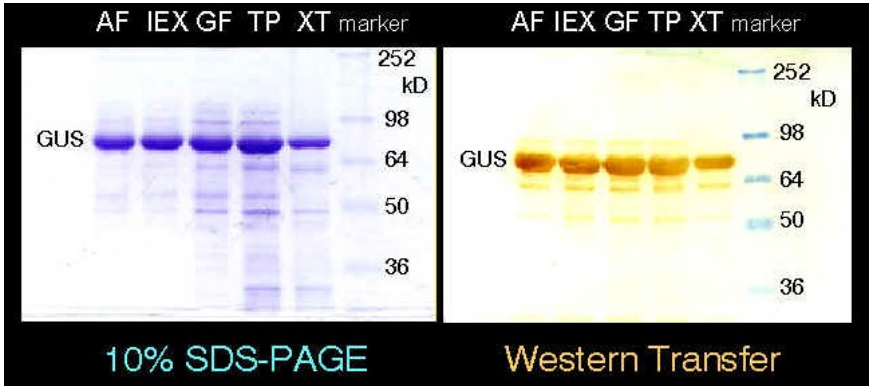
Labeling on sample mRNA as probe
cDNA or oligonucleotide spotted on chips





1. Estimated time to get results: <u>5-7 days</u>
2. Expressed Gene (mRNA) checked: <u>thousands</u>
3. Accuracy: <u>moderate to high</u>

Traditional Protein technique: protein purification and edman degradation



Cut desired band

Peptide N terminal sequencing

Database searching for homolog

1. **Protein purification: <u>necessary</u>**
2. **Protein idetified: <u>1 per purified sample</u>**

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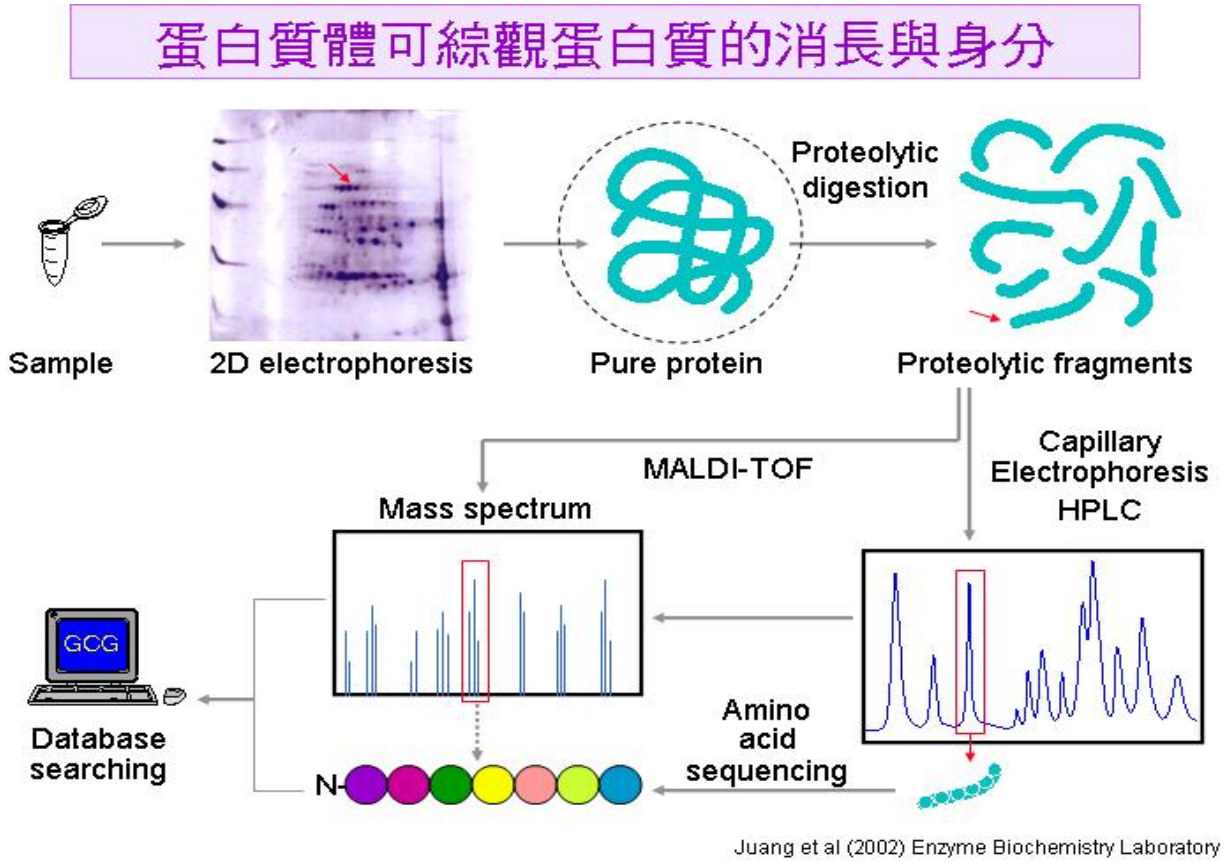
High throughput technique:
2D electrophoresis + Mass spectrometry



1. **Protein purification: <u>not necessary</u>**
2. **Protein idetified: <u>up to thousands per unpurified sample</u>**

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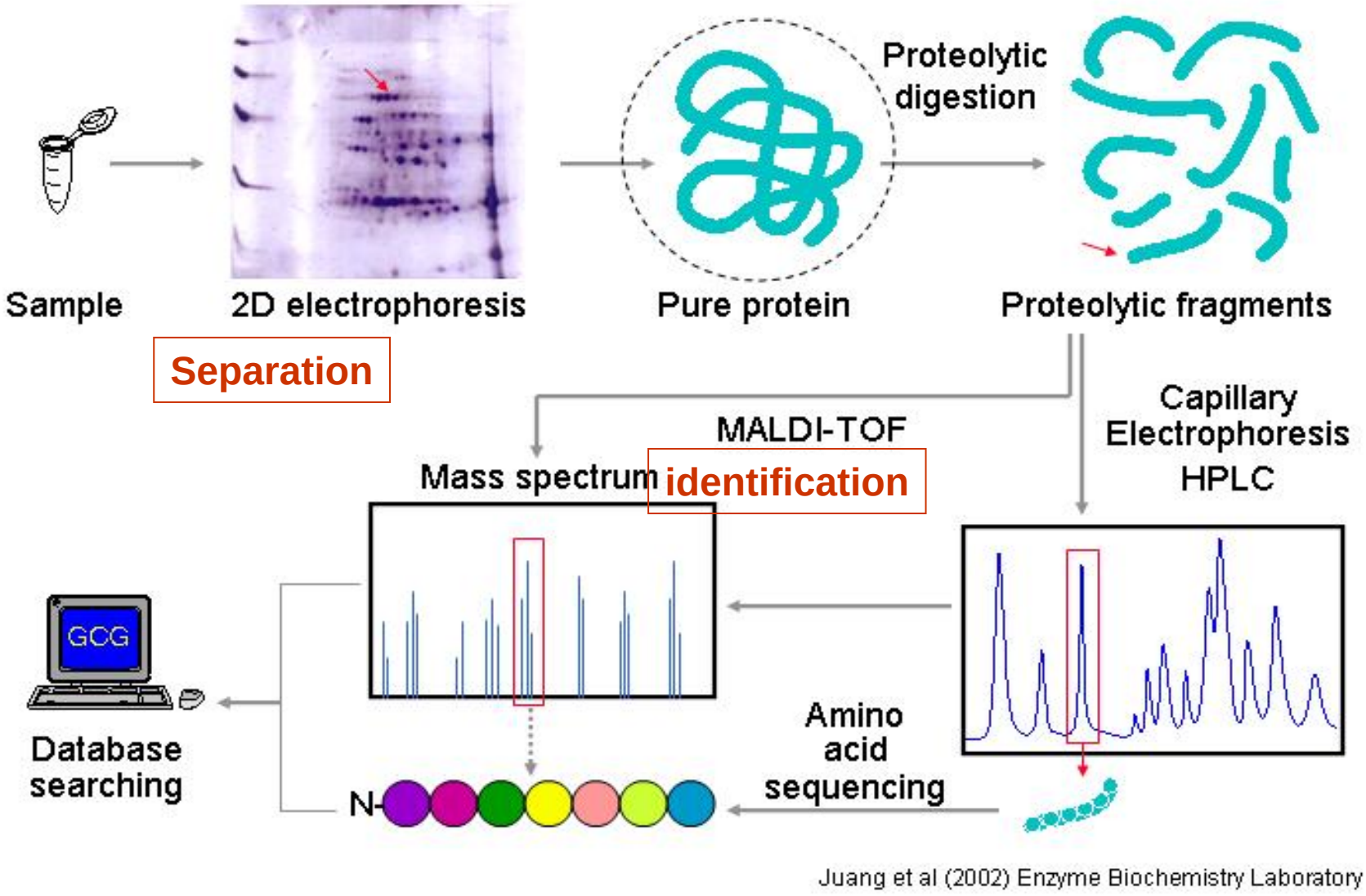
Major techniques in modern proteomics

A. Two dimensional electrophoresis, 2-DE

B. Mass spectrometry

蛋白質體可綜觀蛋白質的消長與身分



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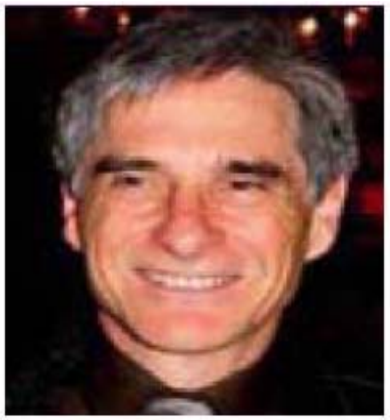
The pioneer of two dimensional electrophoresis

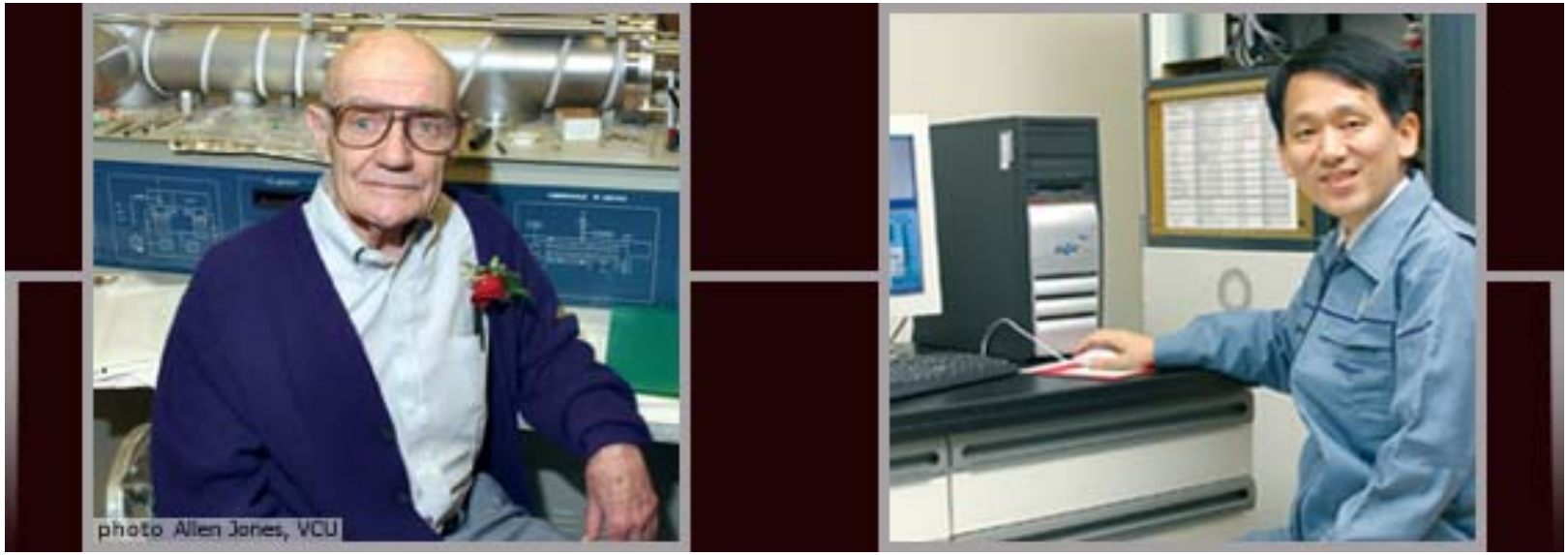


Patrick H. O'Farrell, PhD
Professor, Biochemistry and Biophysics,
UCSF O'Farrell PH (1975)

A technique has been developed for the separation of proteins by two-dimensional polyacrylamide gel electrophoresis. Due to its resolution and sensitivity, this technique is a powerful tool for the analysis and detection of proteins from complex biological sources.

The pioneer of two dimensional electrophoresis

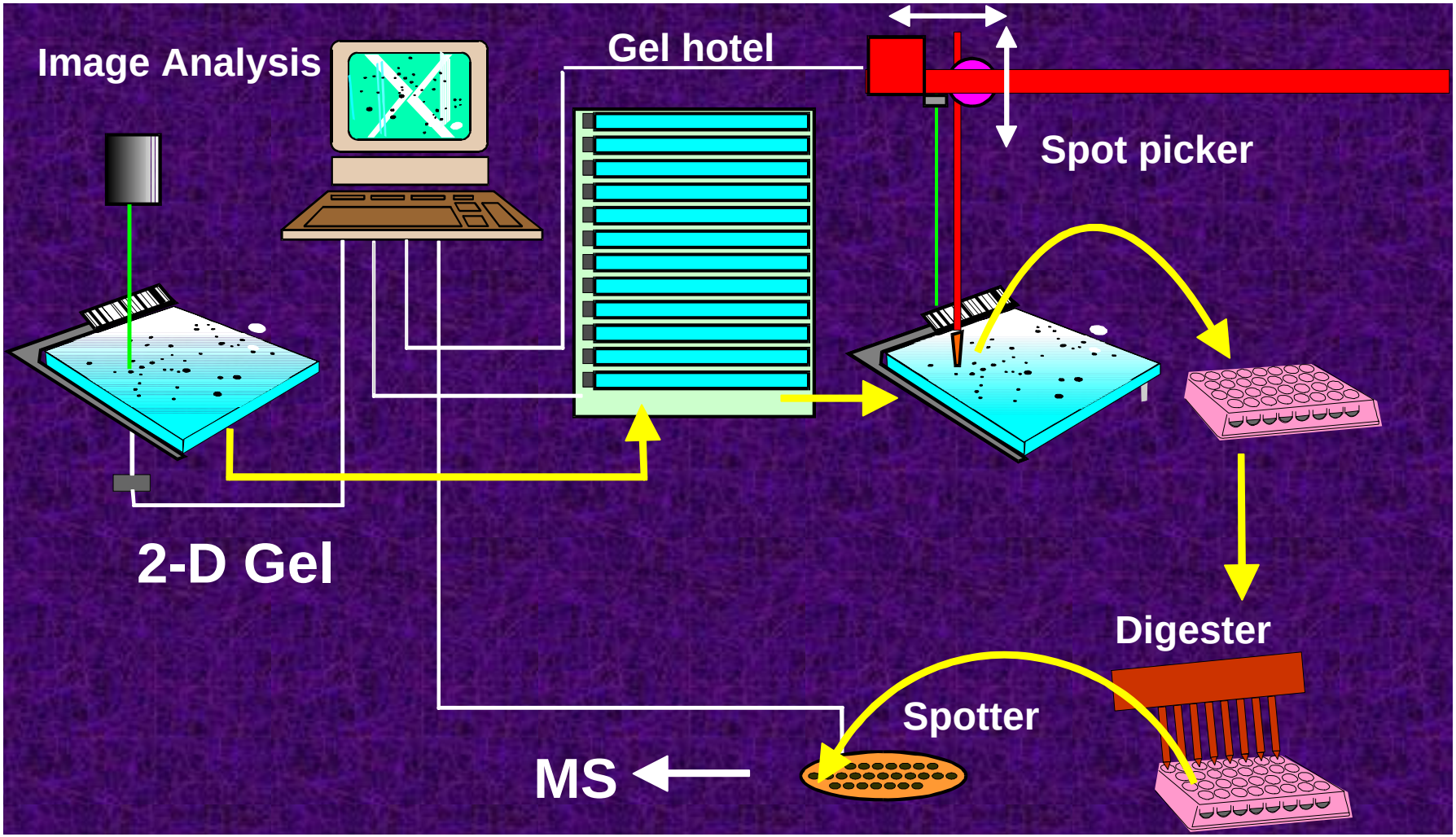
The Nobel Prize in Chemistry 2002



John B. Fenn is the chemist who invented the electrospray method. Today it is used in laboratories all over the world. Fenn has worked mainly as Professor of Chemistry at Yale University, USA, and at Virginia Commonwealth University, USA.

Koichi Tanaka worked as a research engineer at Shimadzu Corp. in Kyoto, Japan . Tanaka's idea was to use the energy from laser light, ingeniously transferred to the proteins, to get them to let go of one another and hover freely. It worked!

Automation process for 2-DE + mass spectrometry



The old way to find protein markers

Takes months to years

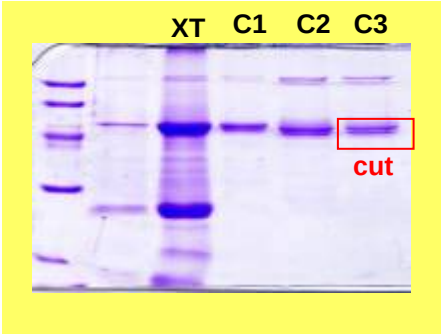
Sample homogenization

Prepare for chromatography

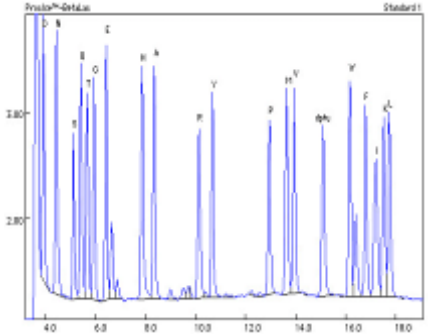
Run Chromatography

Prepare for Chromatography again

Run Chromatography again



Run SDS-PAGE and Transfer to membrane



Sequence N termini (Edman degradation)

Search database for homologous match

?

What's proteomics?

"The analysis of the entire protein complement expressed by a genome, or by a cell or tissue type."

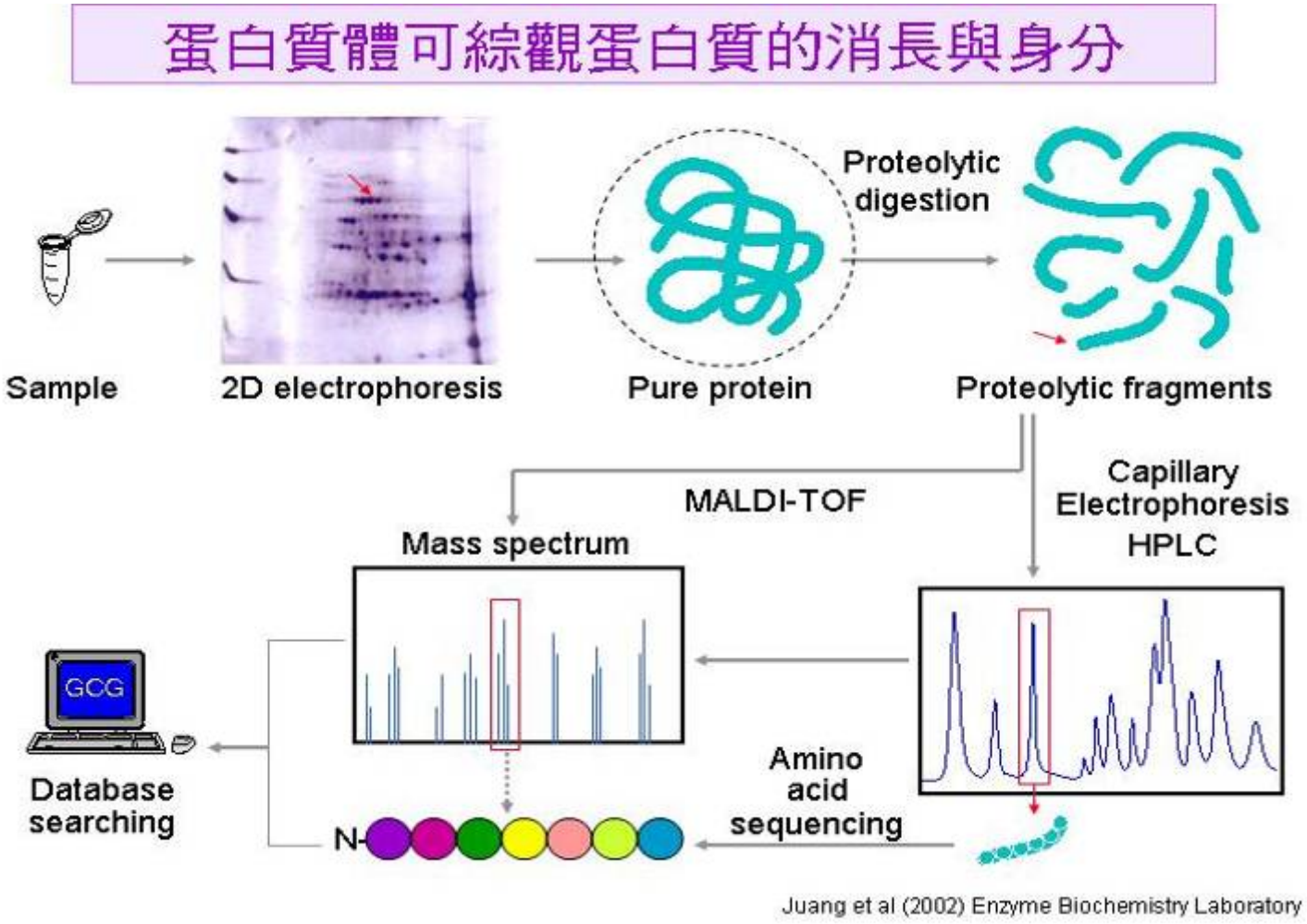
Wasinger VC et al Progress with gene-product mapping of the mollicutes: *Mycoplasma genitalium*. Electrophoresis 16 (1995) 1090-1094

Two MOST applied technologies:

1. 2-D electrophoresis:
separation of complex protein mixtures

2. Mass spectrometry:
Identification and structure analysis

The new way to find protein markers



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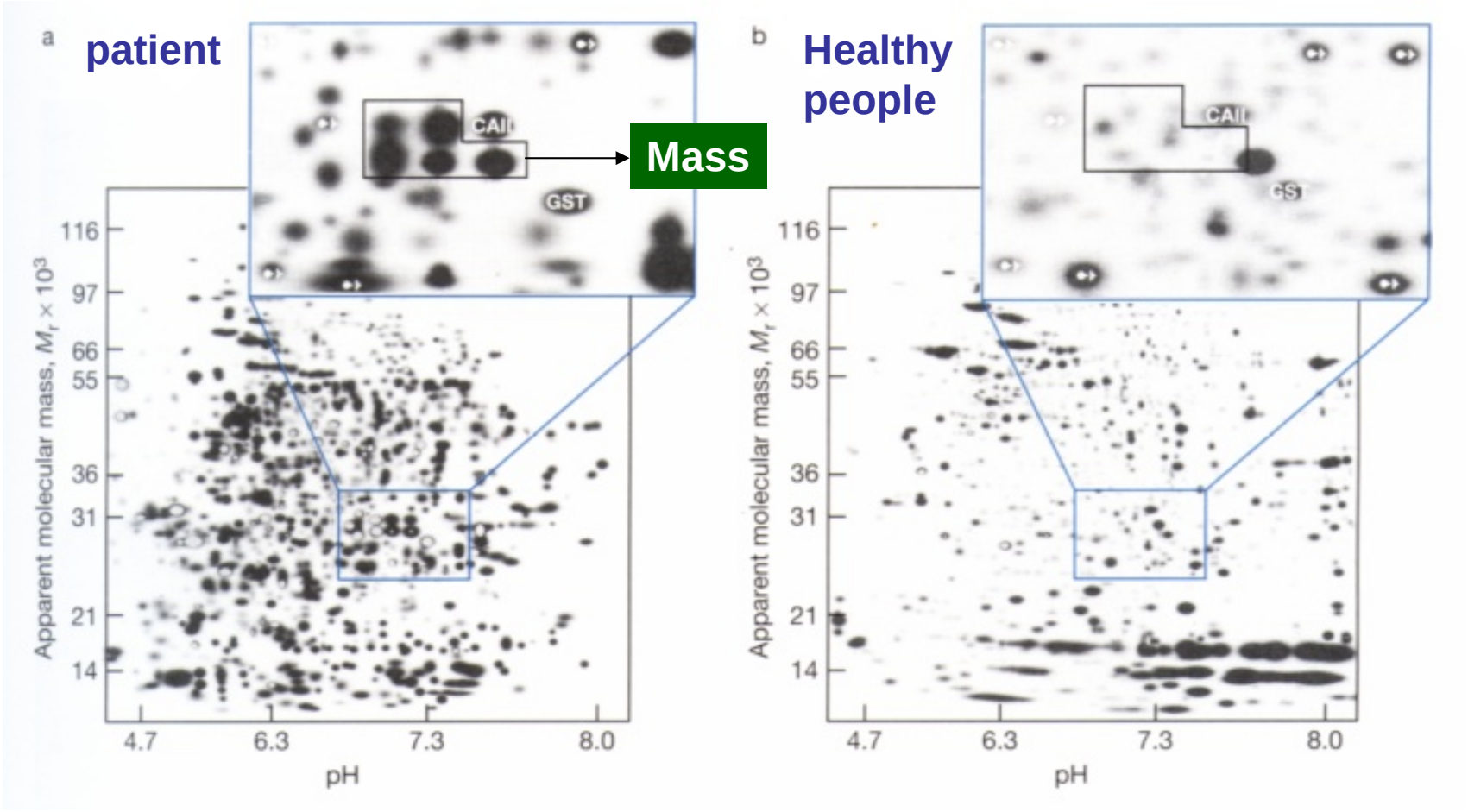
How proteomic techniques help?

