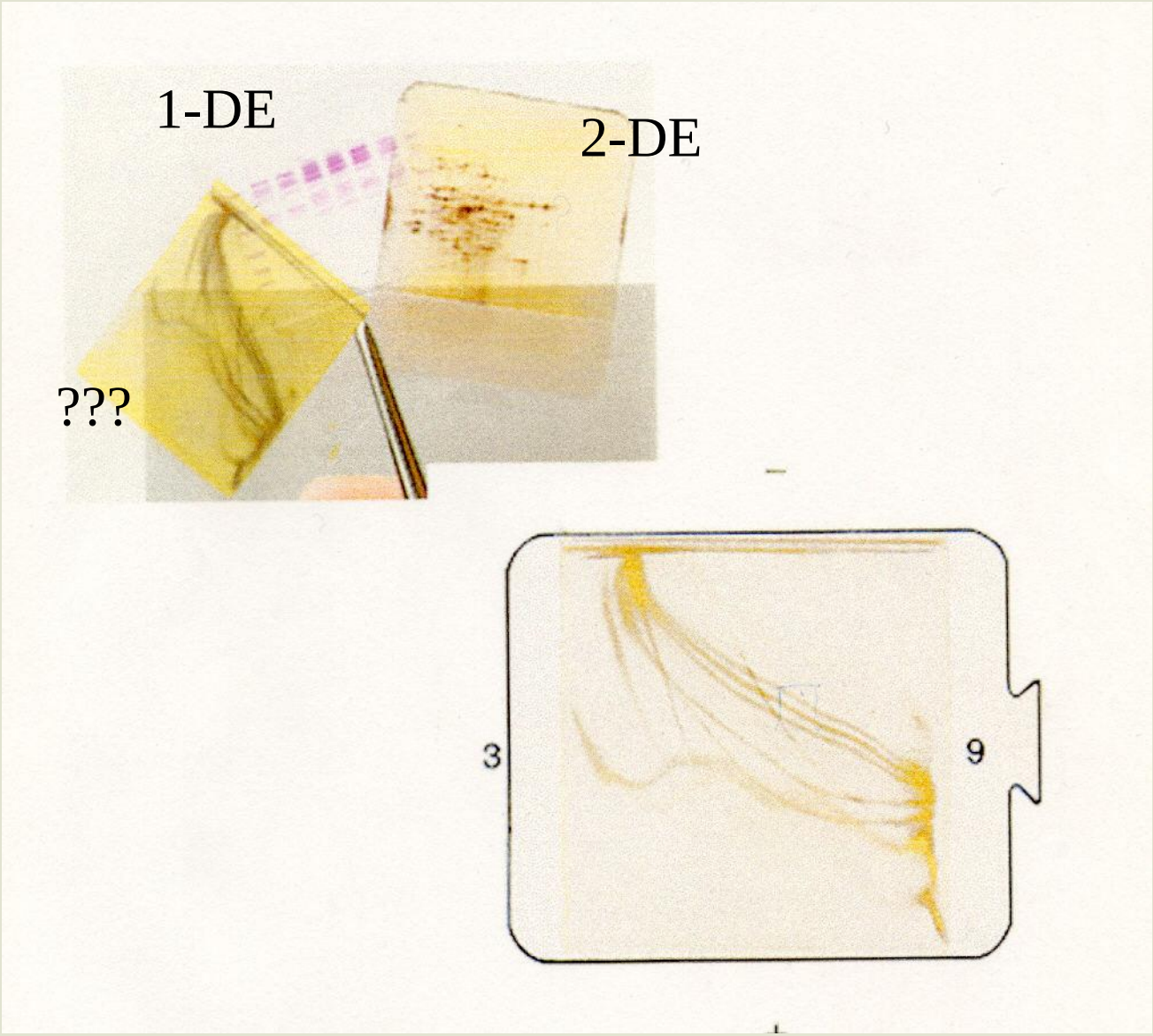

1-DE
2-DE
???
3
9

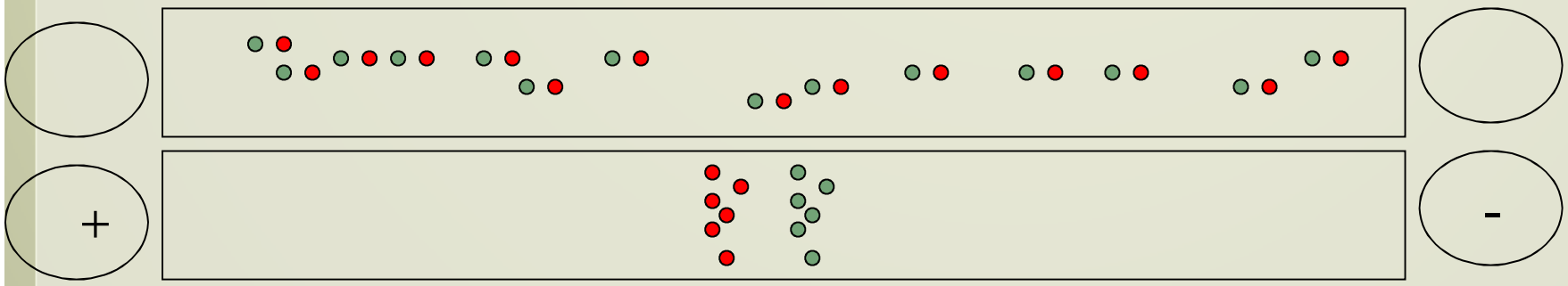
THEORY

Any charged ion or group of ions will migrate when placed in an electric field.