Identification of protein markers from patient samples

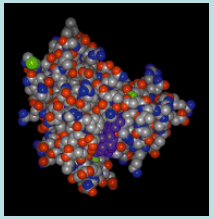


Global profiling proteomics

The identities of proteins

The ID for proteins

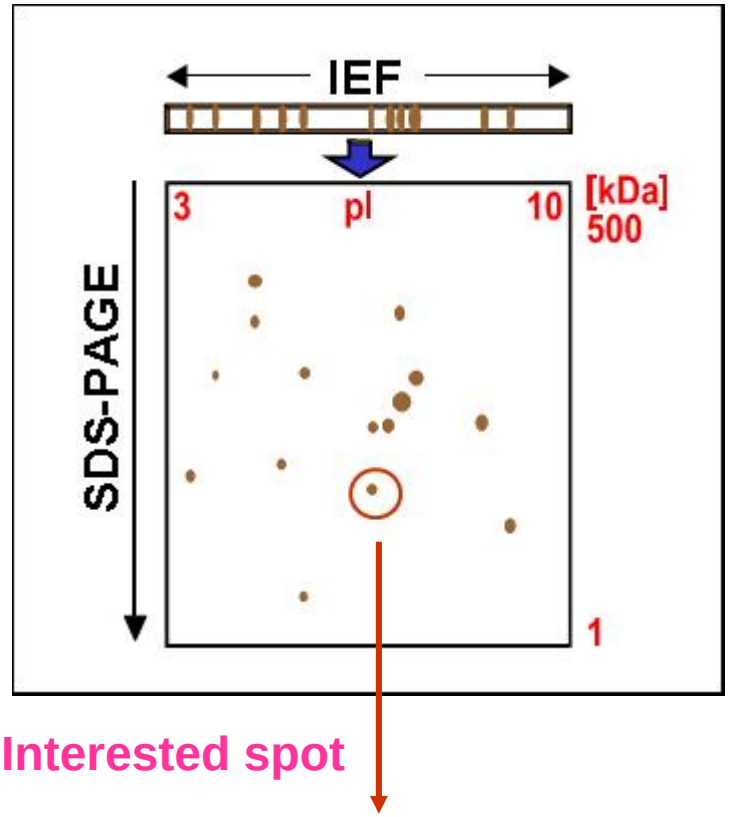
Name: hemoglobin



MW: 20kDa
pI: 5.67
Hydrophobicity: -20?

- Size: molecular weight (utilized in 2-DE)
- Charge: pI (utilized in 2-DE)
- Hydrophobicity

Two dimensional electrophoresis (2-DE)



1. **First dimension:**
denaturing isoelectric focusing
separation according to the **pI**

2. Second dimension:
SDS electrophoresis (SDS-PAGE)
Separation according to the **MW**

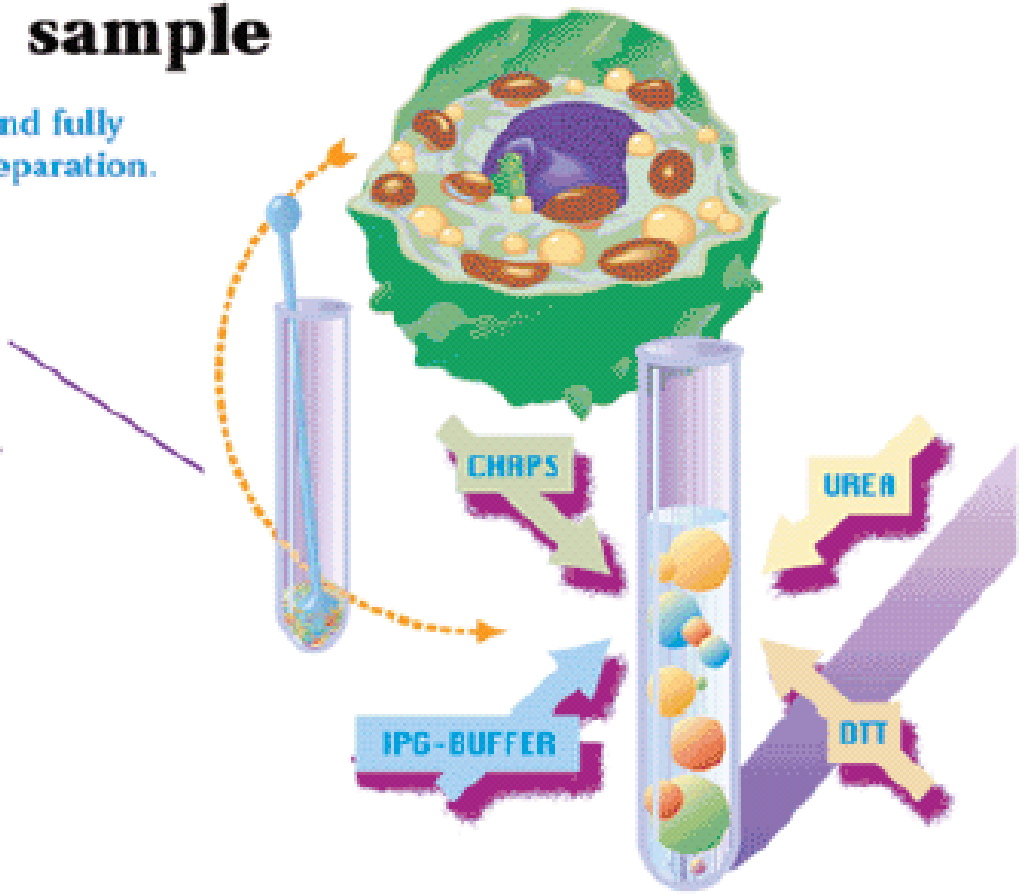
Digest to peptide fragment → **MS analysis**

Run 2-DE, step 1

1. Prepare the sample

Samples are denatured and fully solubilized for optimal separation.

Solubilize your cell extract or sample in urea, a non-ionic detergent, IPG-Buffer and a reducing agent.



Run 2-DE, step 2

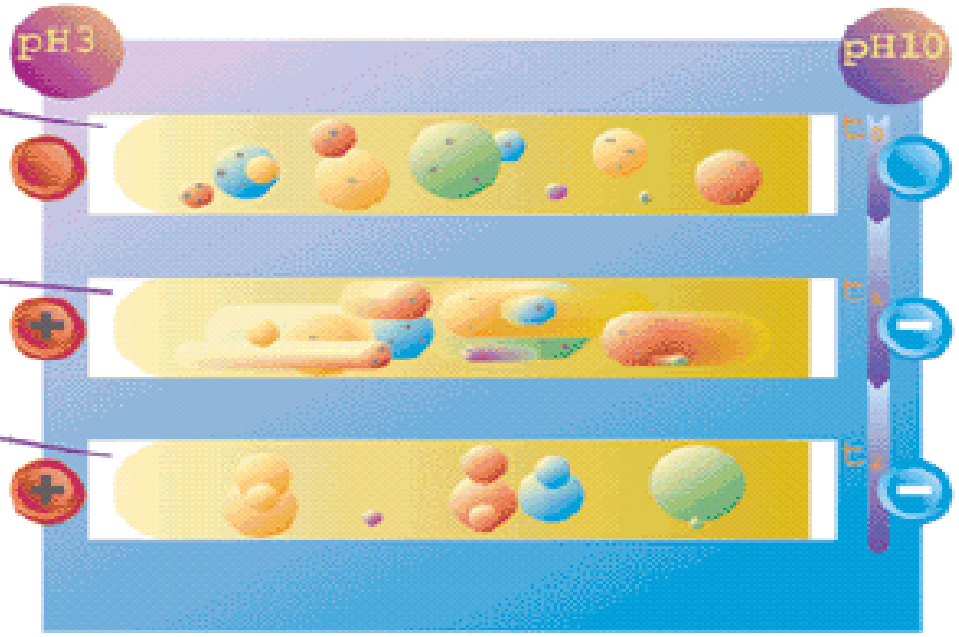
2. Run the first dimension – IEF

In the first dimension, proteins separate by isoelectric point (pI) in the immobilized pH gradient (IPG) of the Immobiline DryStrip gel.

A. Apply the sample to the IPG strip either in a defined zone or distributed throughout the entire strip.

B. Apply the electric field. The charged proteins migrate within the pH gradient.

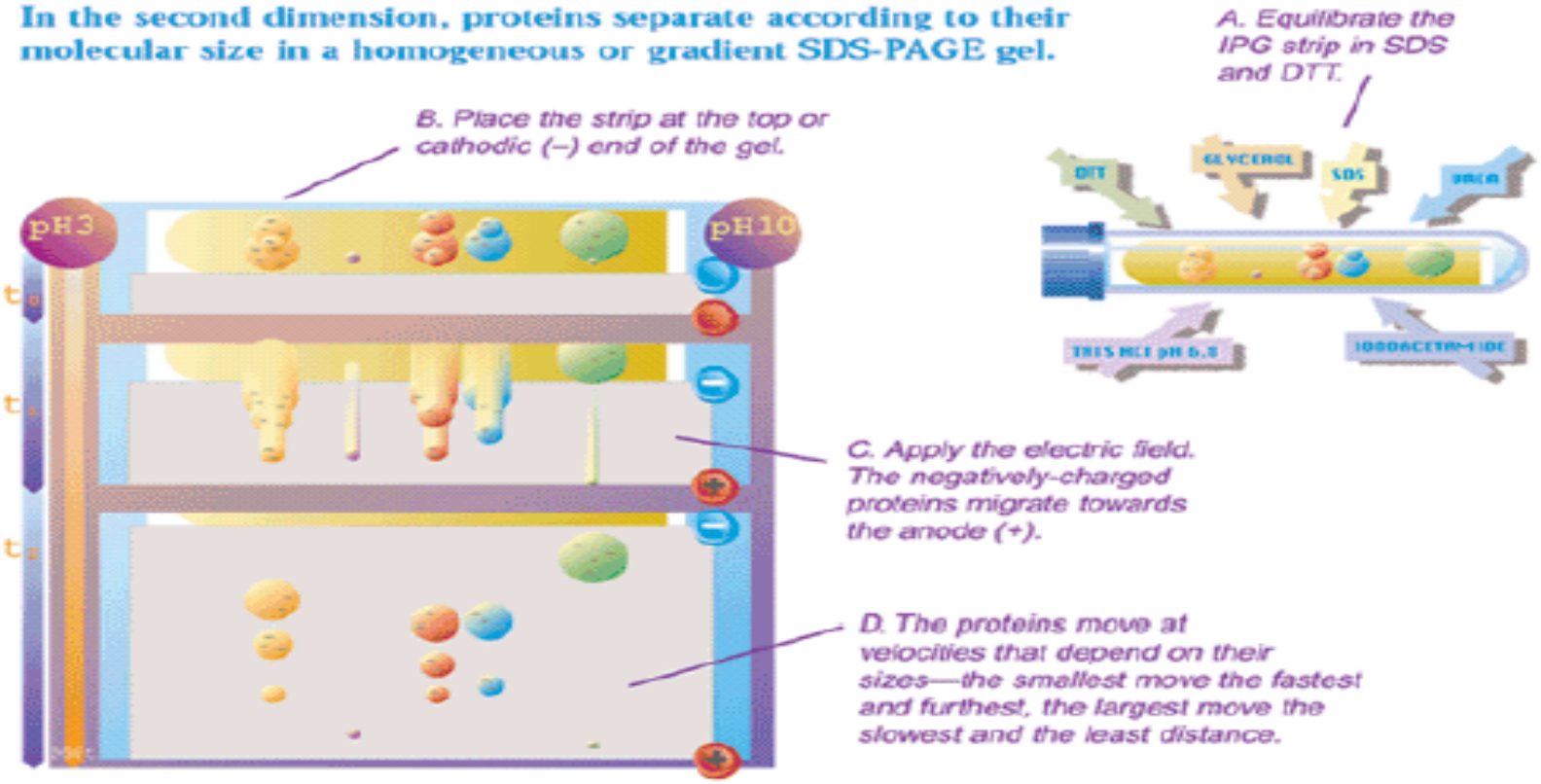
C. When the proteins reach the pH in the gradient equal to their pIs, their net charge drops to zero and they focus as distinct zones. Because the pH gradient in the strip is immobilized, no further migration can occur.



Run 2-DE, step 3

3. Run the second dimension – SDS-PAGE

In the second dimension, proteins separate according to their molecular size in a homogeneous or gradient SDS-PAGE gel.

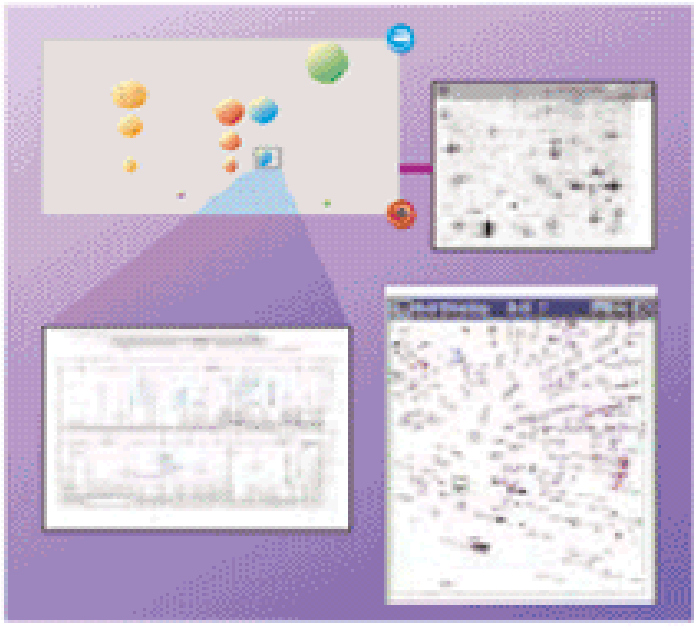


Run 2-DE, step 4

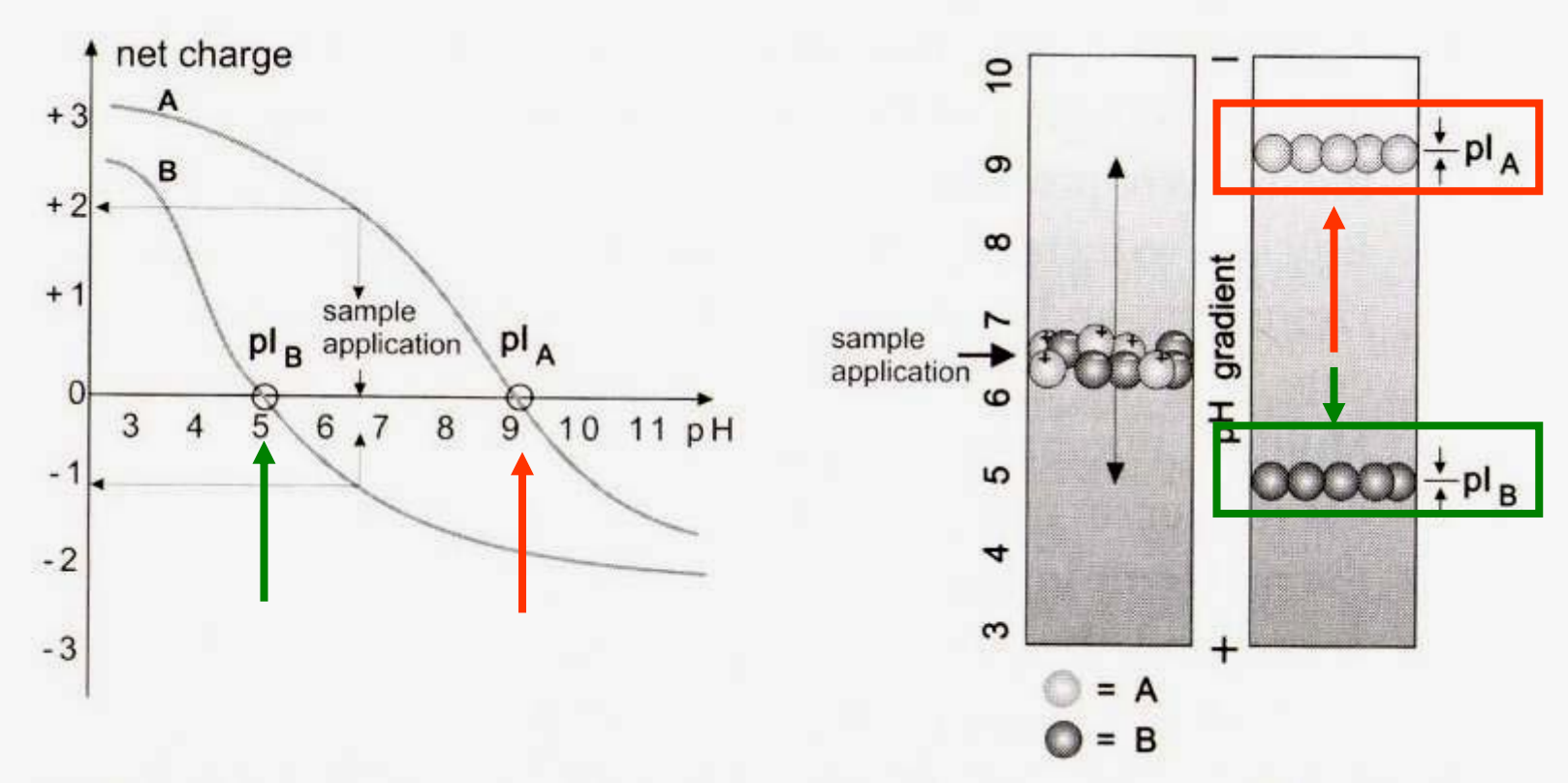
4. Visualize and analyze

Detect separated proteins by autoradiography, staining, or immunodetection after blotting onto a membrane. An array of powerful tools and techniques is available to compare samples and identify proteins of interest:

- Image analysis software to determine spot position and abundance, compare gel images, create databases, and search for patterns;
- Mass spectrometry to determine masses with high precision, peptide fragment fingerprints, amino acid sequences, and nature and site of post-translational modifications.



The theory of IEF



The IEF is a very high resolution separation method, and the pI of a protein can be measured.

How pH gradient forms, Immobilized pH gradient, IPG

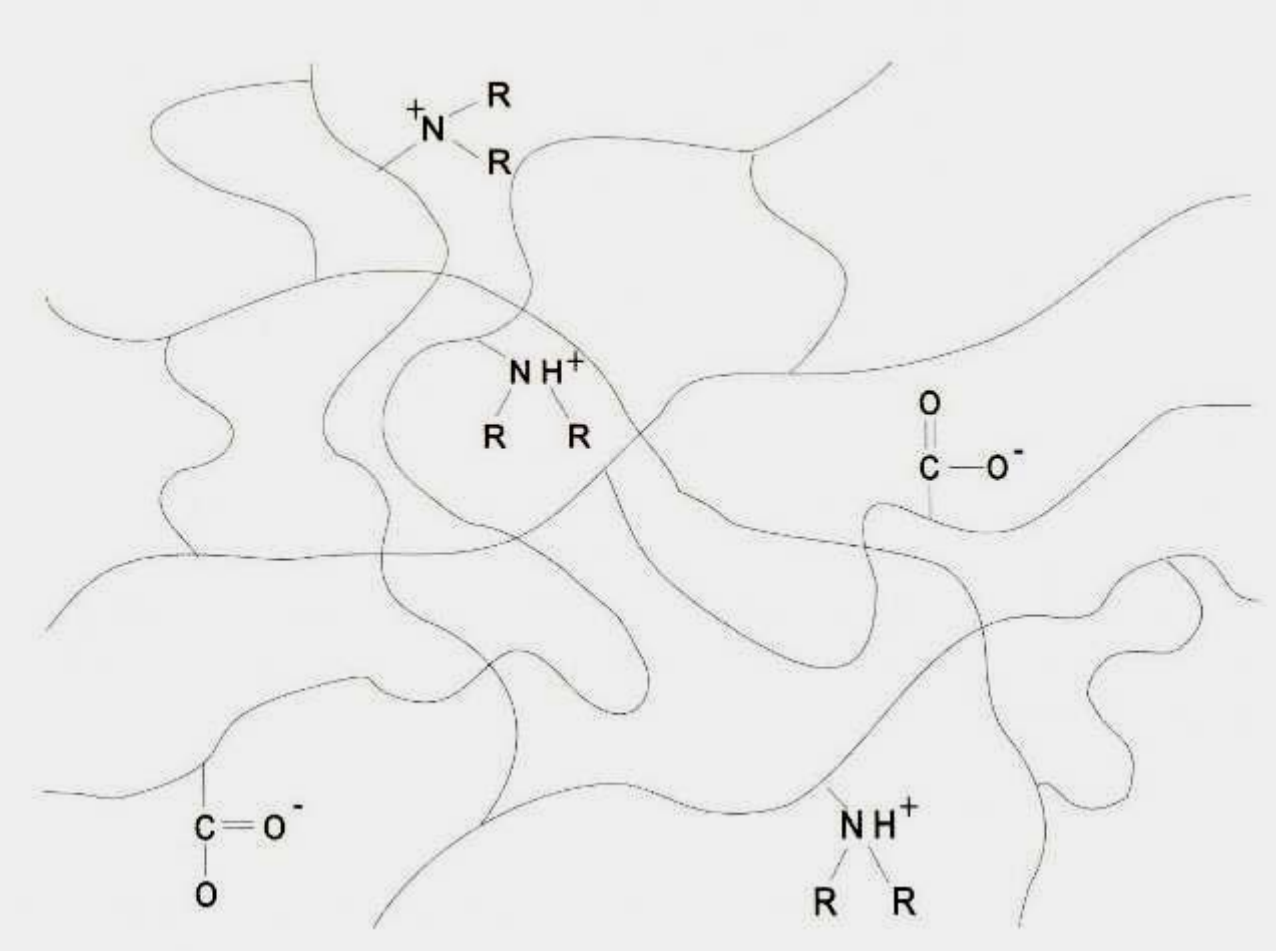
- First developed by Righetti ,(1990).
- Immobilized pH gradient generated by buffering acrylamide derivatives (Immobilines)
- Immobilines are weak acid or weak base.