→ pH - - - - - IEP (pI)

Migration is dependent on charge density

→ Charge/Mass



Zone Electrophoresis

Free solution: diffusion problems

→ Supporting media
(Paper, NZ, ...etc)

Agarose, Starch, Polyacrylamide ....

Agarose (Natural product)

-large pore size

-separation mainly based on charge density
(suitable for large molecules e.g. DNA)

Starch and PAA

→ sieving effect

Starch is also a biological product
(= product variation considerable)

PAA is a synthetic product

- pH stable
- chemically inert
- transparent
- pore size variability
- etc

(PAA gels are formed by free-radical polymerization of acrylamide and a co-monomer crosslinker)

Catalysts for Polymerization

- APS (ammonium persulfate)
- Riboflavin

- TEMED (N,N,N,N-tetramethylene diamine)
- DMAPN (3-dimethylamino-propionate)

(TEMED catalyses the formation of free radicals from persulfate)

→ These initiate the polymerization

Attention: Since TEMED is required as a free base, do not use low pH.

Attention: TEMED ↑ or APS ↑ = polymerization rate ↑

Oxygen inhibits the polymerization (no radicalization)
(degas the solutions before polymerization!!)

<u>How to play around with the pore size??</u>

1) Pore size ↓ if AA concentration ↑ (usually 2.5% - 16%)

2) AA 2.5% lower limit (fluidic!)
(Add 0.5% Agarose if you go under this limit)

3) AA 20% and more = polymerization extremely fast

→ Separation of molecules higher than 2000 Da

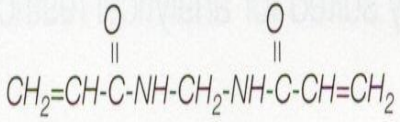
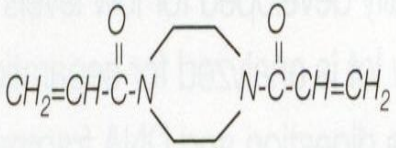
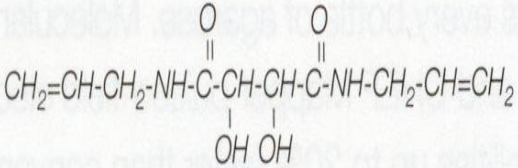
The Crosslinker??

Effects:
- pore size
- swelling of the gel
- stiffness
- brittleness
- etc

Polymerization without a crosslinker will result in random polymerization (viscous solution)

crosslinker ↑ = pore size ↓

Crosslinkers

Crosslinker	Formal Name	Structure	Application
Bis	N,N'-methylene-bis-acrylamide		General crosslinker in PAGE techniques.
PDA	Piperazine diacrylamide		Reduction of silver stain background in SDS-PAGE and 2-D gels, increased resolution, and higher gel strength.
DATD	N,N'-diallyl-tartardiamide		Increase pore size of IEF gels where molecular sieving is a problem. Used in scintillation counting: 1,2 diol structure is solubilizable with periodic acid.

%T and %C

PAA gels are described in terms of two parameters that determine the pore size. The total monomer concentration or %T is defined as:

$$\%T = \frac{\text{gr AA} + \text{gr CL}}{\text{total vol}} \times 100$$

the weight percentage of CL is:

$$\%C = \frac{\text{gr CL}}{\text{gr AA} + \text{gr CL}} \times 100$$

With higher poportions of Bis-AA:

- the polymerchains become crosslinked into increasingly large bundles with large spaces between.

= Effective pore size ↑

Usual AA/CL ratios:

19:1	5.0 %(CL)	DNA sequencing
29:1	3.3 %(CL)	Protein separation
37,5:1	2.7 %(CL)	Protein separation

Methods Used in 1-Dimensional Electrophoresis

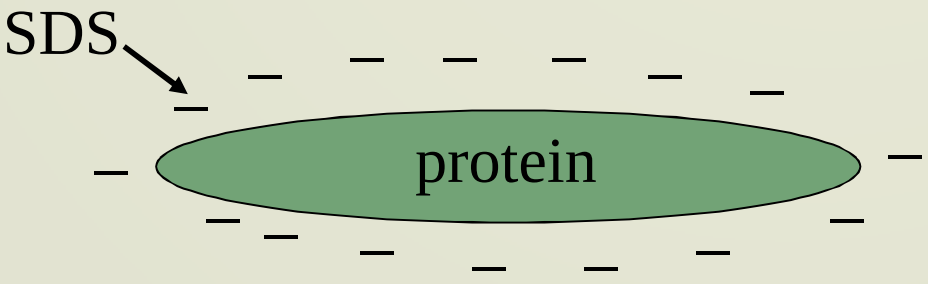
Buffer Systems:

Dissociating system (Läemmli gels, Schägger gels)

Usually SDS (sodium dodecyl sulphate)

- most proteins bind to SDS (appr. 1,4 gr SDS / 1 gr protein)

→ SDS-polypeptide complexes have identical charge densities (molecular weight determination possible)



UREA

- 6-8 M concentration necessary
- UREA does not bind to the protein = must be kept present during electrophoresis in all solutions

→ Separation is based on both charge density and size = no molecular weight determination possible

Att: Not as good as SDS in dissociating proteins
(Up to 50% of protein mixture may fail to enter the gel)

(90% of even crude cell lysate will enter the gel if SDS is used!)