General structure

R = amino or carboxylic groups

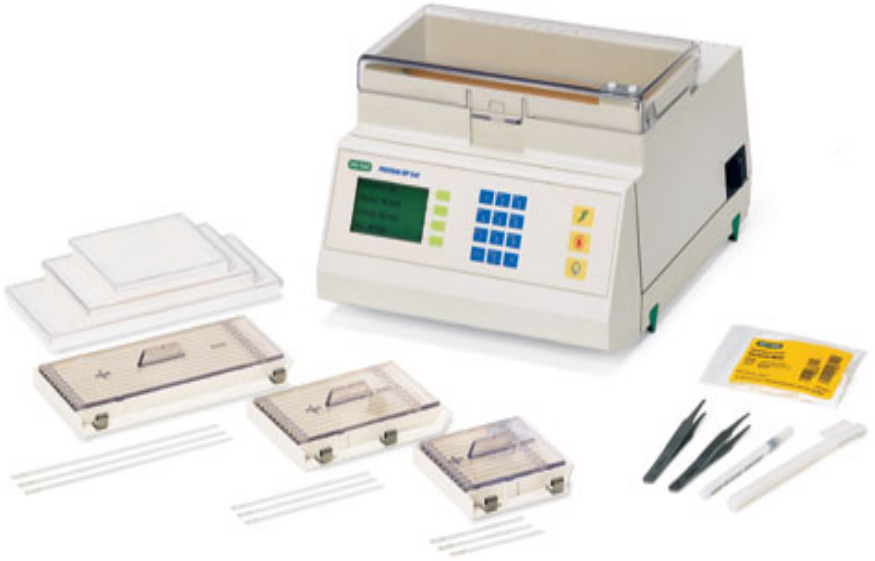
Acrylamide

Schematic drawing of IPG matrix



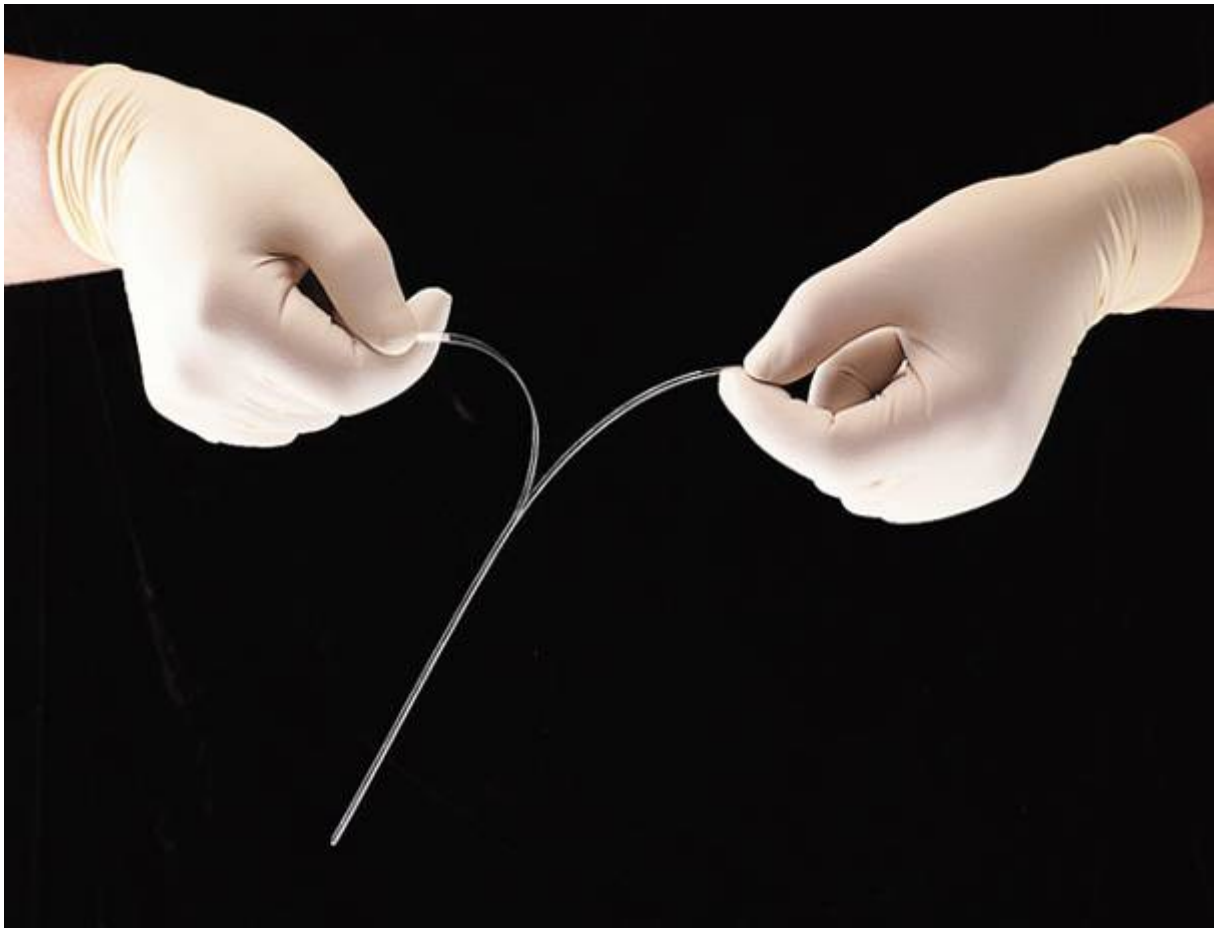
First dimension electrophoresis instrument

Amersham Biosciences



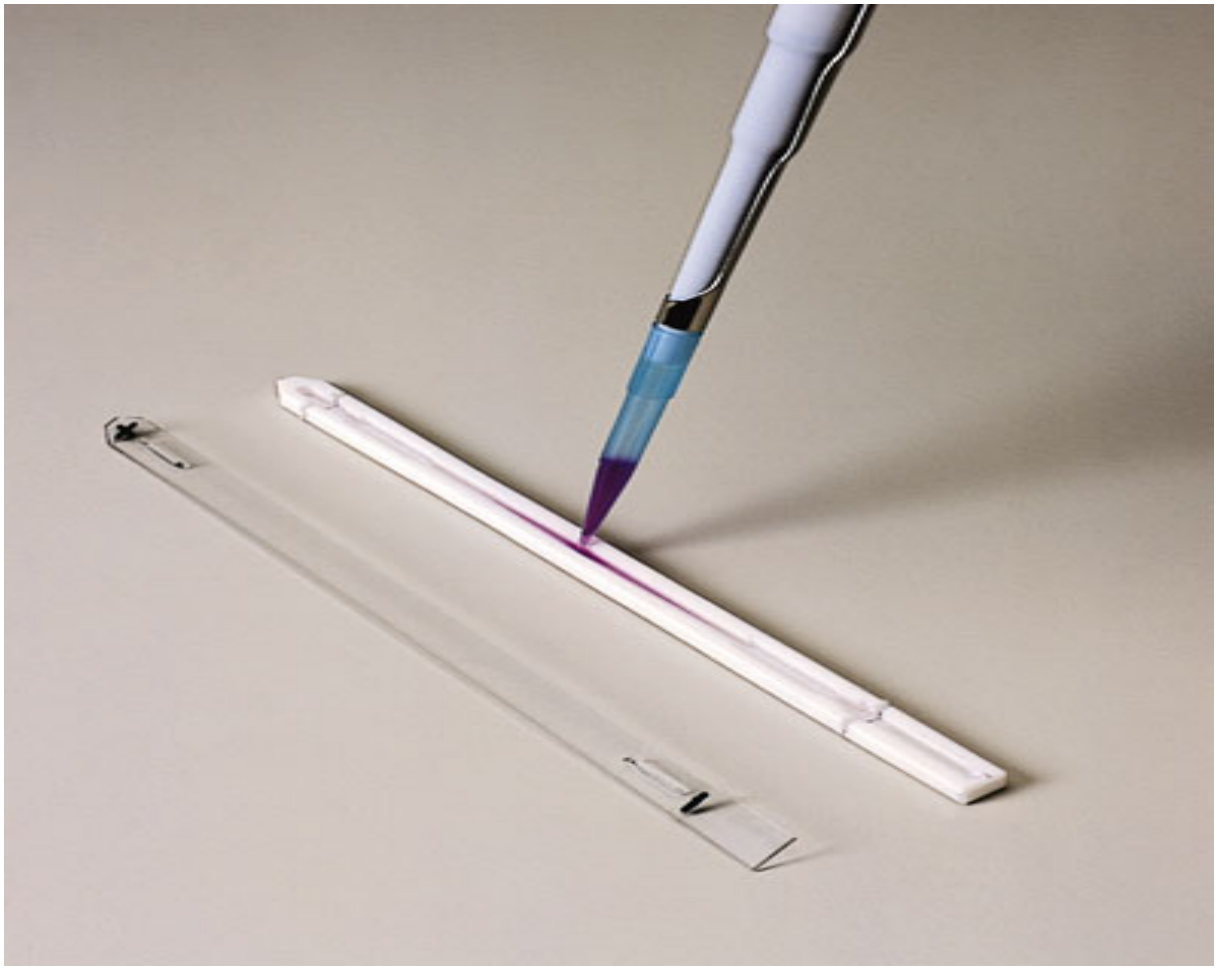
Bio-Rad

Run IEF, STEP 1



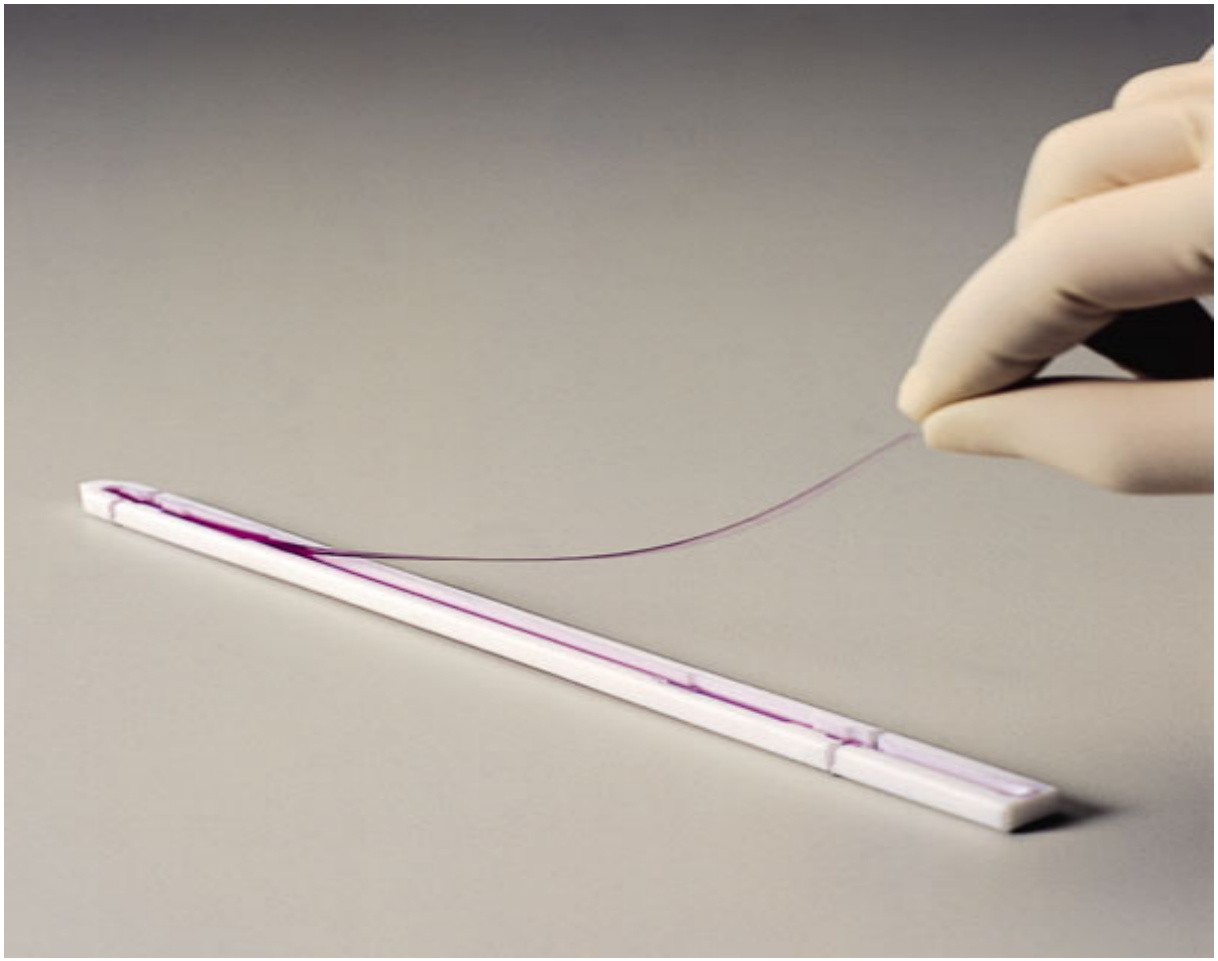
1. Remove protective film from Immobiline™ DryStrip gel.

Run IEF, STEP 2



2 . Apply rehydration solution to the Strip Holder.

Run IEF, STEP 3

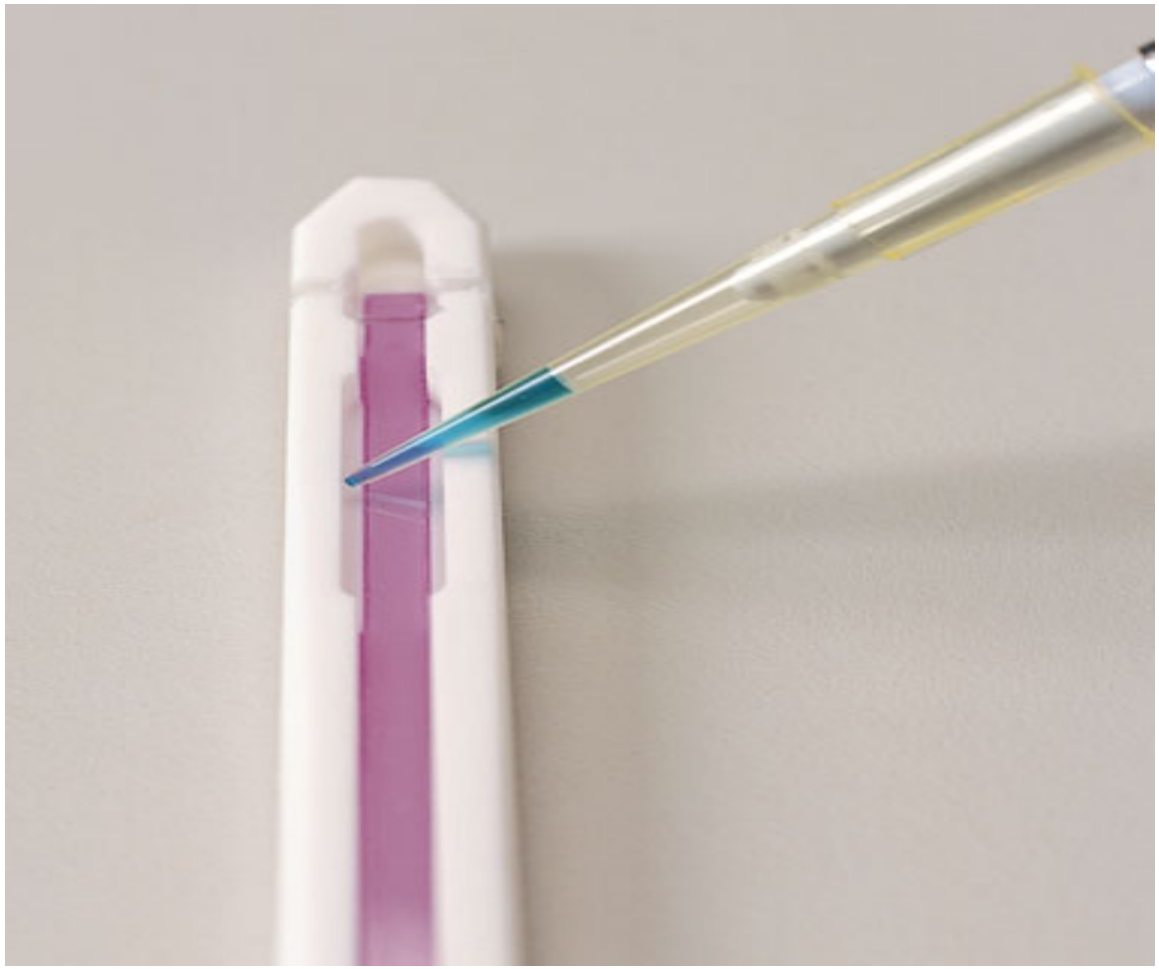


3. Wet entire length of IPG strip in rehydration solution by placing IPG strip in strip holder (gel facing down).

Run IEF, STEP 4

4. Gently lay entire IPG strip in the strip holder, placing the end of IPG strip over cathodic electrode.

Run IEF, STEP 5

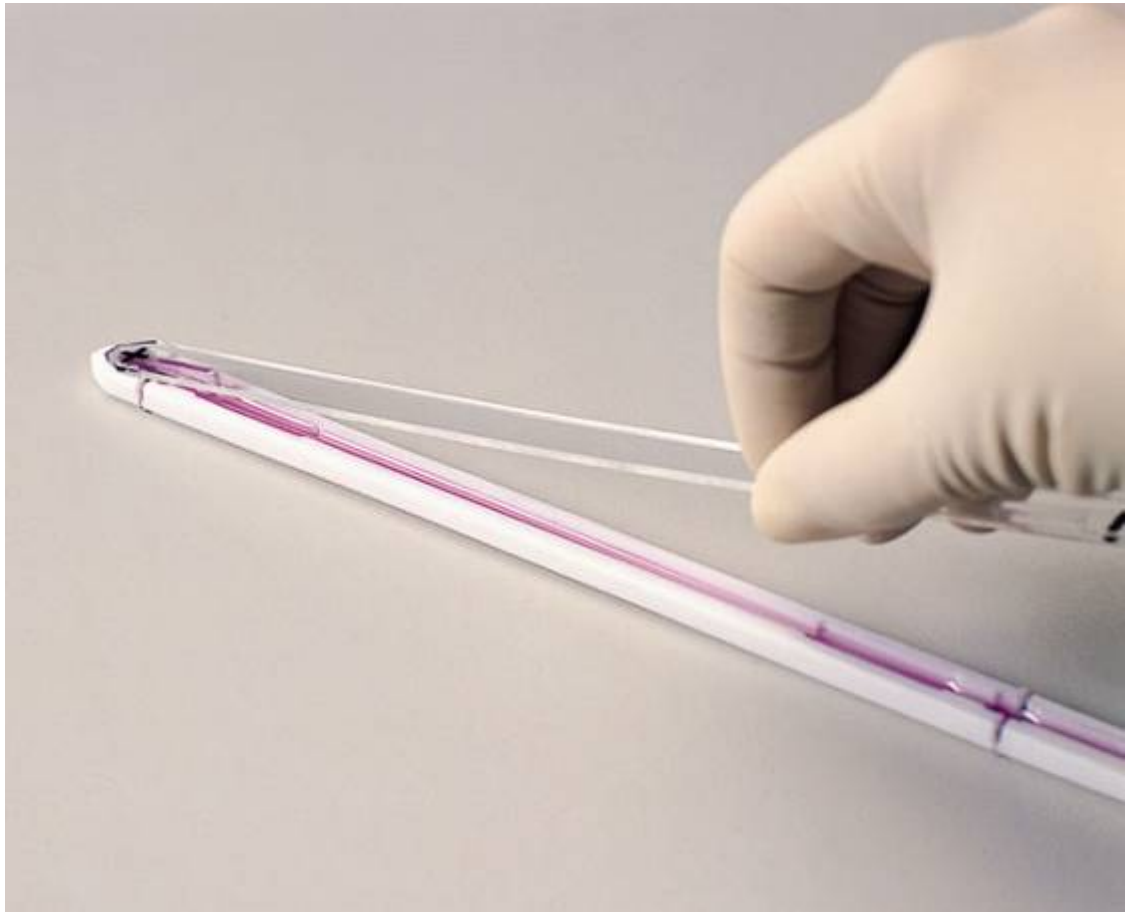


5. Protein sample can be applied at sample application well following the rehydration step if the protein sample was not included in the rehydration solution.

Run IEF, STEP 6

6. Carefully apply DryStrip Cover Fluid along entire length of IPG strip.

Run IEF, STEP 7

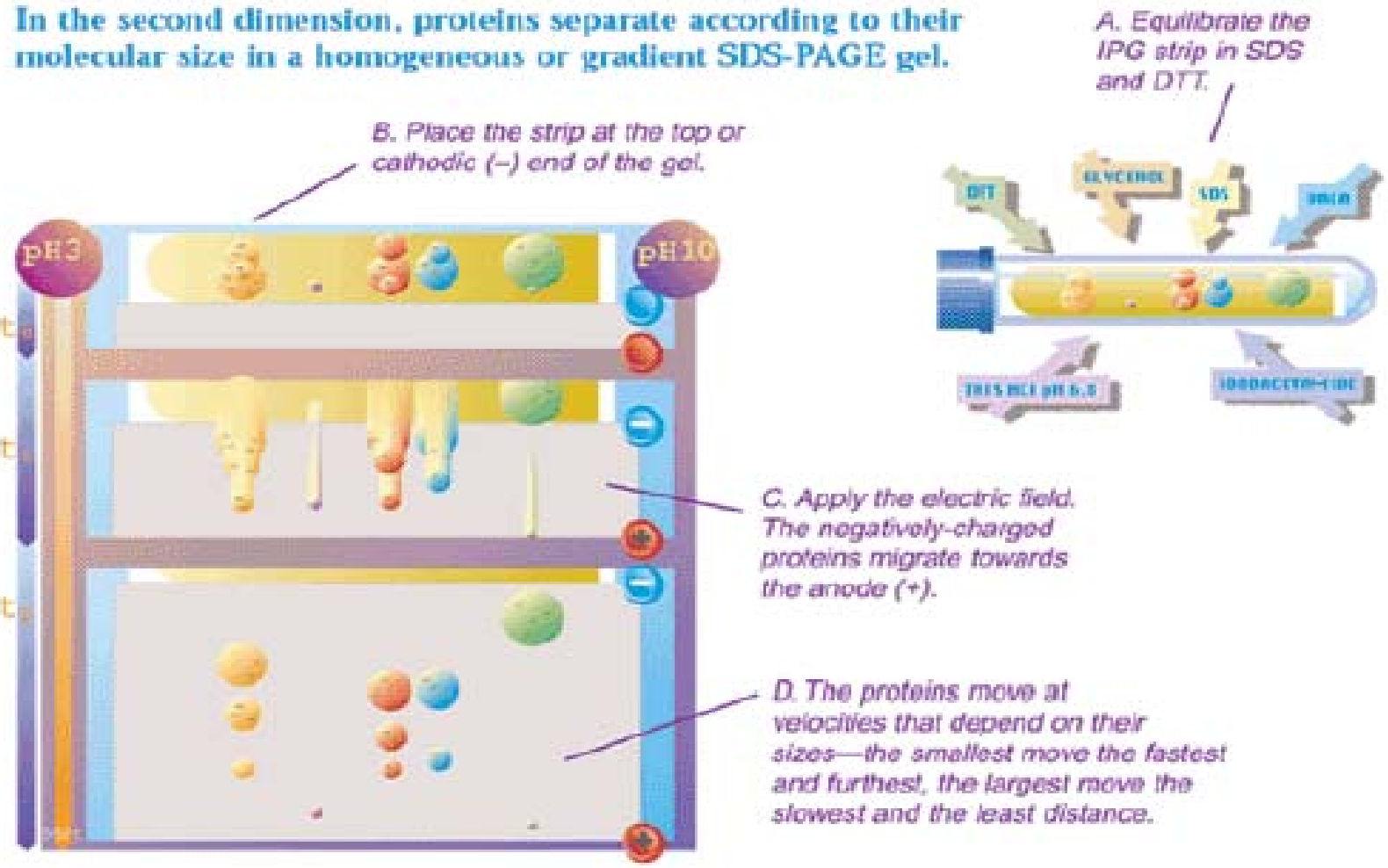


7. Place cover on strip holder.

Run IEF, STEP 8

8. Place assembled strip holder on Ettan™ IPGphor™ platform

From IEF to SDS-PAGE



Choice of electrophoresis systems

Hoefer mini VE requires power supply EPS301 to complete the system.

8 x 10 cm

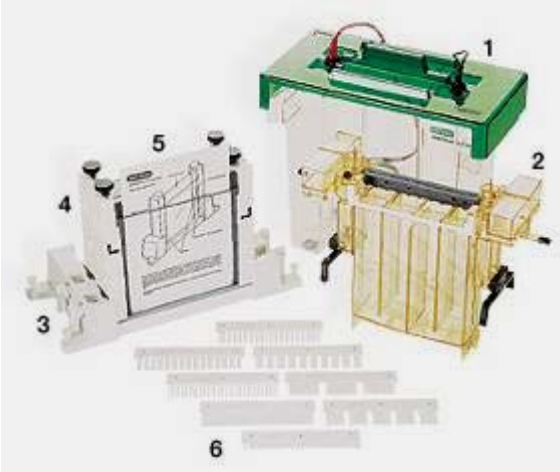
SE 600 requires MultiTemp III and power supply EPS601 to complete the system.

16 x 16 cm

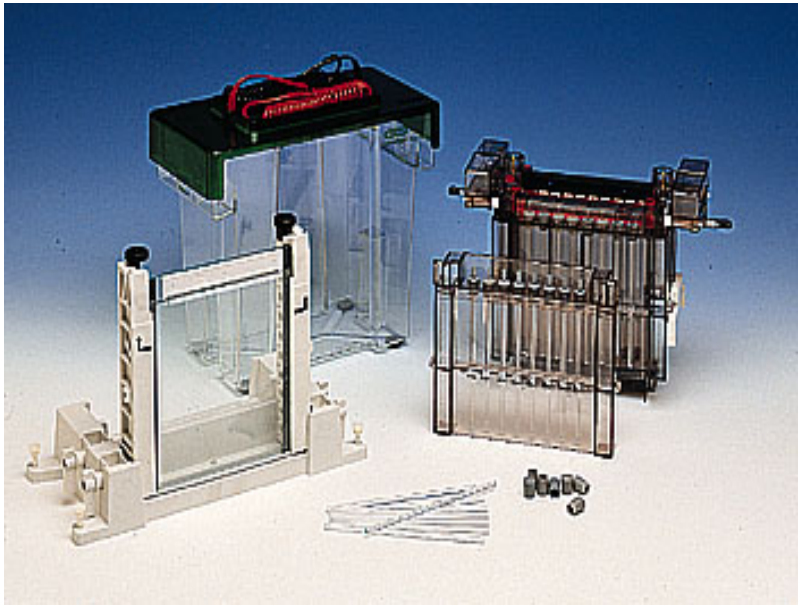
Amersham Biosciences

23 x 20 cm

Choice of electrophoresis systems



Bio-Rad



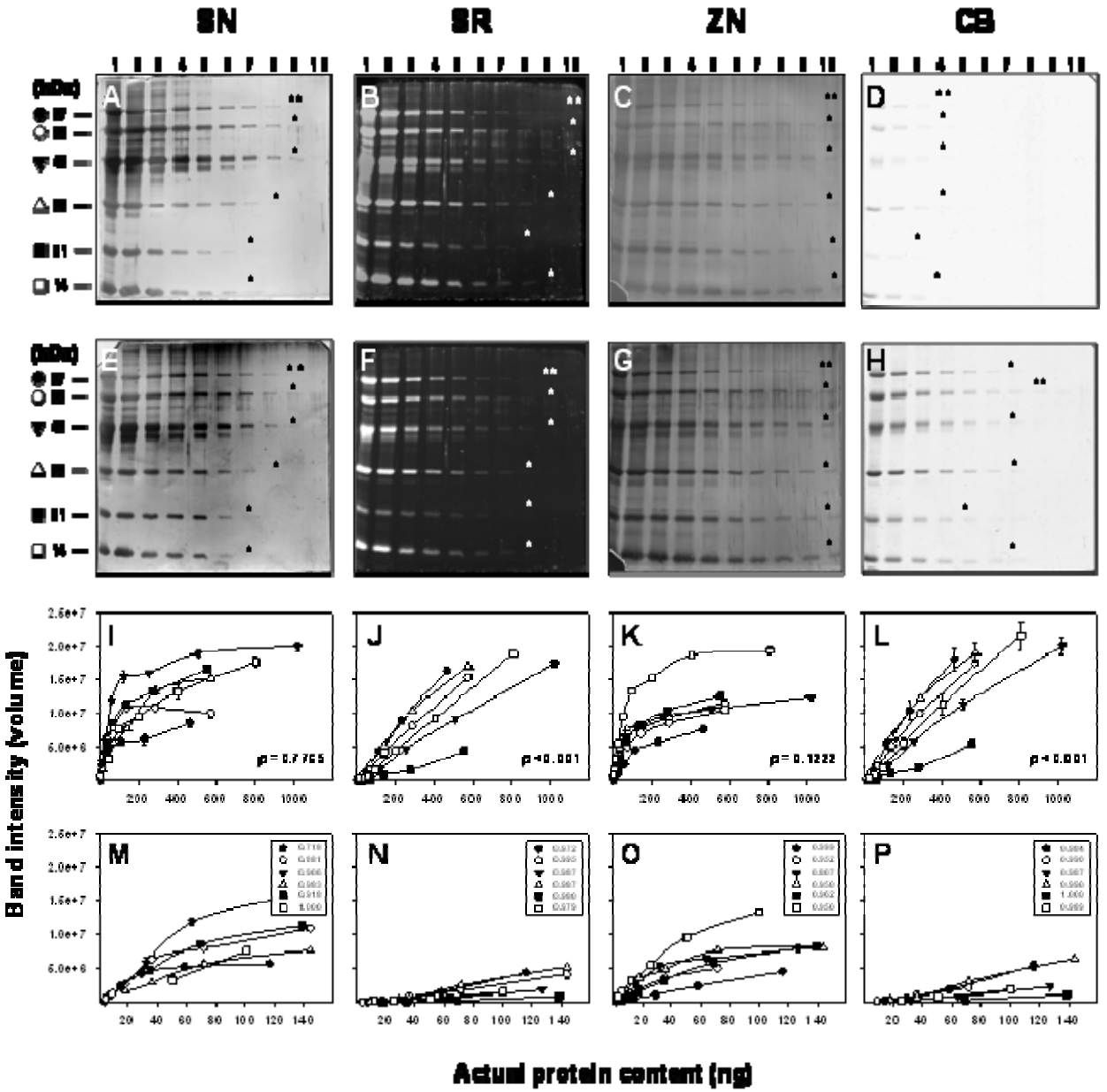
Protein gel staining methods

Comparison of staining methods				
Methods	VisPRO 5 minutes Protein Stain	Sypro Ruby	Silver Stain	CBR Stain
Preparation of solutions	0 min	6 min	20 min	0 min
Fixing Step	0 min	1 hr	1hr~overnight	0 min
Stain	5 min	overnight	3 hr	30 min~ overnight
Image Dispaly*	30 sec	30 min		30 min~ overnight
Total Time	5 min 30 sec	18 hr	4~20 hr	1hr~8hr
Irratated or toxic chemical	no	Acetic acid、Methanol	Acetic acid、Silver Nitrate、Glutaraldehyde	Acetic acid、Methanol
Sensitivity	<1ng	1 ng	1 ng	50 ng
Quantitative range	1-100 ng	1-1000 ng	1-100ng	50-500 ng
Capability to downstream applications	Yes	Yes	No (Protein Cross link)	Yes but limit
*including operating、developing and destaining Time				

Advantage of using zinc reverse staining

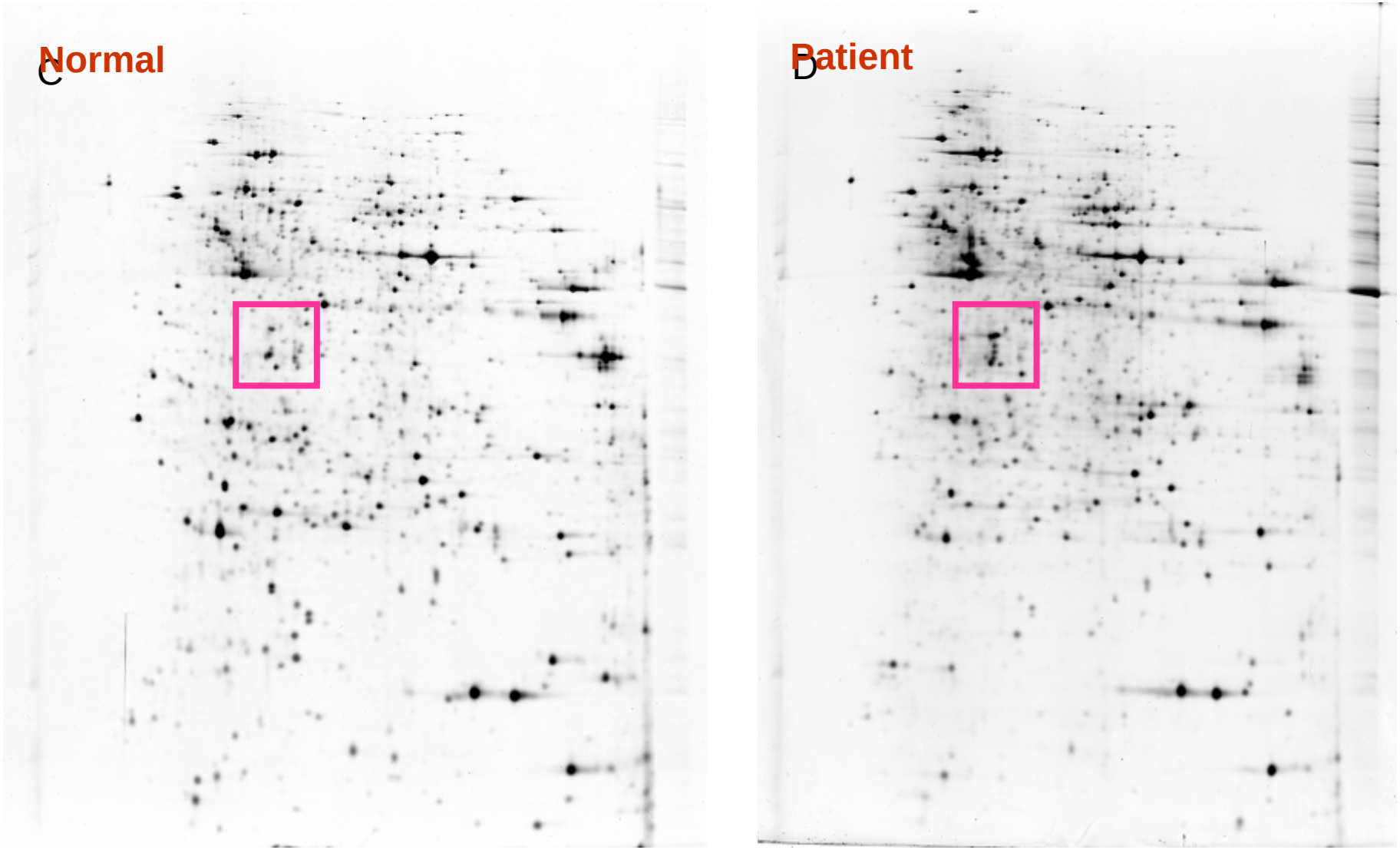
1. Fast (5mins)
2. Convenient (only 2 reagents)
3. Sensitivity (1ng)
4. Compatible to mass spectrometry
5. Re-stainable by all other methods
6. Recover of protein is possible
7. Economic
8. Less toxic (require no heavy metal or organic solvent)

Comparison of staining sensitivity of four methods



2008 Proteomics Lin et. al,

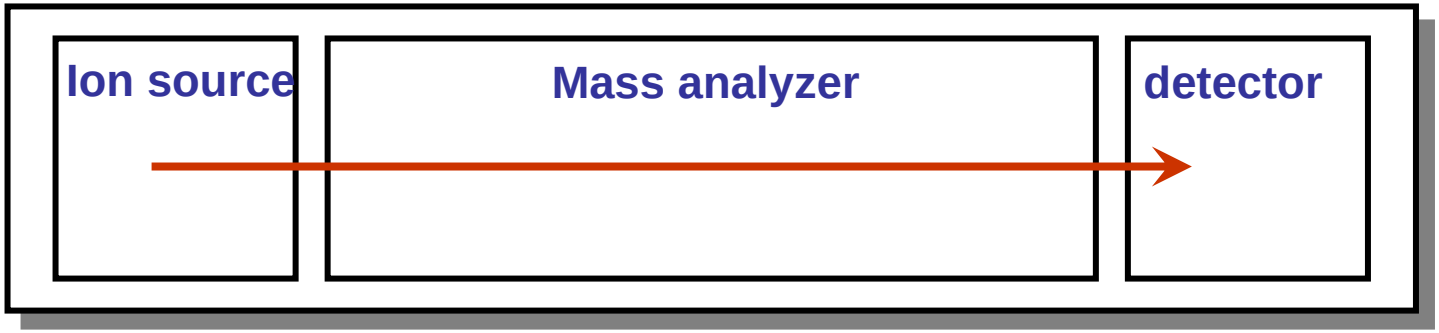
Find the different protein spots on 2-DE gels



Prepare the protein spots for analysis by mass

1. Washing staining material from the gel
2. Equilibrium of gel to protease buffer condition
3. Reduction and alkylation of proteins
4. Digestion of proteins to peptides
5. Extraction of peptides
6. Purification of peptides (Optional)

Principle of mass spectrometry in proteomics



Ion source: ionize peptide/protein to gas ions

Mass analysis: analyze ion according to mass/charge (m/z)

Detection: detect the prescence of ions

femtomole –attomole (10^{-15} – 10^{-18} mole)

Commonly used Mass Spectrometer in Proteomics

MALDI-TOF

Matrix Assisted Laser Desorption Ionization Time Of Flight

ESI tandem MS (with HPLC, LC tandem MS or LC MS/MS)

Electro Spray Ionization Mass Spectrometry

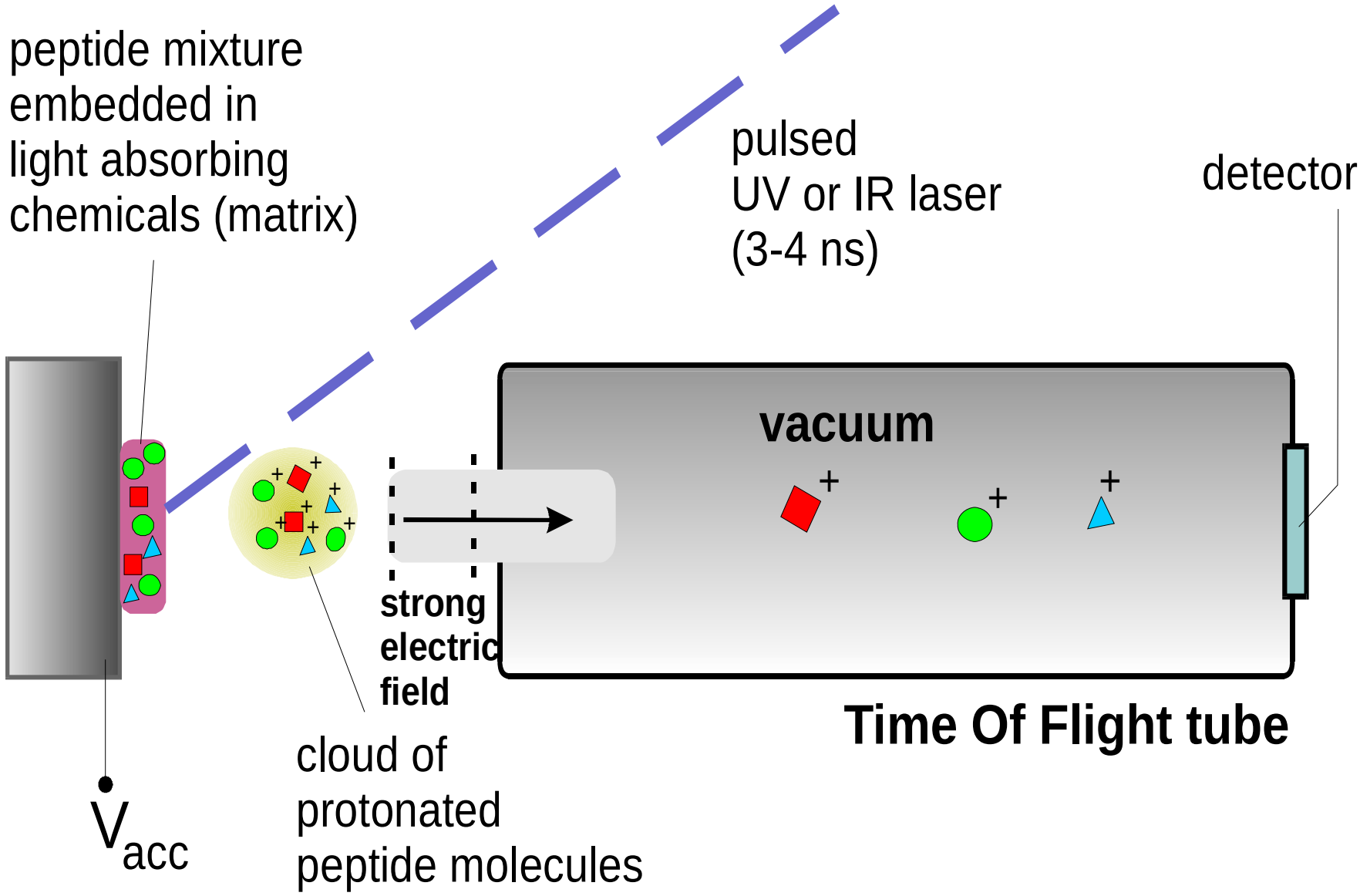
Commercial available MALDI-TOF

Microflex ™, Bruker

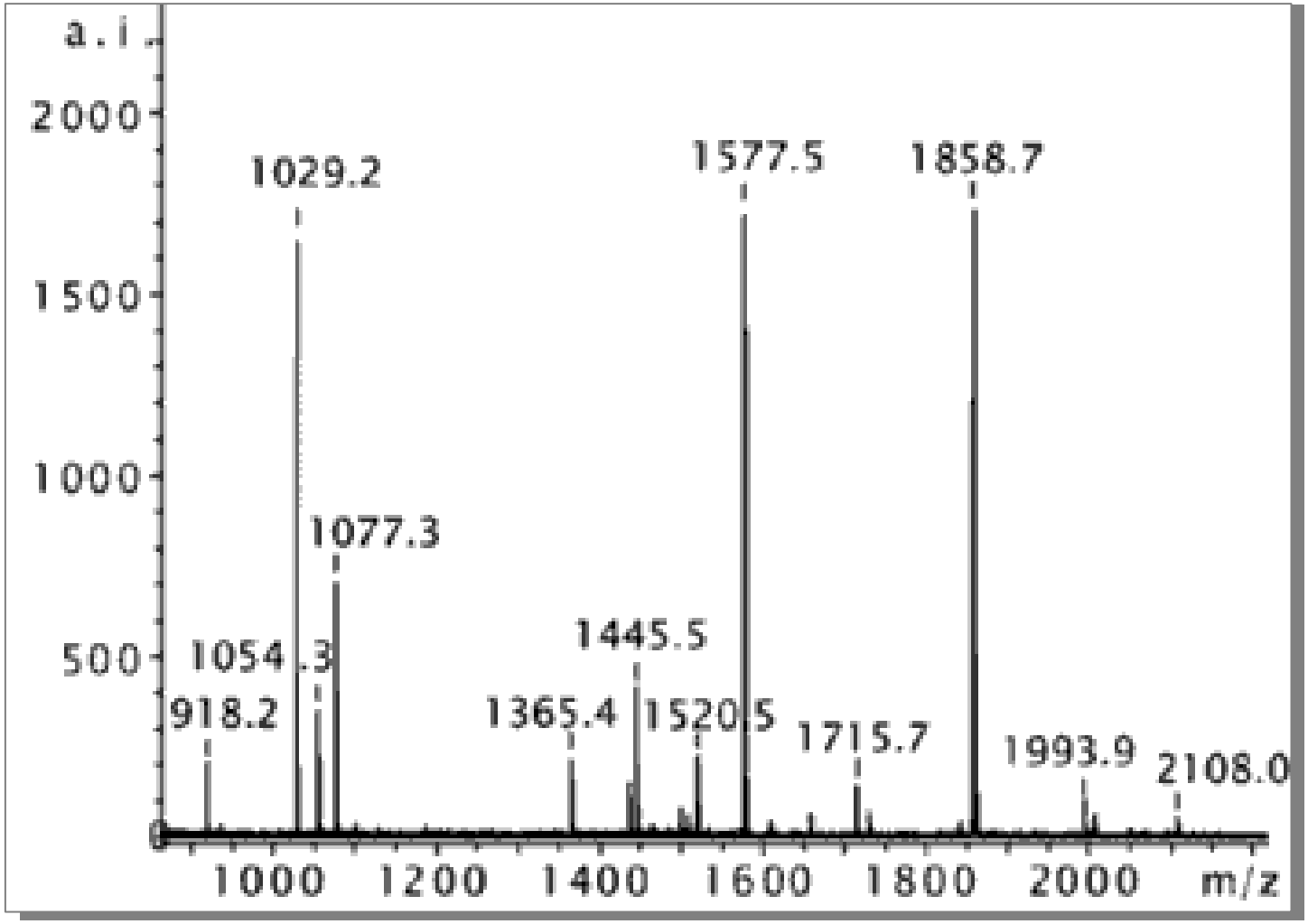
Voyager DE-PRO™, ABI

MALDI micro™, Micromass

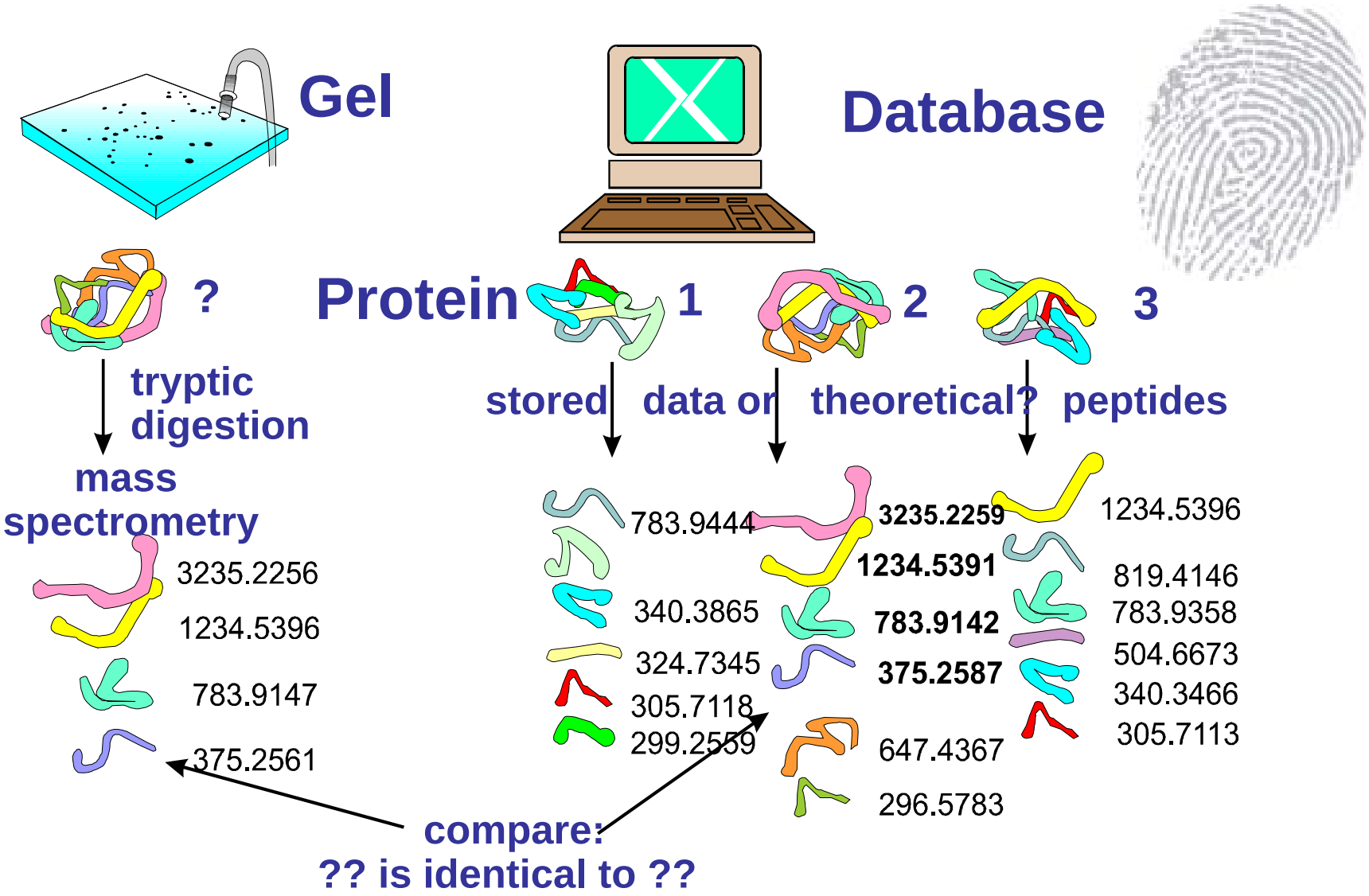
Principle of MALDI-TOF mass spectrometry



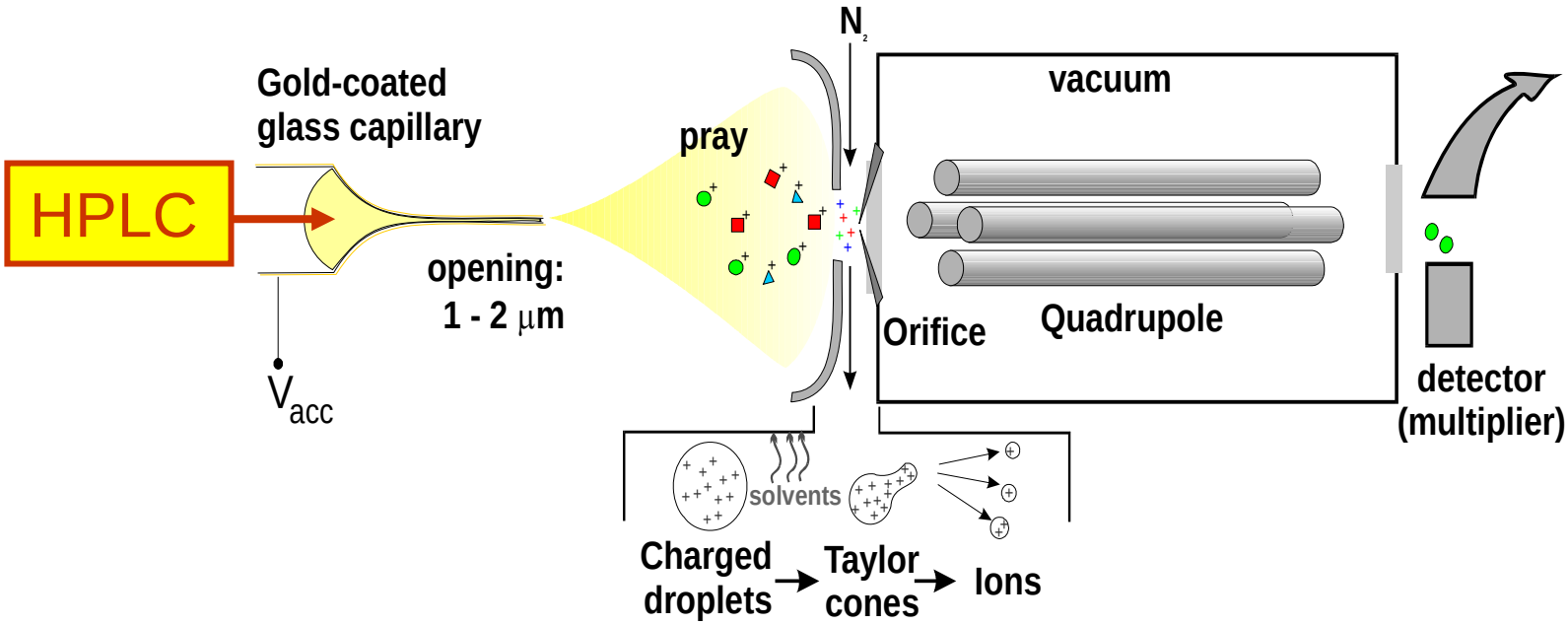
Typical result from MALDI-Tof (spectrum)



Peptide mass fingerprinting (PMF)

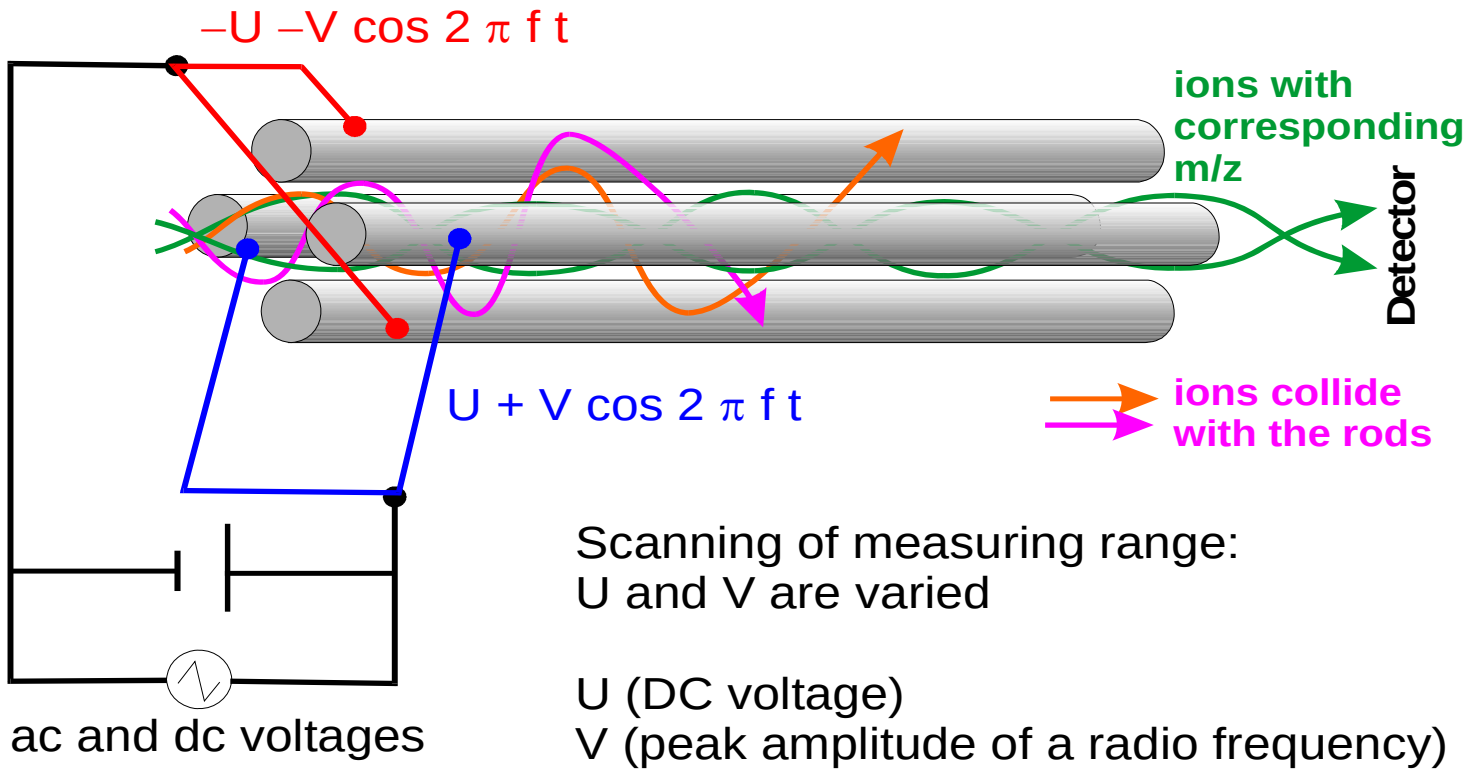


ESI Quadrupole MS



- Nano electrospray: >30 min spray time for 1 μL sample
- Highly charged molecules are selected by ac modulation of transverse fields

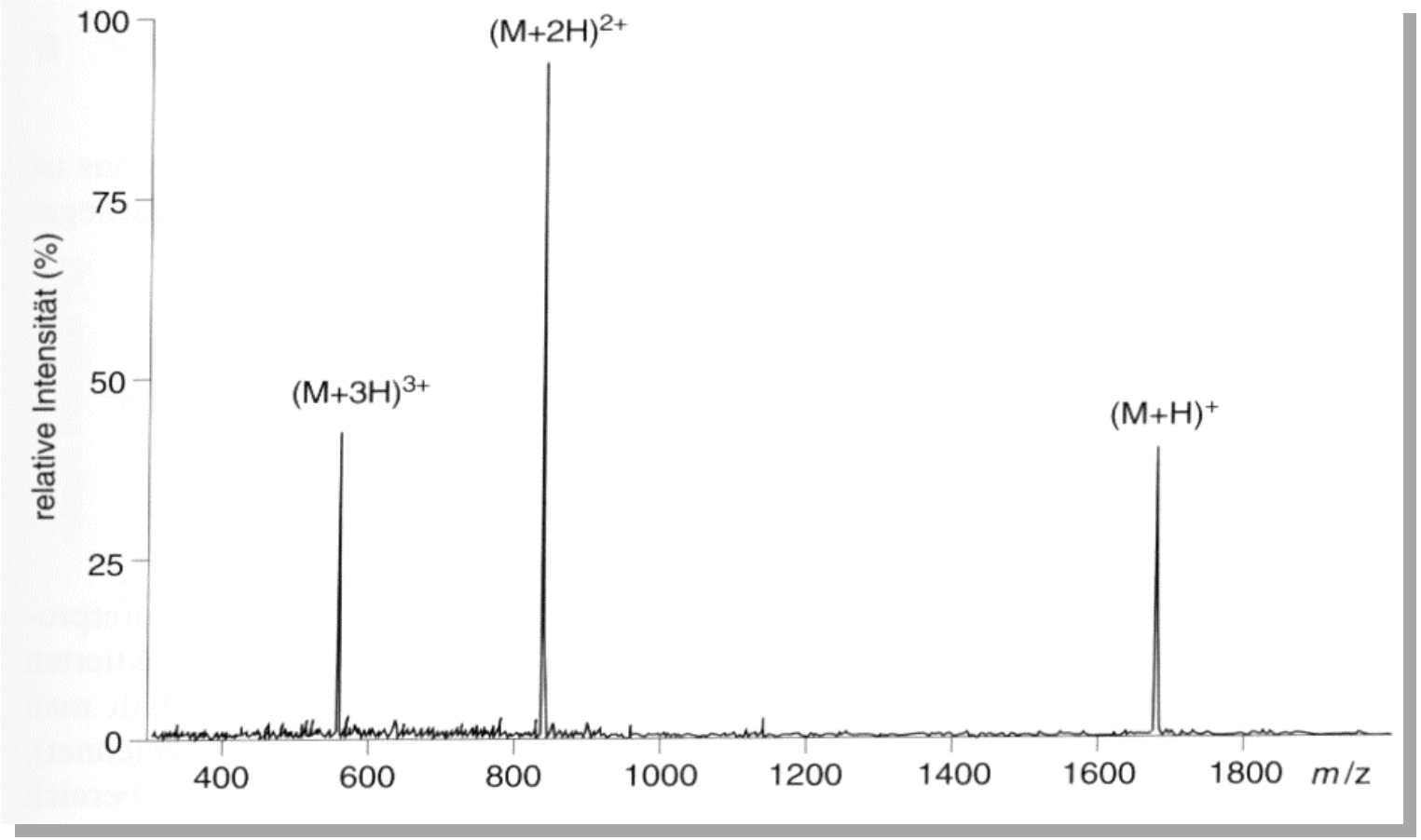
Principle of Quadrupole mass filter



Scanning of measuring range:
U and V are varied

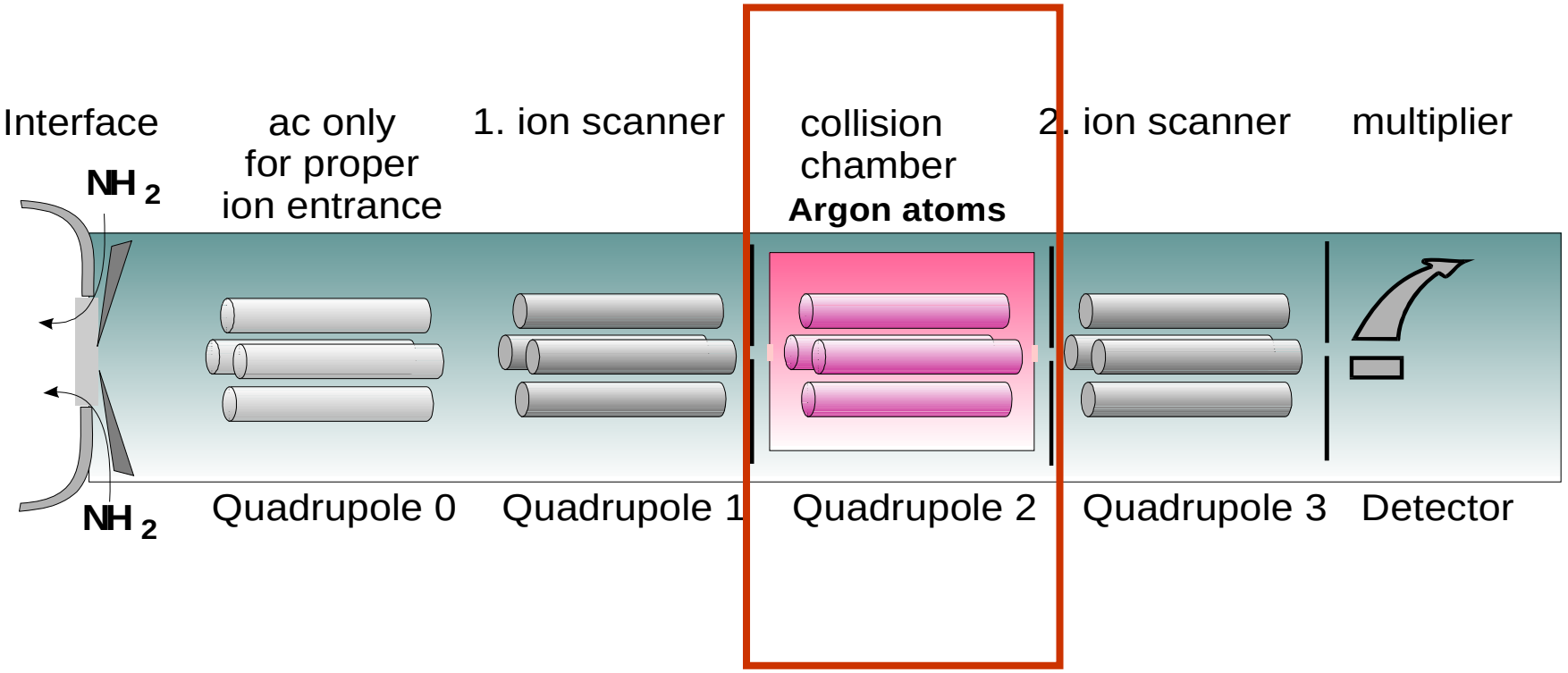
U (DC voltage)
V (peak amplitude of a radio frequency)

Typical result from ESI Quadrupole MS



From Eckerskorn in “Bioanalytik”, Lottspeich and Zorbas (Eds)

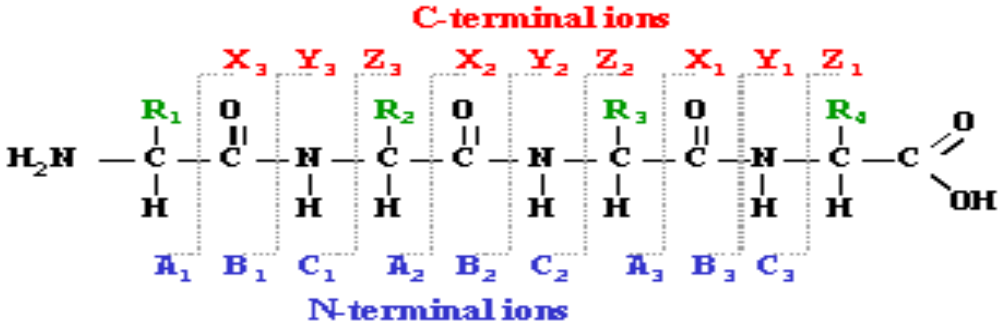
Triple Quadrupole Mass Spectrometer



CID: **C**ollision **I**nduced **D**issociation

for acquiring Molecular weight and Structural information

Nomenclature for CID fragments

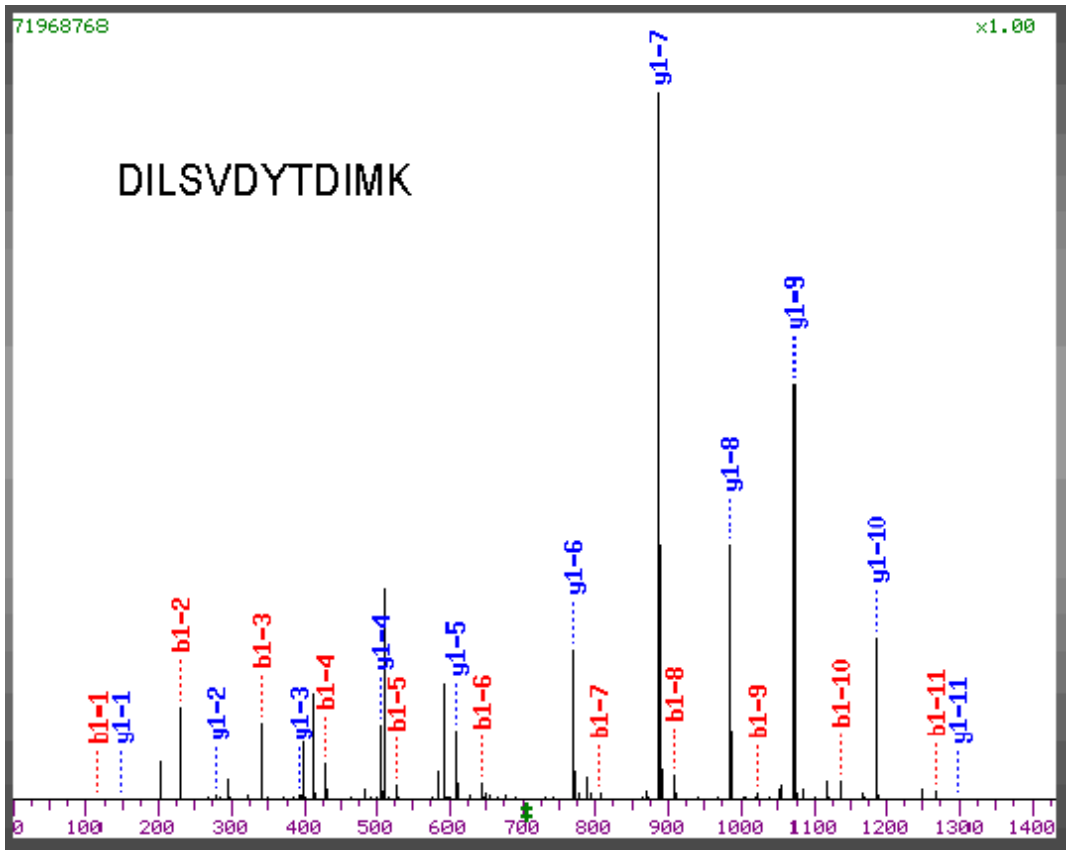


S-P-A-F-D-S-I-M-A-E-T-L-K

(protonated mass 1410.6)

mass+	b-ions	y-ions	mass+
88.1	S	PAFDSIMAETLK	1323.6
185.2	SP	AFDSIMAETLK	1226.4
256.3	SPA	FDSIMAETLK	1155.4
403.5	SPAF	DSIMAETLK	1008.2
518.5	SPAFD	SIMAETLK	893.1
605.6	SPAFDS	IMAETLK	806.0
718.8	SPAFDSI	MAETLK	692.3
850.0	SPAFDSIM	AETLK	561.7
921.1	SPAFDSIMA	ETLK	490.6
1050.2	SPAFDSIMAE	TLK	361.5
1151.3	SPAFDSIMAET	LK	260.4
1264.4	SPAFDSIMAETL	K	147.2

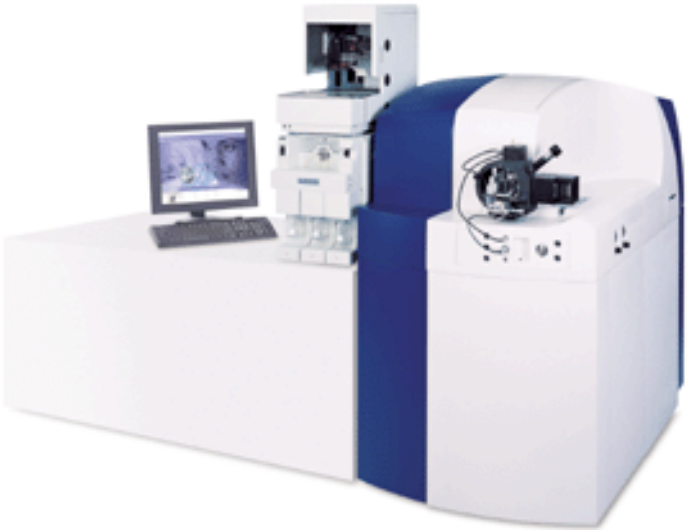
CID mass spectrum



Note: Not all b or y ions will present in the spectrum

Commercial LC/MS/MS

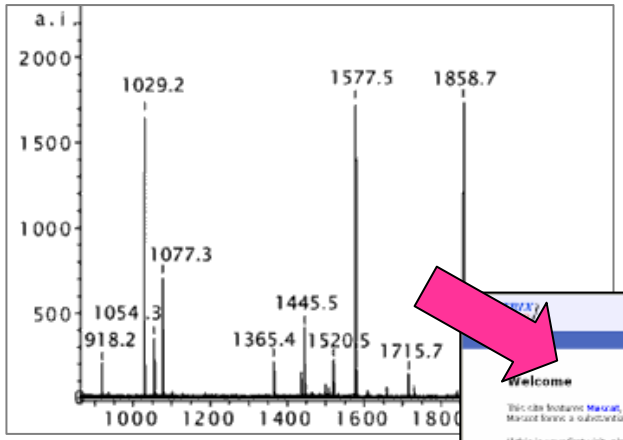
API 4000, API



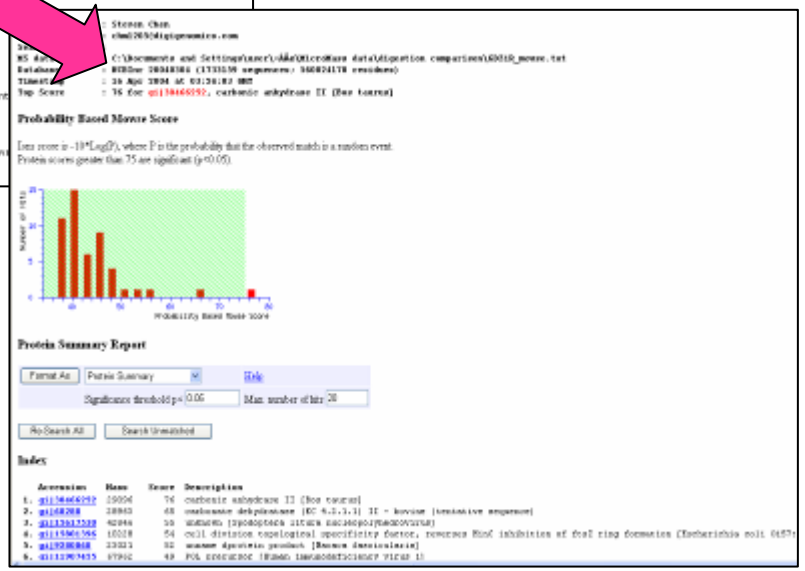
Q-Tof ultima API, Micromass

HCT plus, Bruker

Identification of protein (MASCOT)



http://www.matrixscience.com/



Other proteomic techniques

Applications of Proteomics

1. Protein Complexes Mining
2. Yeast Two-hybrid system (*in vivo* PIP)
3. Phage display and cell surface display system (*in vitro* PIP)
4. Protein Arrays
5. SELDI protein chips (Ciphergen)
6. Multi-dimensional HPLC (MDLC)

1. Protein Complexes Mining

1. Proteome Complex Mining

- A “functional” proteomics approach

- A proteome complex mining example (purification of kinases)

  1. ATP is immobilized to beads in “protein kinase” conformation
  2. Total protein is mixed the beads and the mixture “washed”
  3. Remaining proteins isolated and identified…protein kinases, and purine dependent metabolic enzymes

Proteome complex mining by affinity capture

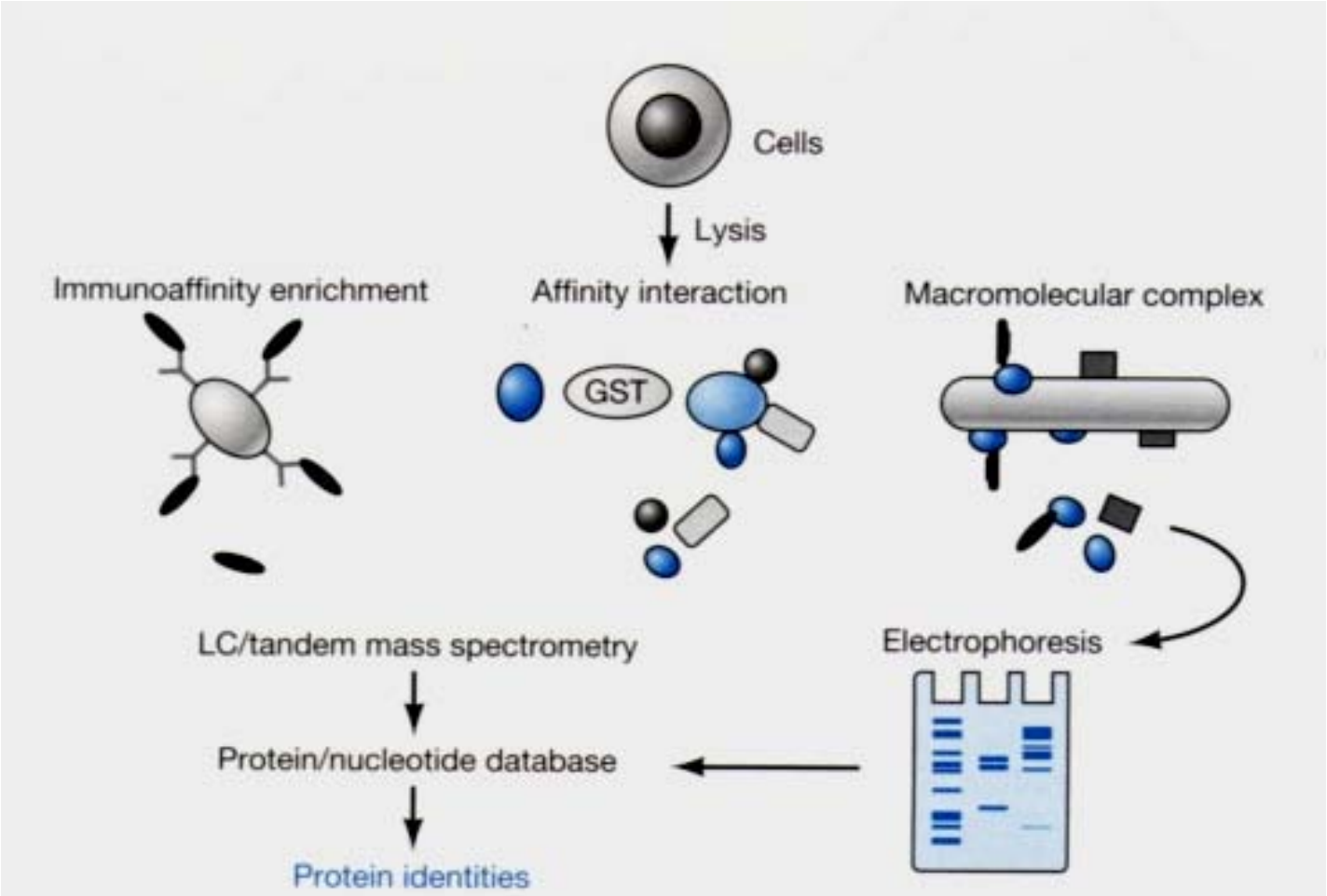
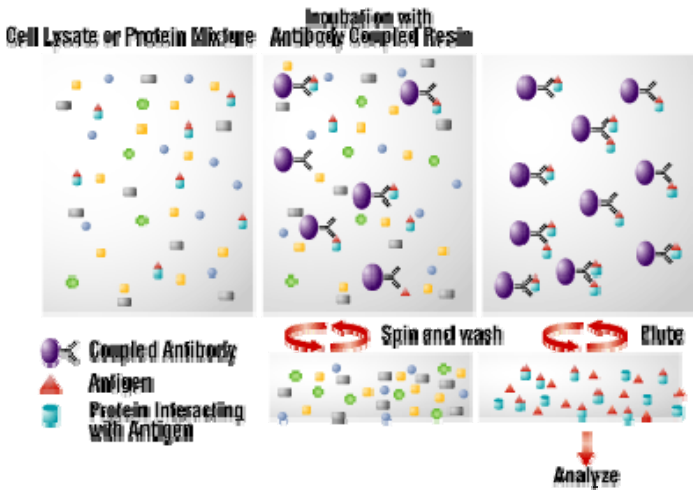


FIGURE 1.9. Cell mapping: Affinity capture methods.

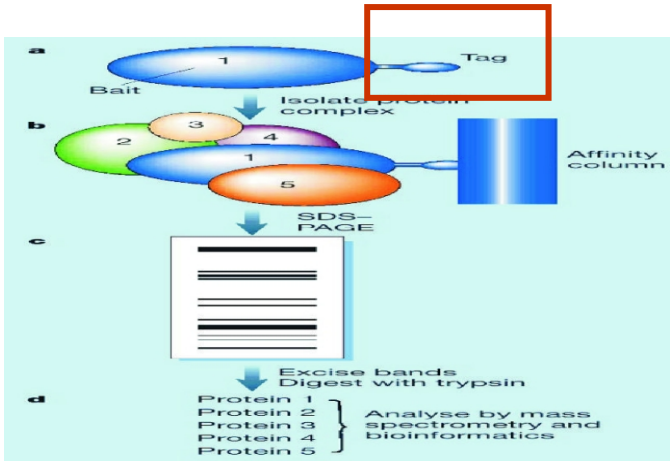
Affinity capture methods

- Coimmunoprecipitation (Adams et al. 2002, eg. Anti p53 antibody)
- Coprecipitation (Seraphin et al. 2003, eg. V5 epitope)
- Protein affinity-interaction chromatography (Einarson and Orlinick 2002, eg. GST fusion protein)
- Isolation of intact multi-protein complexes (eg. Nuclear pore complexes, ribosome complexes, and spliceosomies)

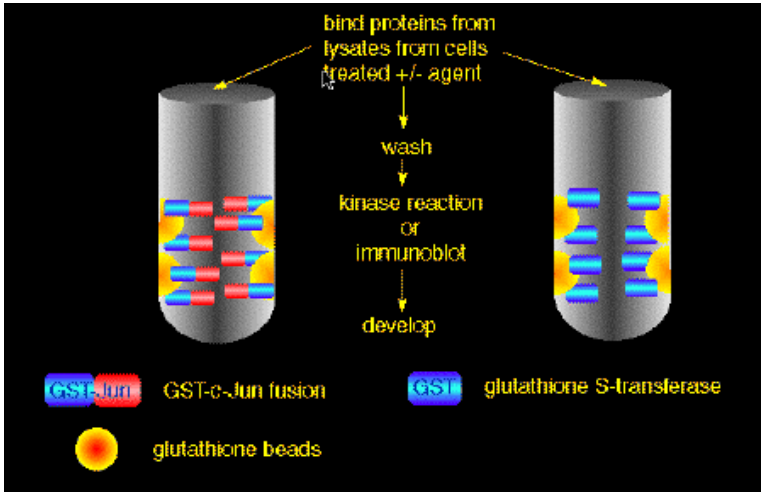
Affinity capture methods



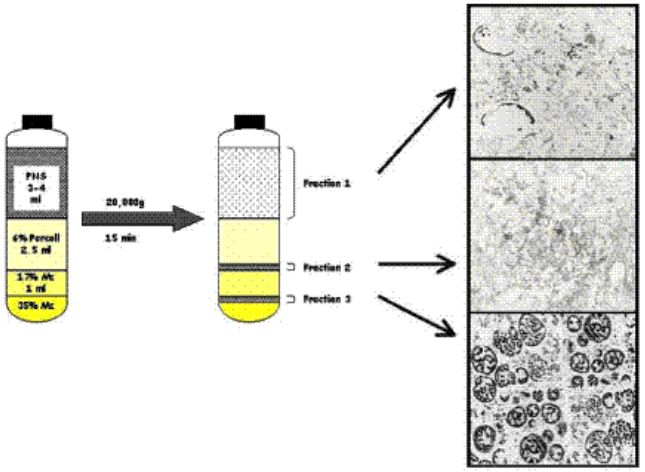
Coimmunoprecipitation



Coprecipitation



Protein affinity-interaction

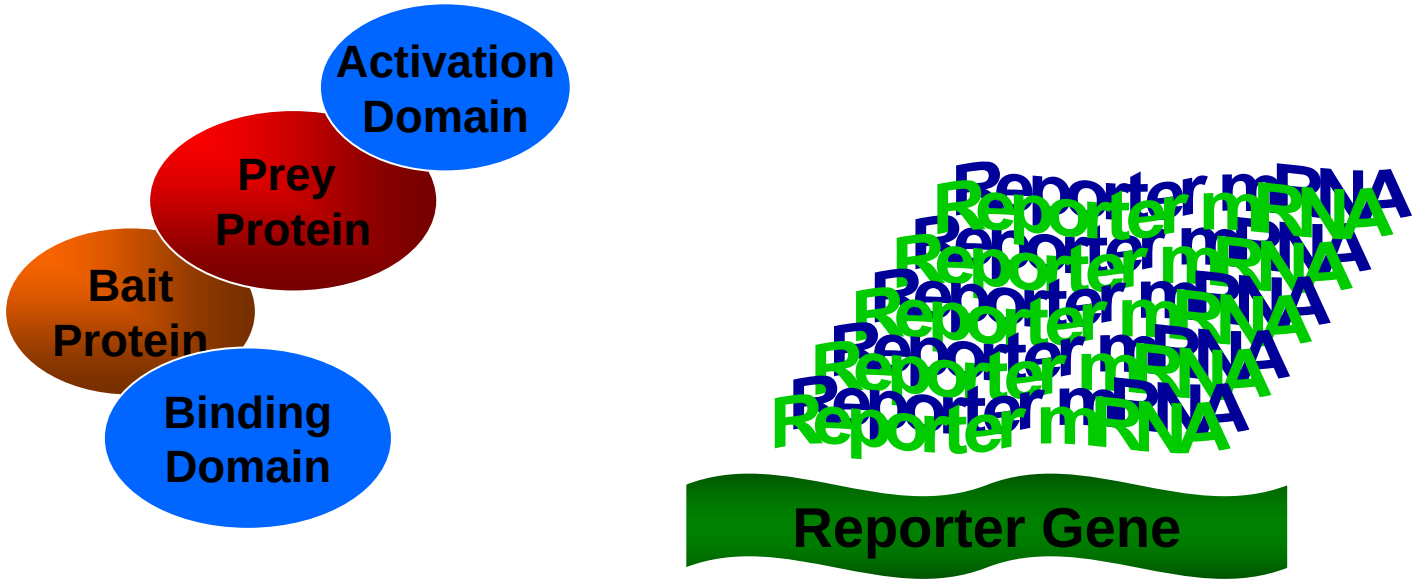


Isolation of intact multi-protein complexes

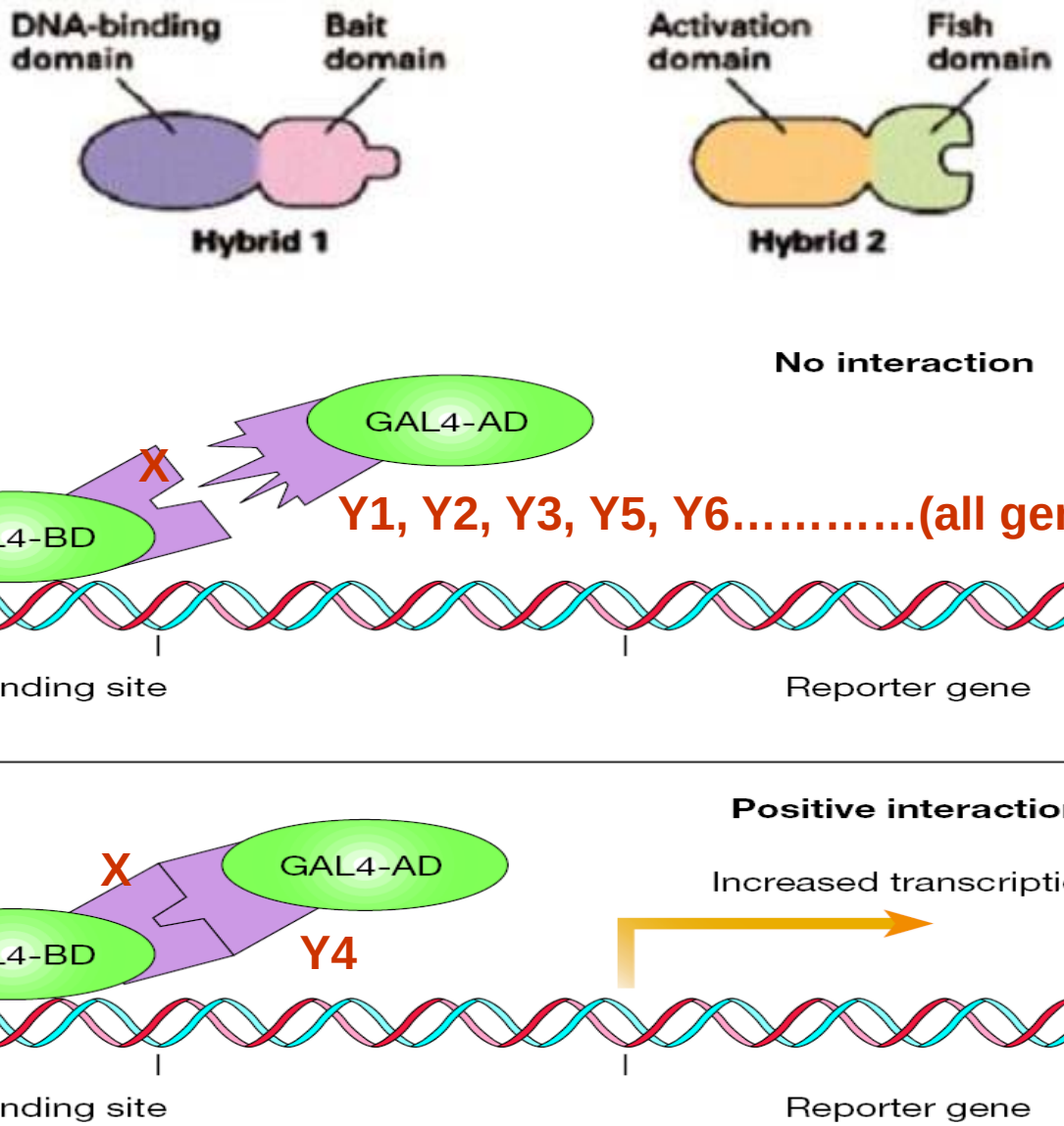
2. Yeast Two-hybrid system (*in vivo* PIP)

2. Yeast Two-Hybrid System (*in vivo*)

- Interaction of **bait** and **prey** proteins localizes the activation domain to the reporter gene, thus activating transcription.
- Since the reporter gene typically codes for a survival factor, yeast colonies will grow only when an interaction occurs.

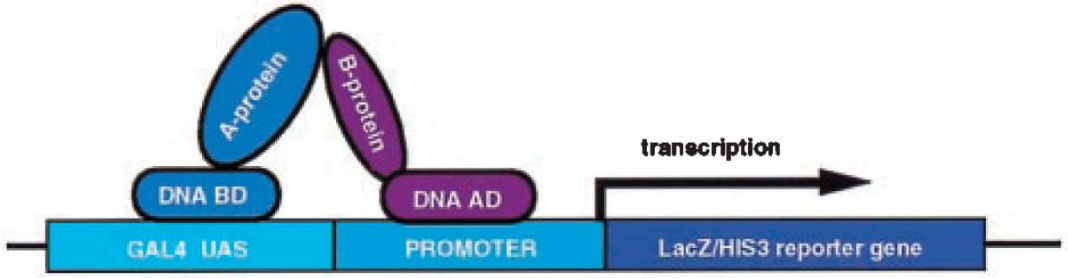


Yeast 2 hybrid system, contd.

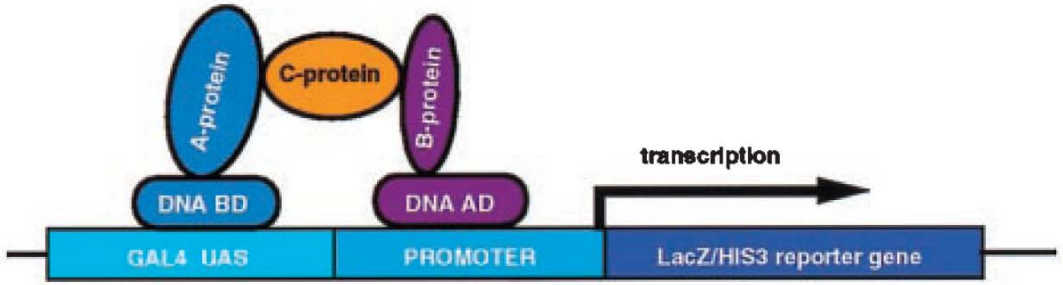


More complex Yeast 2/3/4 hybrid system

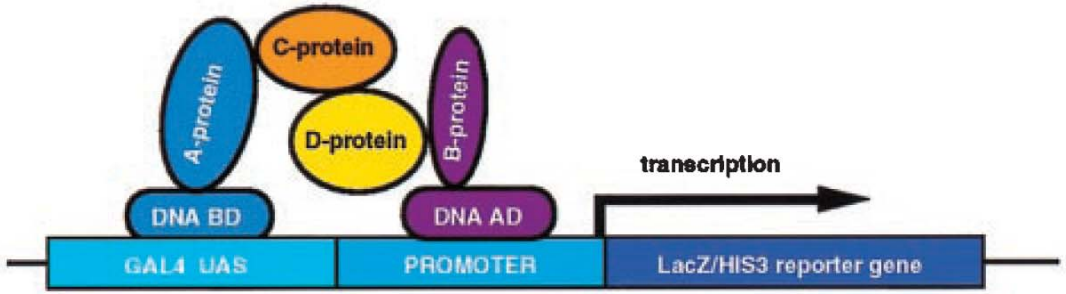
(1) Interaction of two proteins



(2) Interaction of three proteins

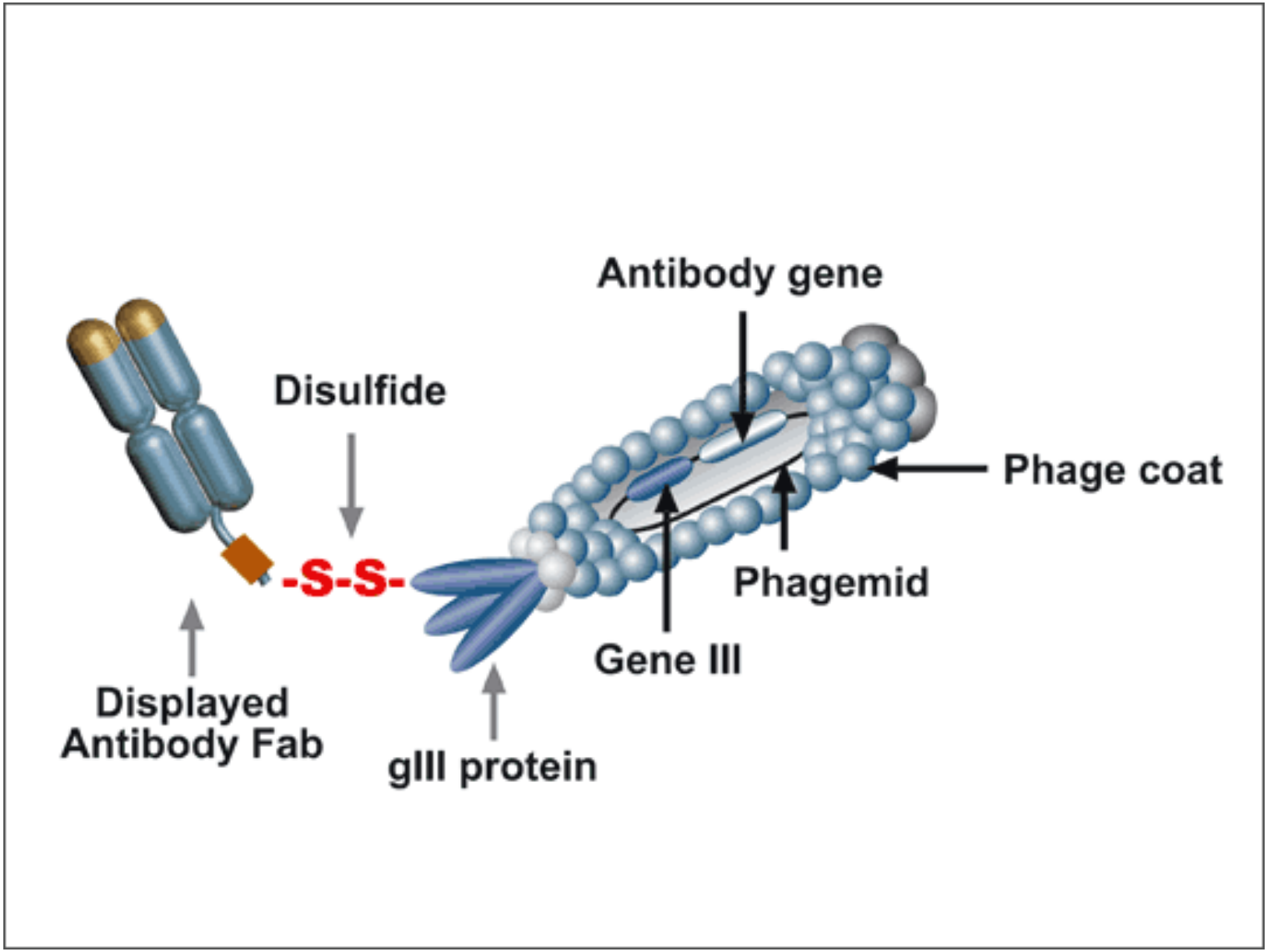


(3) Interaction of four proteins

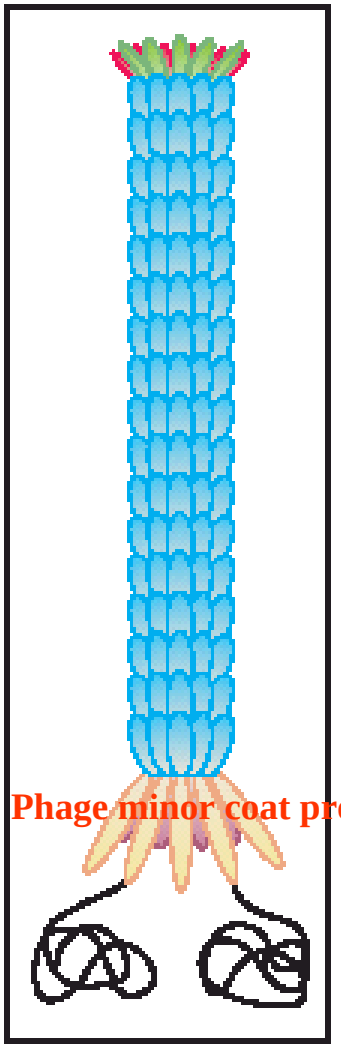


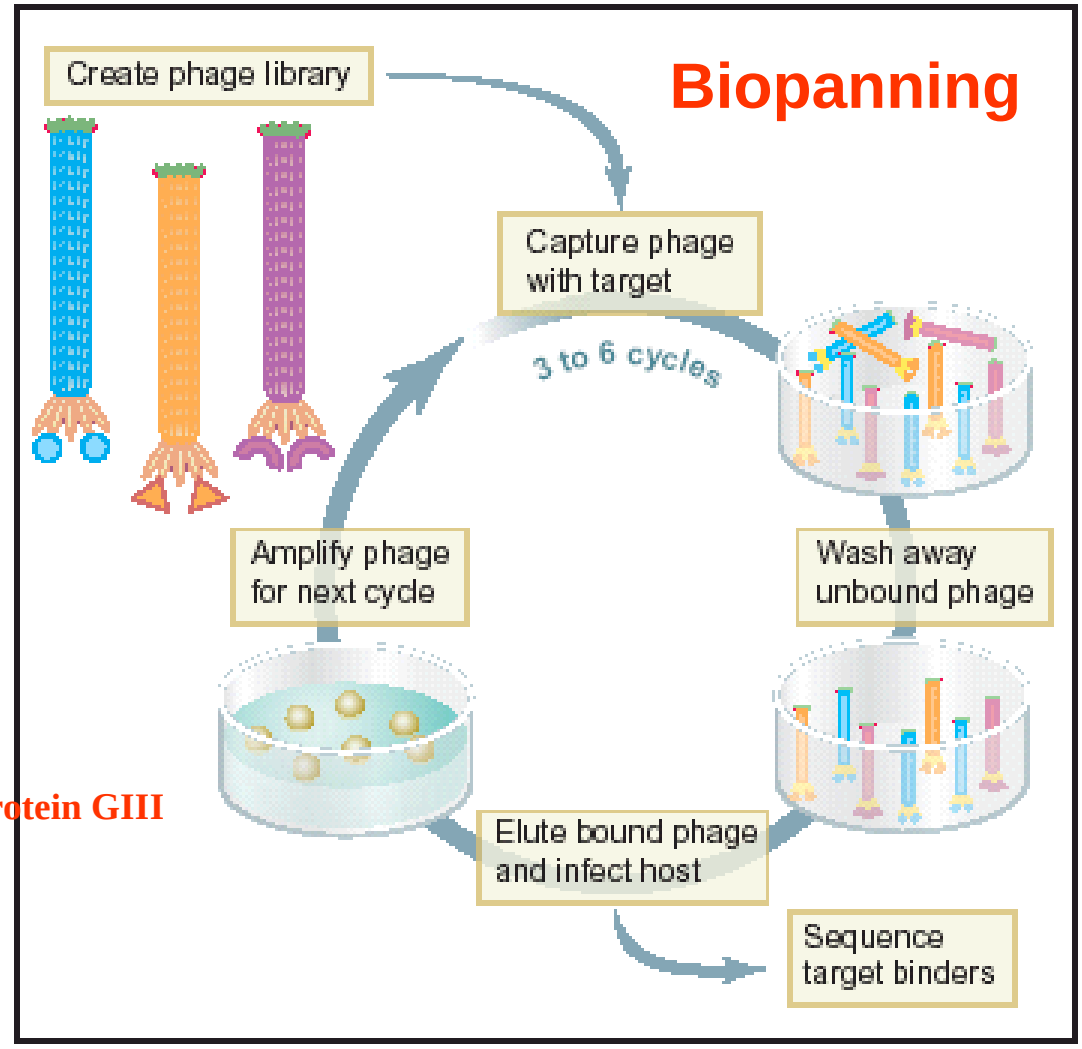
3. Phage display and cell surface display system (*in vitro PIP*)

3. Phage display system (*in vitro*)



3. Phage display system (*in vitro*)



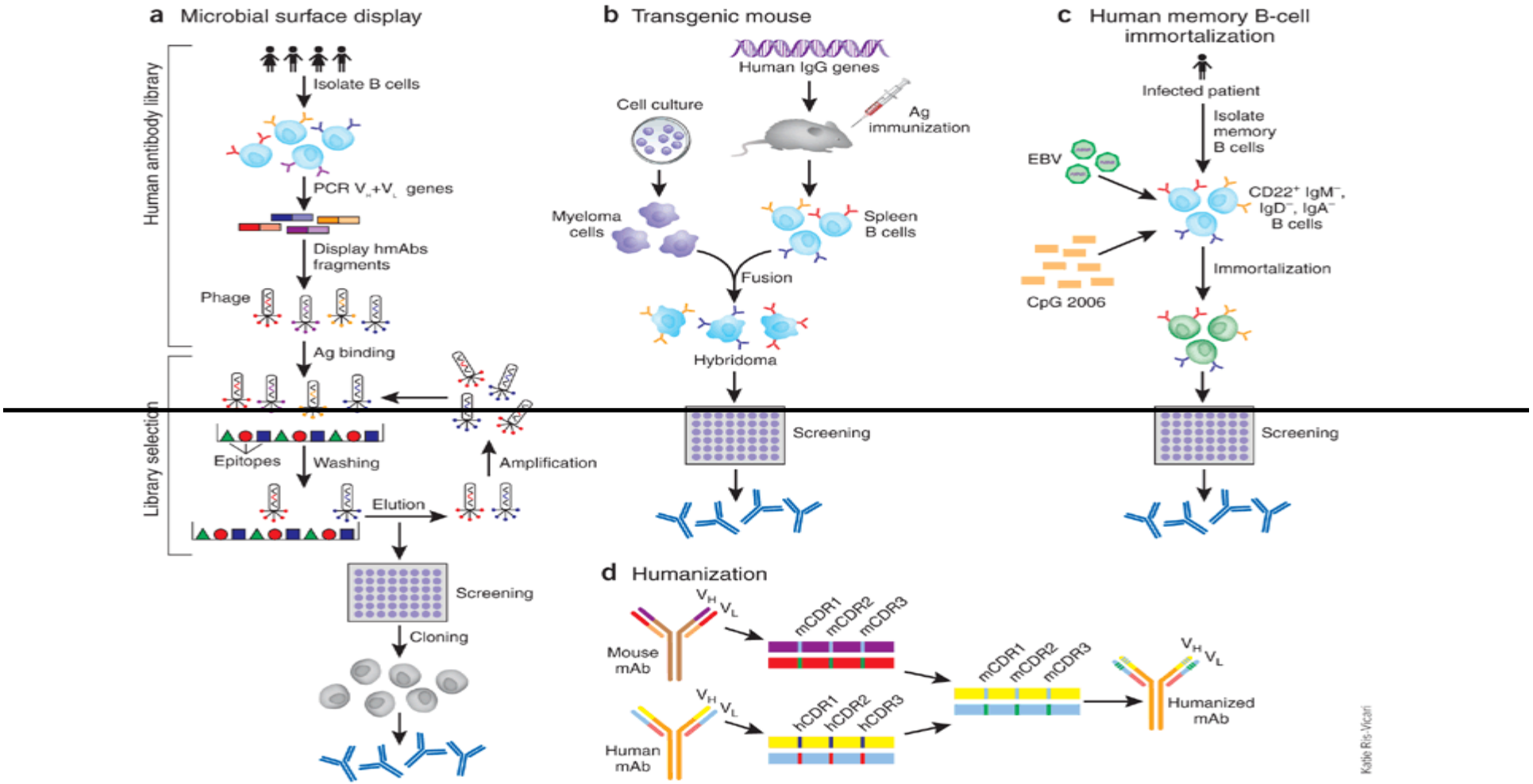


Applications for Phage display system

VERSATILITY OF PHAGE DISPLAY LIBRARIES

Library	Common Applictions
Random peptides	Map protein-peptide interactions
	Identify specific binding reagents Diagnostics Receptor antagonists or agonists Enzyme inhibitors Mimics of epitopes and natural ligands (mimitopes)
	Determine protease substrate specificity
	Create vaccine
	Identify cell- or tissue-specific markers
Antibody fragments	Identify high-affinity specific binding reagents Human therapeutics and diagnostics Receptor antagonists or agonists Enzyme inhibitors
	Identify cell- or tissue-specific markers
cDNA libraries	Identify naturally occurring protein complexes
	Determine enzyme-substrate specificity
Protein fragments or variants	Map protein-protein interactions and epitopes
	Change protein function Enhance binding affinity Alter binding specificity Alter substrate specificity or enzyme kinetics

Human antibody techniques



The growth and potential of human antiviral monoclonal antibody therapeutics

Wayne A Marasco & Jianhua Sui

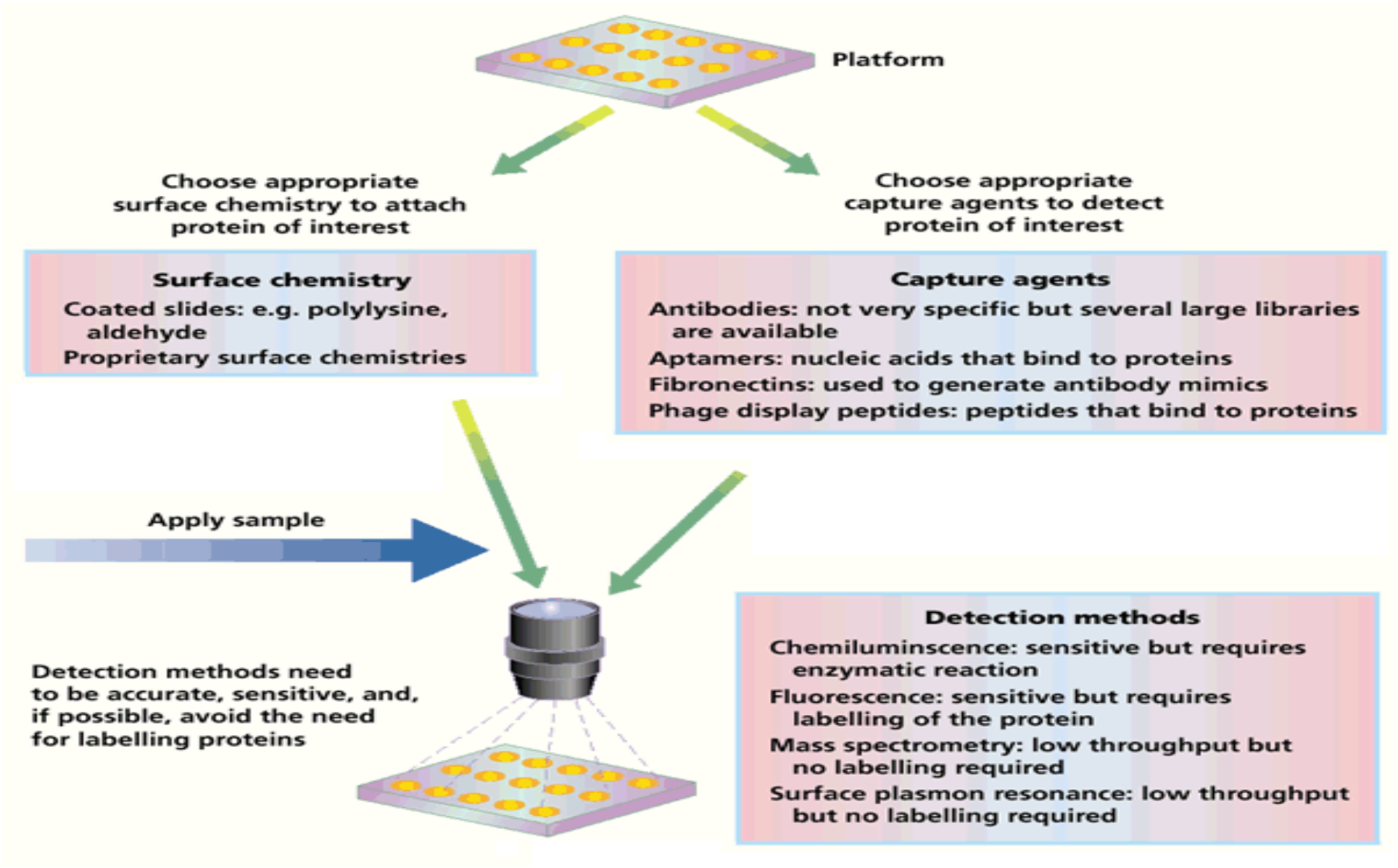
Nature Biotechnology 25, 1421 - 1434 (2007) Published online: 7 December 2007

4. Protein Arrays

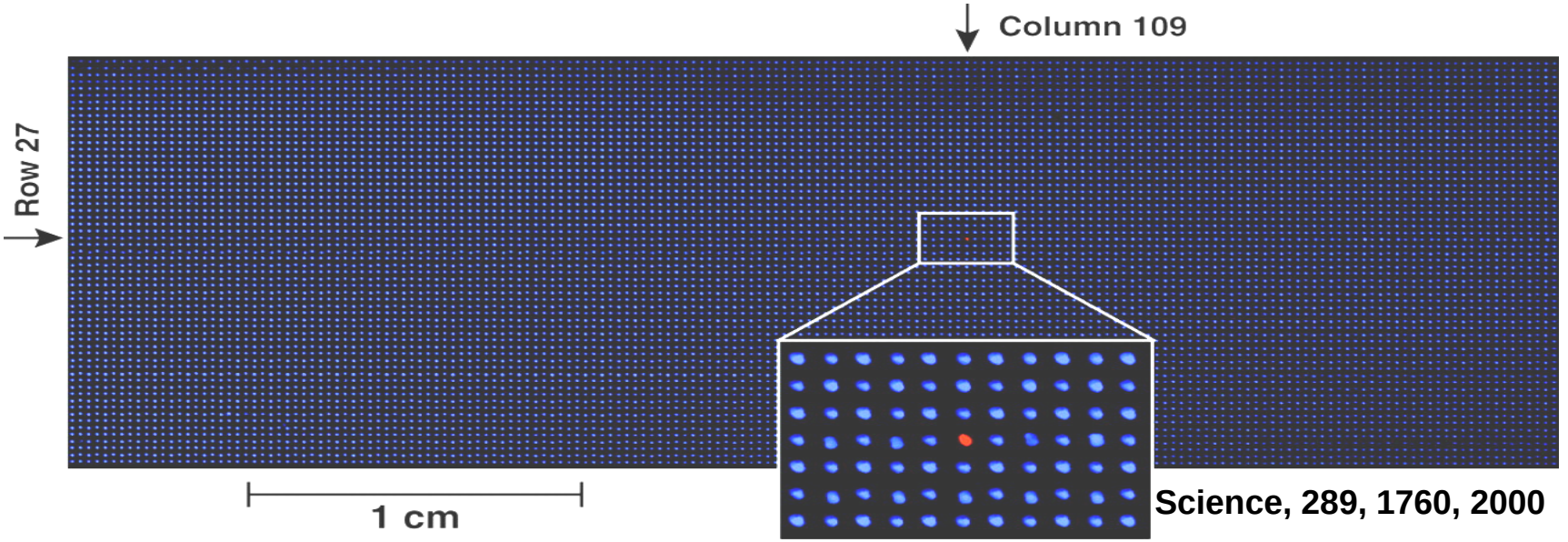
4. Protein (micro) arrays

- Another Functional Proteomics Approach
- Same concept as a DNA Array
- Has been published in a peer-reviewed journal
- Too much expectation lies in with.

Technological Components for Protein Chips



Protein Microarrays

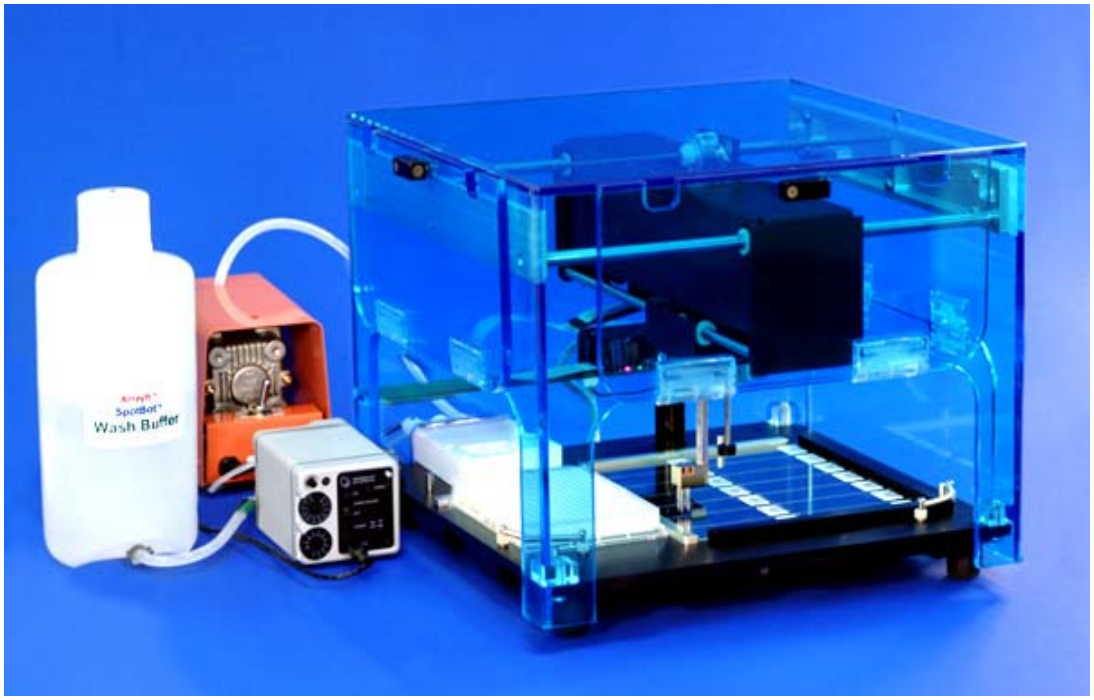


Science, 289, 1760, 2000

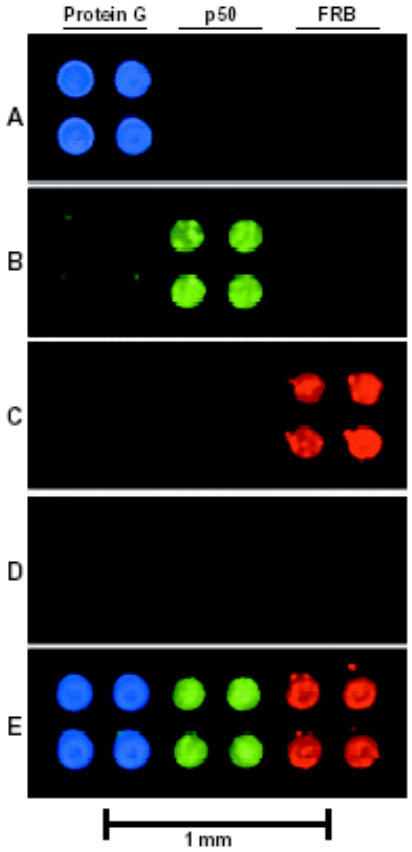
- Microspotting of proteins on aldehyde glass slide
- 150~200 μm in diameter (100 μg/mL)
- 10,799 spots of Protein G (1,600 spots/cm^2)
- A single spot of FRB (FKBP12-rapamycin binding)

Protein Microarray

G. MacBeath and S.L. Schreiber, 2000, Science 289:1760



Spotting platform and protein microarray



What protein microarray can do?

1. Protein / protein interaction
2. Enzyme / substrate interaction (transient)
3. Protein / small molecule interaction
4. Protein / lipid interaction
5. Protein / glycan interaction
6. Protein / Ab interaction

Reference: 1. G. MacBeath and S.L. Schreiber, 2000, Science 289:1760

2. H.Zhu et al, 2001 Science 293:2101

3. Ziauddin J and Sabatini DM, 2001 Nature 411:107

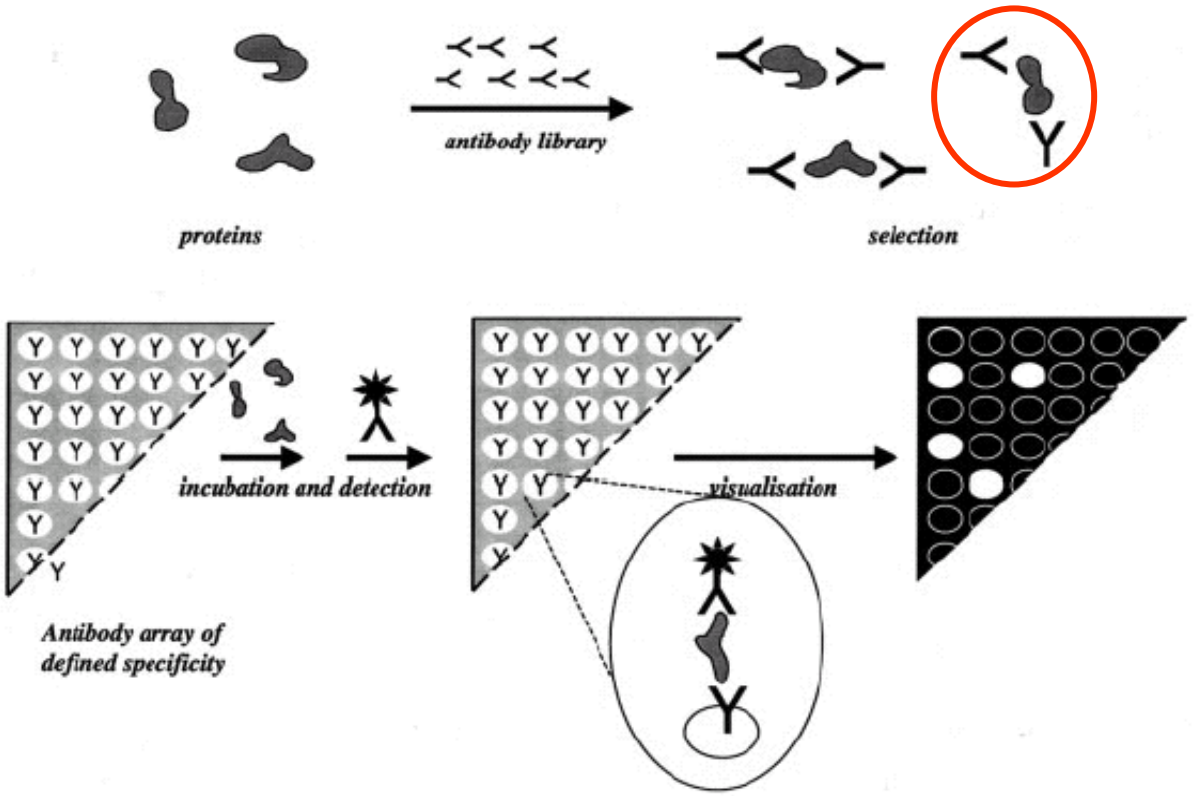
Application of protein microarray

TABLE 1.7. Protein arrays: Classes of capture molecules

Capture molecules	Source	Technique	References
mAb	mouse	hybridoma	Goldman (2000)
scFv/Fab diabodies	antibody libraries	phage display, in vitro evolution	Gao et al. (1999); Ryu and Nam (2000); Krebs et al. (2001); Lecerf et al. (2001); Raum el al. (2001)
Affinity binding agents	recombinant fibronectin structures	in vitro evolution	Kreider (2000)
Affibodies	microorganism	heterologous expression	Gunneriussion et al. (1999a,b)
Aptamers (DNA/RNA/ peptide)	library	SELEX/mRNA display, in vitro evolution	Jayasena (1999); Brody and Gold (2000); Hoppe-Seyler and Butz (2000); Lee and Walt (2000); Lohse and Wright (2001); Wilson et al. (2001)
Receptor ligands	synthetic	combinatorial chemistry	MacBeath et al. (1999); Lee and Walt (2000)
Substrates of enzymes	synthetic; pro- and eukaryotic organisms	protein purification, recombinant protein technology (bacterial fusion proteins, baculo-virus, peptide synthesis)	Arenkov et al. (2000); MacBeath and Schreiber (2000); Zhu et al.(2000)

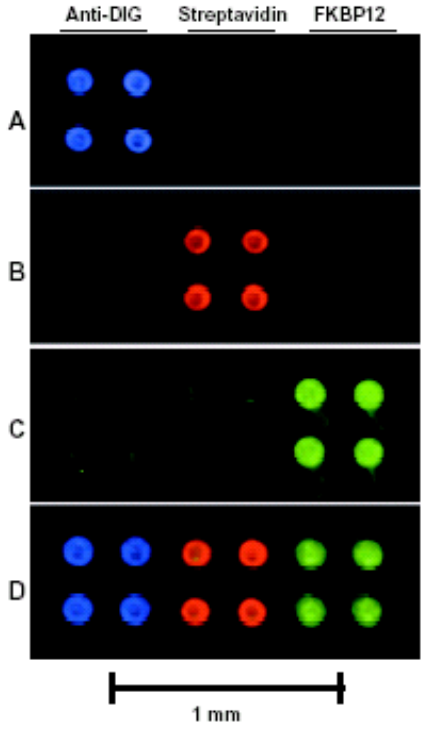
This table summarizes classes of molecules that have the potential to be used or are actually used as capture molecules in protein microarray systems. Abbreviations: (Fab) Antigen-binding fragment; (scFv) single-chain variable region fragment; (mAb) monoclonal antibody.
Reproduced, with permission, from Templin et al. (2002).

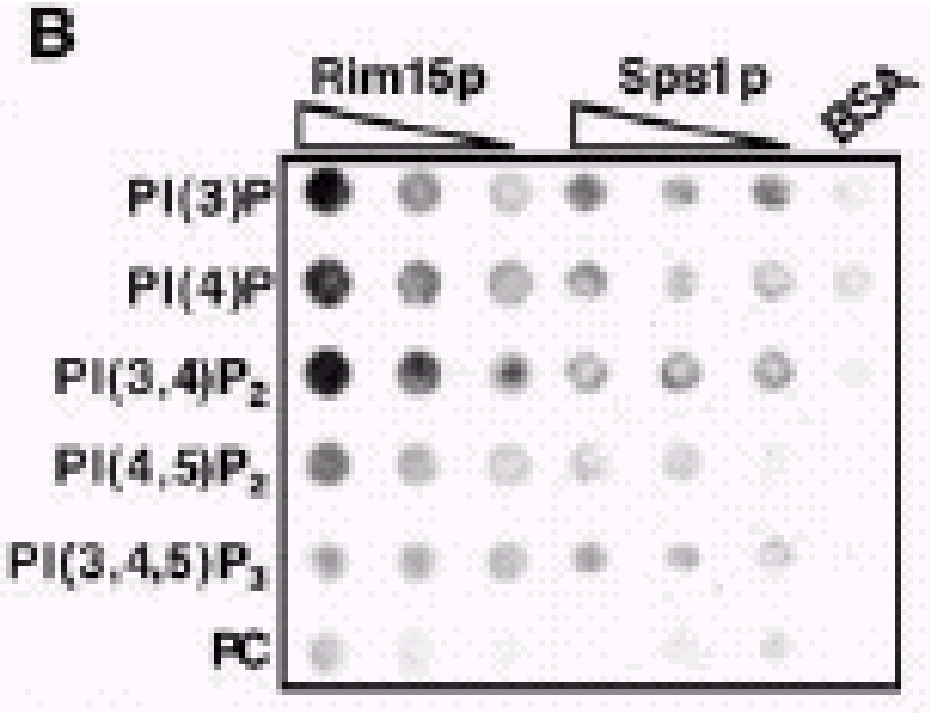
Protein microarrays (Ab arrays)



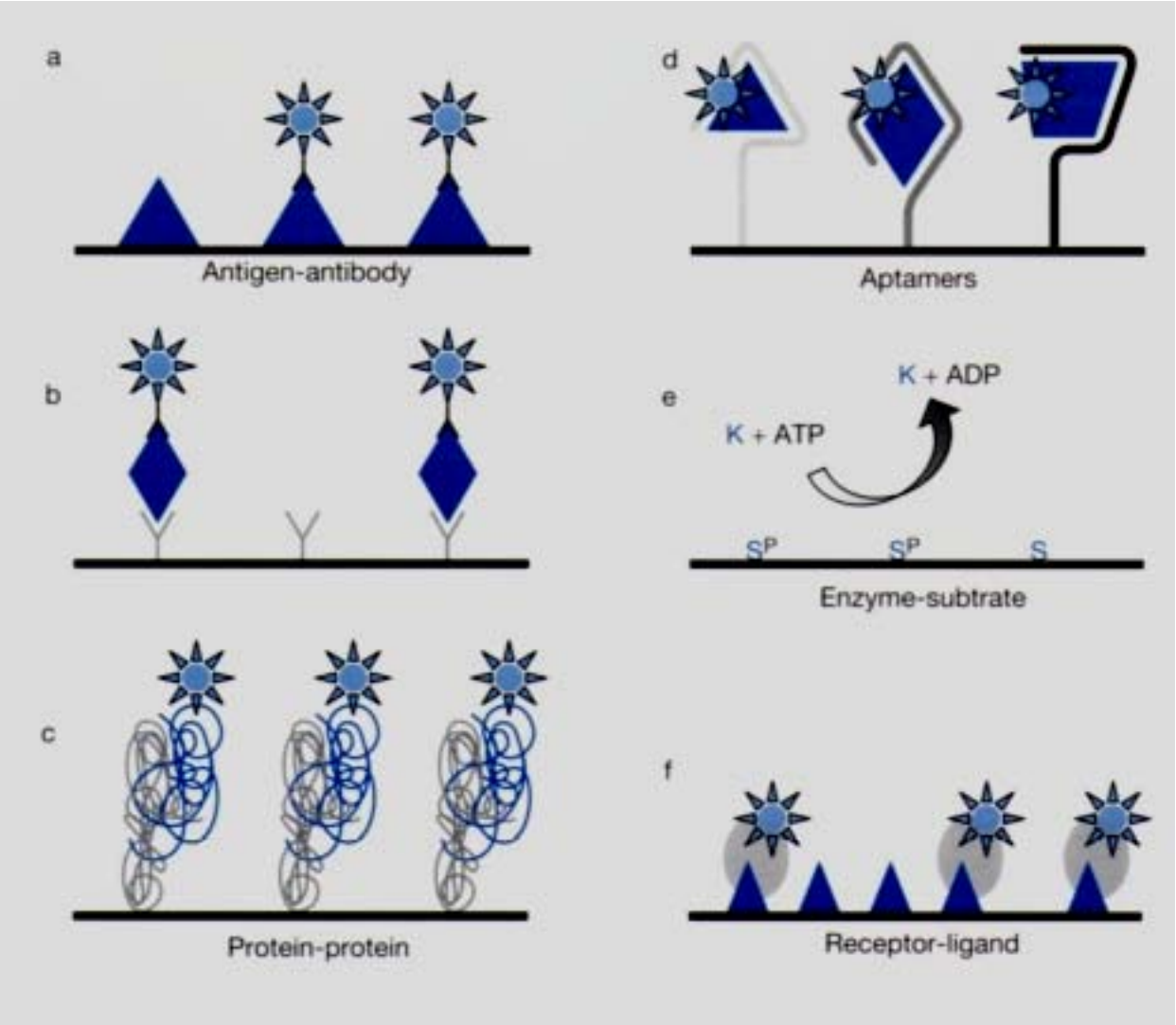
Face the real world

The true spot quality from real experiment

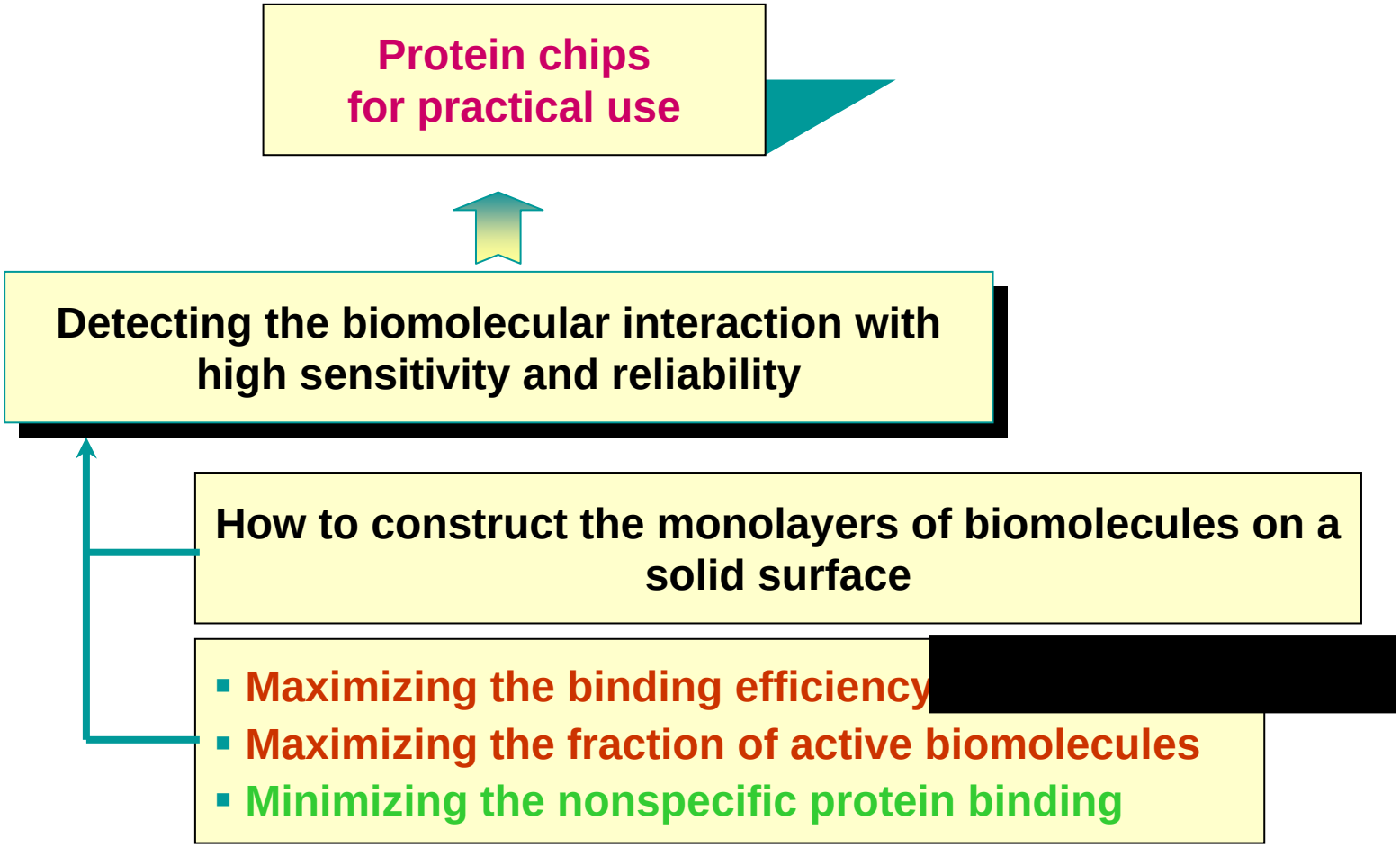




Class of capture molecule for protein microarray



Core Technologies in Protein Chip

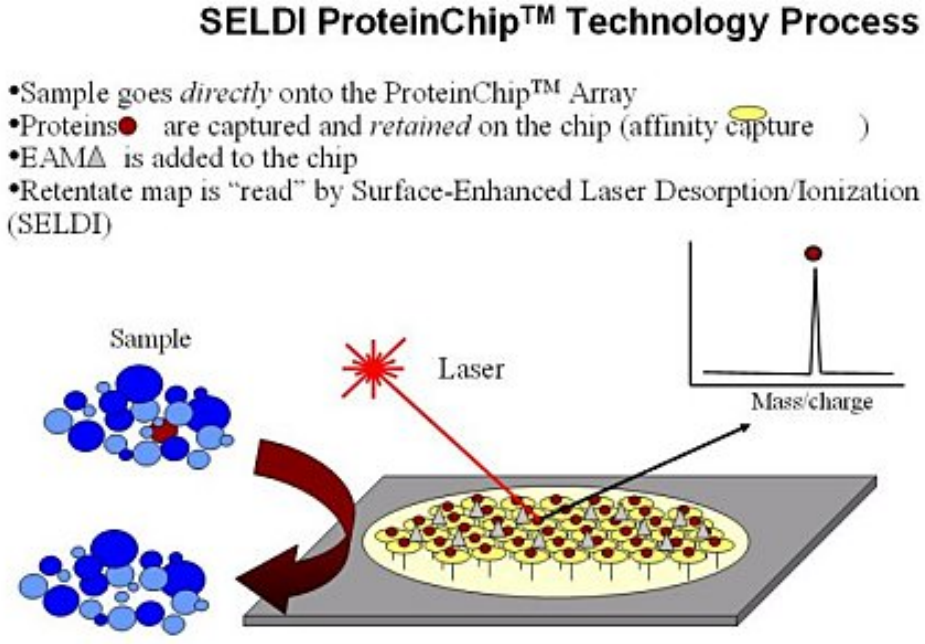


5. SELDI protein chips

5. SELDI protein chip

SELDI – surface enhanced laser desorption/ ionization

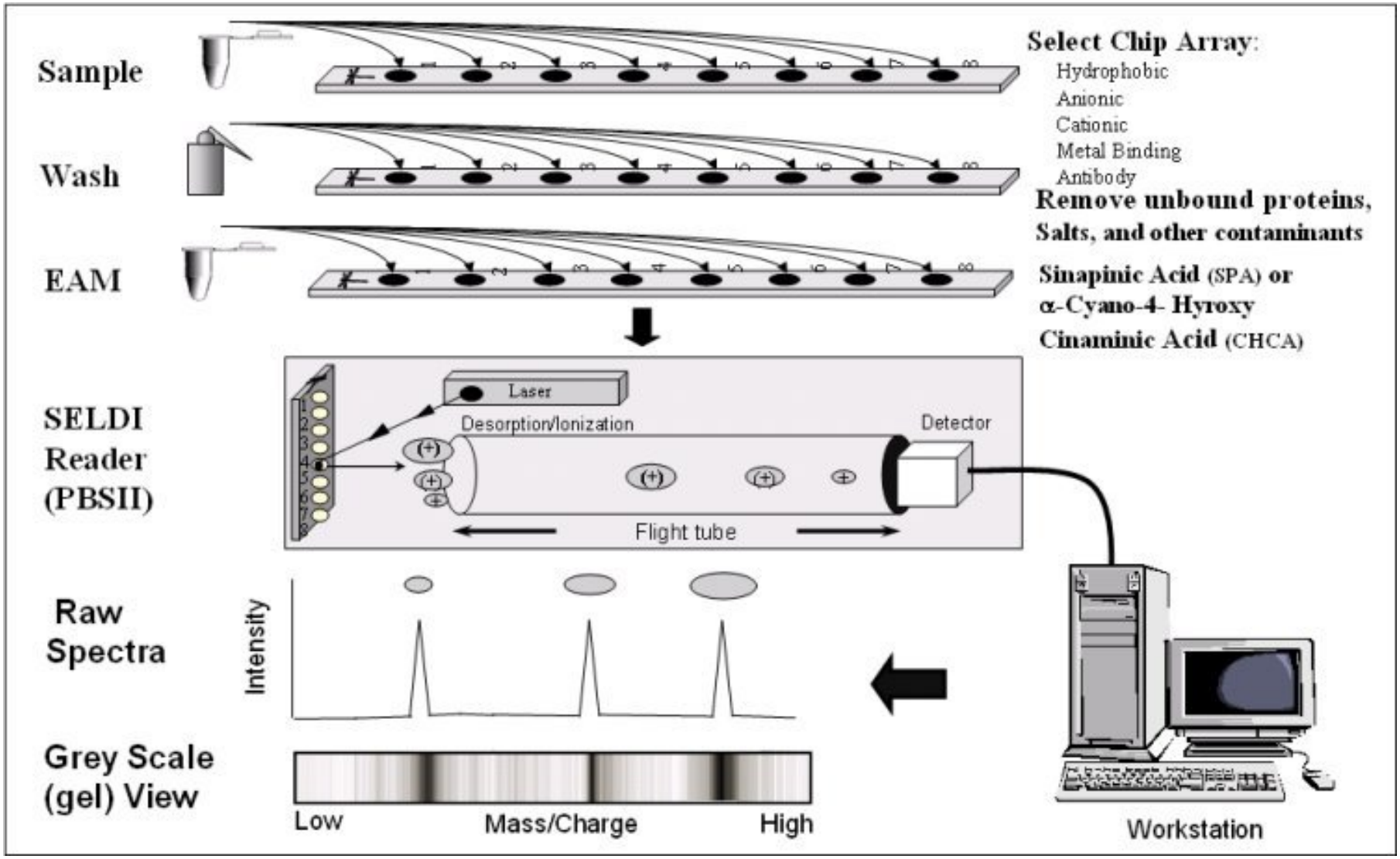
Protein chips



Types of protein chip

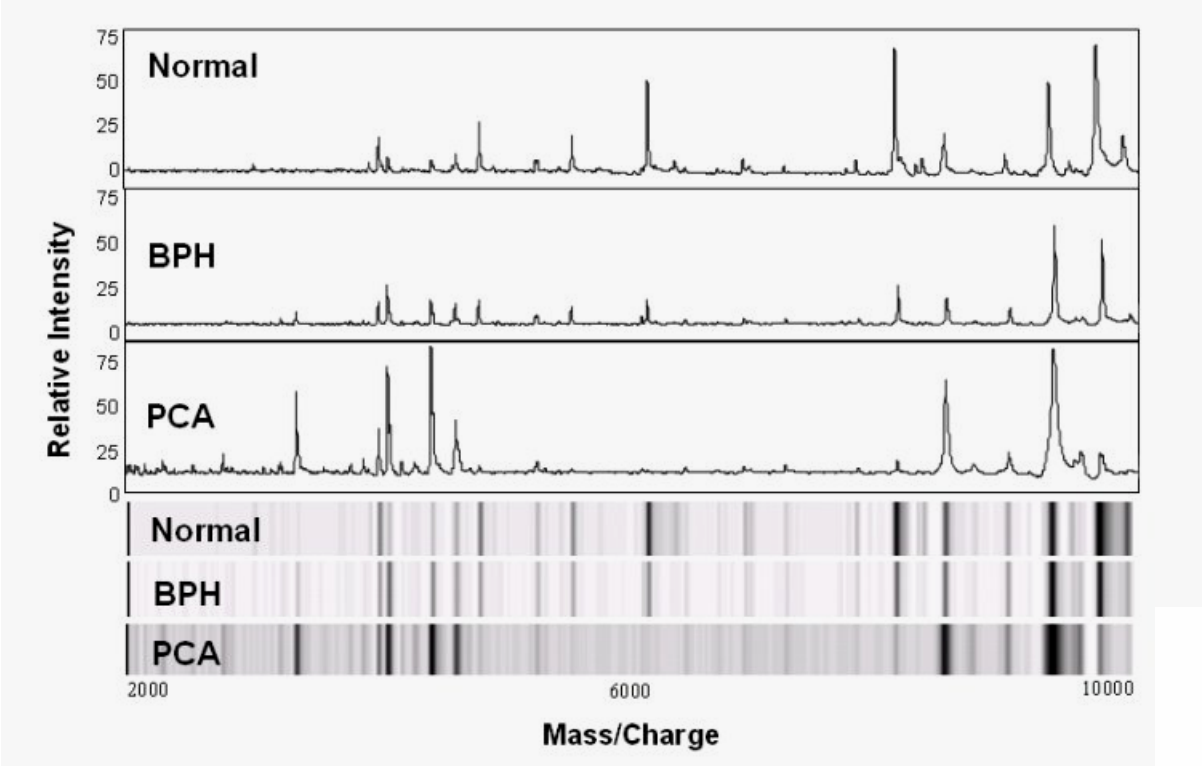
IMAC30뿌immobilized metal affinity capture array with a nitriloacetic acid (NTA) surface with an updated hydrophobic barrier coating.
IMAC3뿌mmobilized metal affinity capture array with a nitriloacetic acid (NTA) surface.
CM10삯weak cation exchange array with carboxylate functionality, with an updated hydrophobic barrier coating
WCX2삯weak cation exchange array with carboxylate functionality.
Q10뿔strong anion exchange array with quaternary amine functionality, with an updated hydrophobic barrier coating.
SAX2뿔strong anion exchange array with quaternary amine functionality.
H50빵bind proteins through reversed phase or hydrophobic interaction chromatography with an updated hydrophobic barrier coating
H4 mimic reversed phase chromatography with C16 functionality.
NP20뿔mimic normal phase chromatography with silicate functionality
Au빠old chips to be used directly for MALDI-based experiments

Experimental procedure of SELDI protein chip



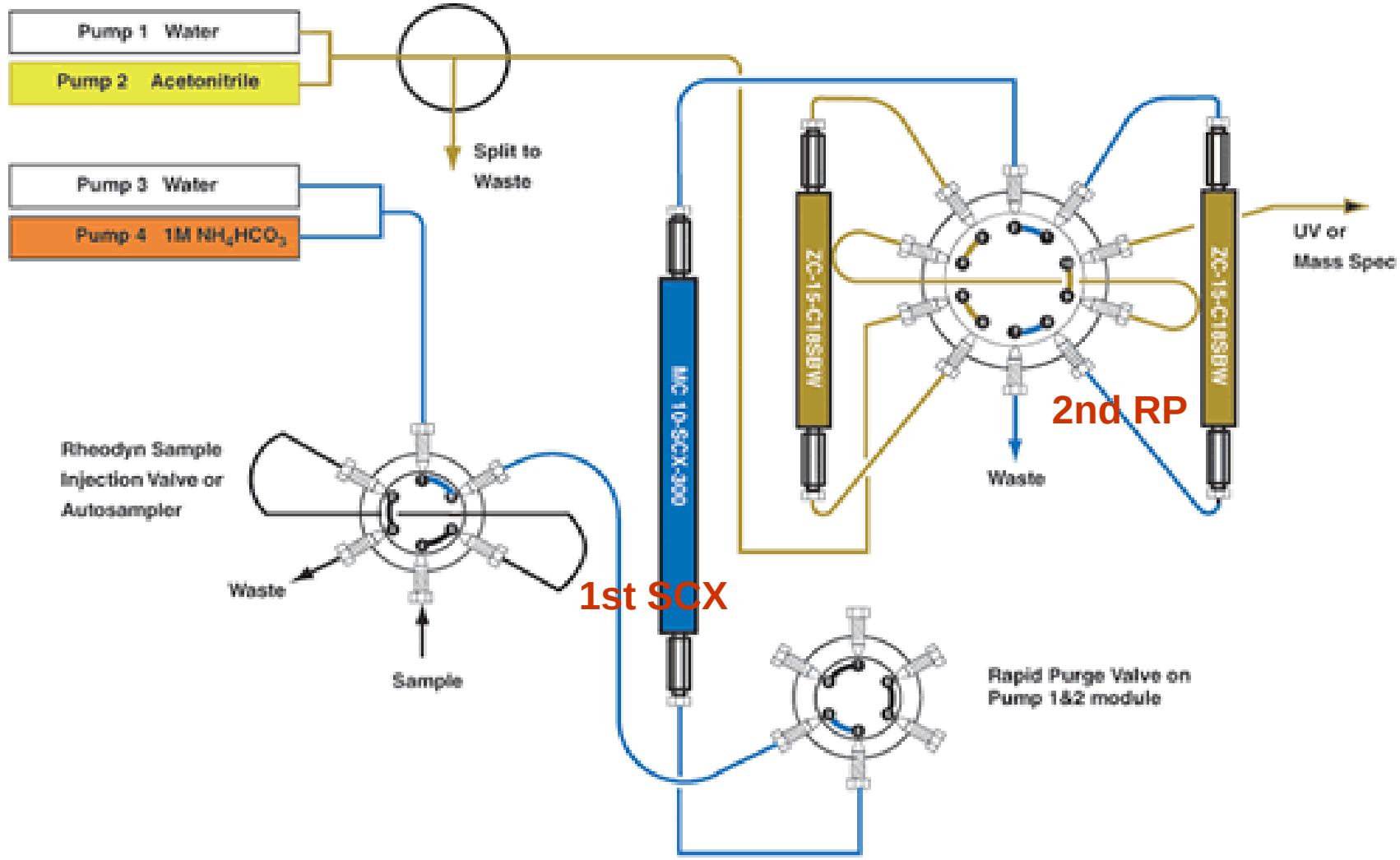
SELDI protein chip, application

Representative “raw” spectra and “gel-view” (grey-scale) of serum from a normal donor, and from patients with either BPH (benign prostate hyperplasia) or prostate cancer (PCA) using the IMAC3-Cu chip chemistry (Virginia Prostate Center).



6. Multi-dimensional HPLC (MDLC)

Configuration of MDLC



Ultra-Plus II Setup for High Throughput 2-D Proteomics (Sample Loading)

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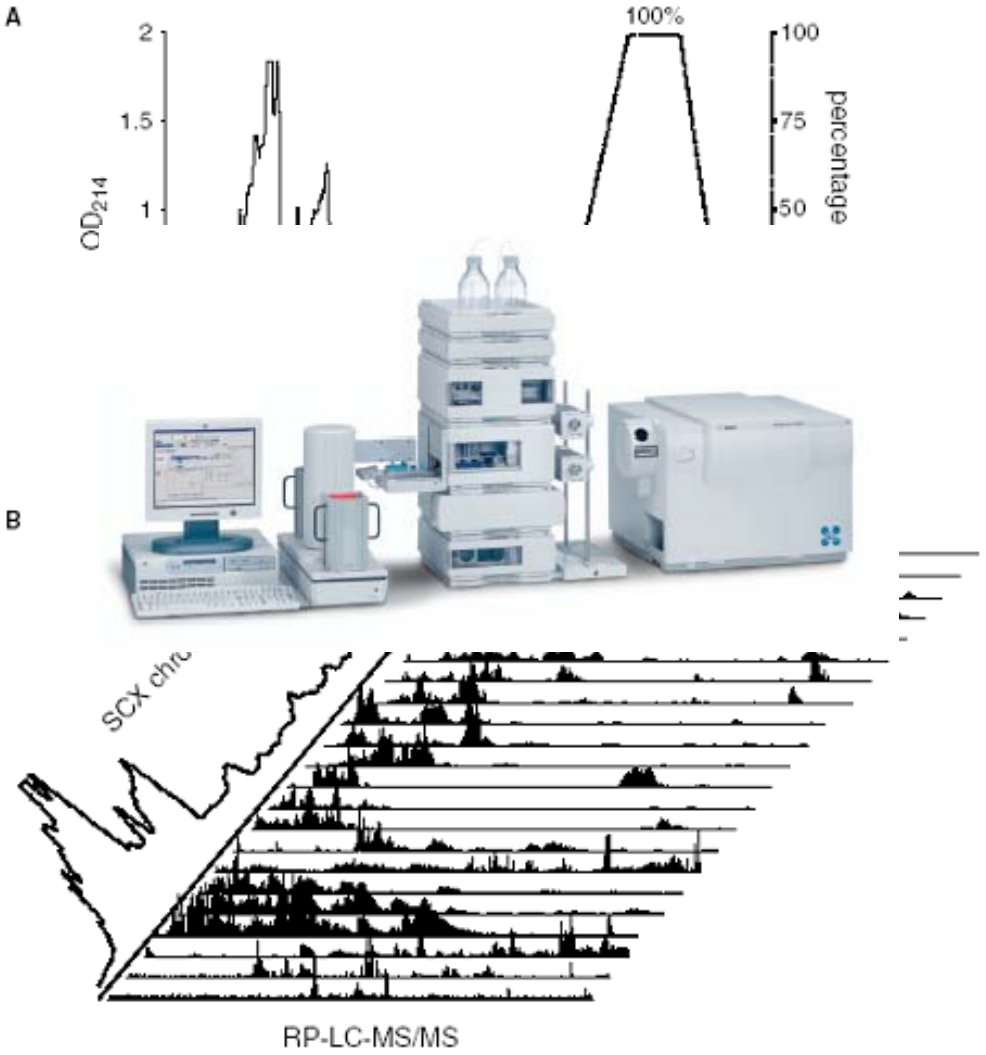
An analysis result by MDLC



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Agilent 1100 series