Non-Dissociating system

= native gel electrophoresis (No SDS; No reducing egents)

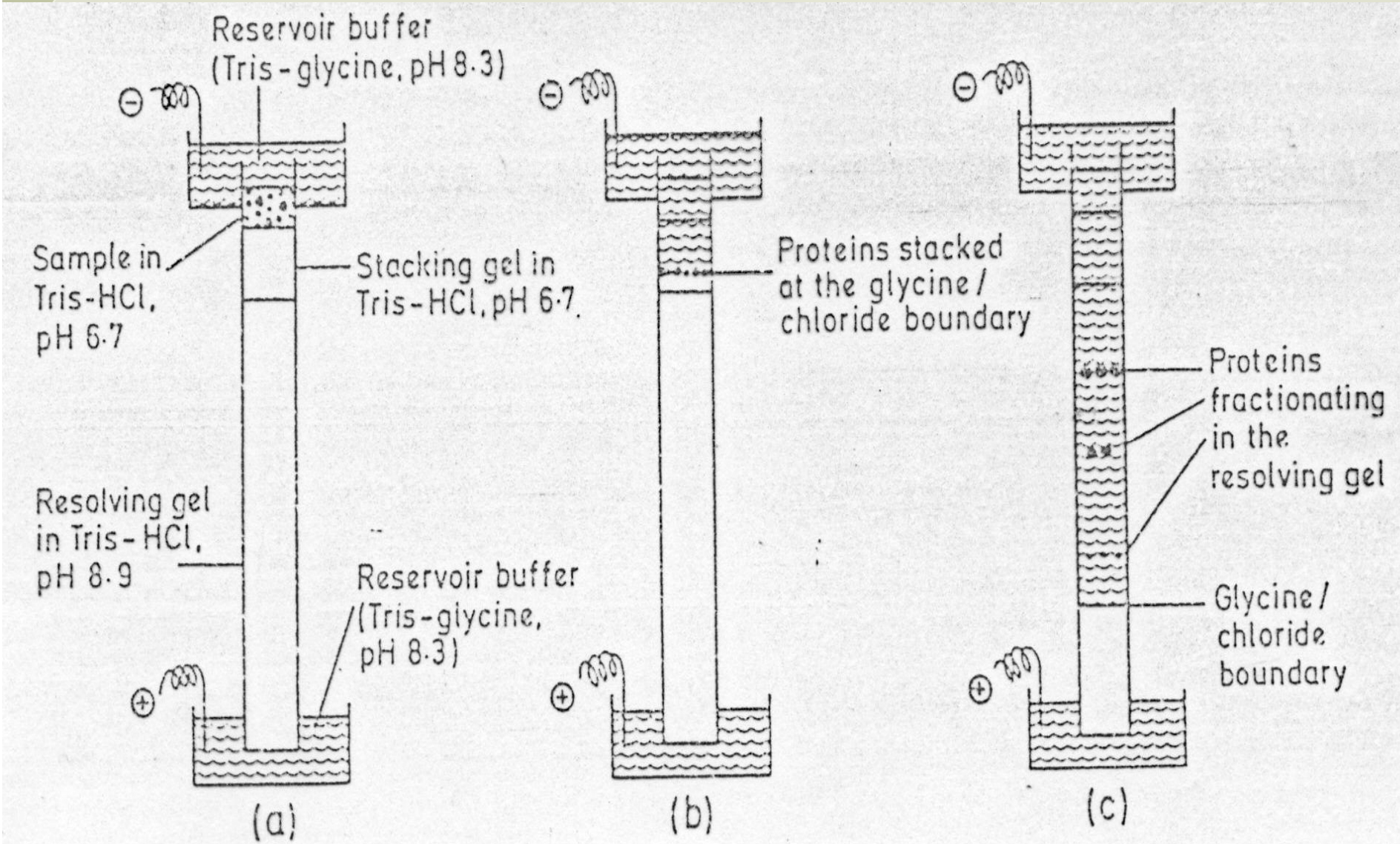
Continuous or Discontinuous buffer systems

Continuous system: same buffer ions in sample buffer and electrode reservoirs at constant pH (various conc.)

Discontinuous system: Different buffer ions in the gel and electrode reservoirs. Discontinuities in pH and conc. (e.g. Lämmli system)

→ Possibilty for stacking... Relative large amounts of dilute samples can be applied to the gel.

The stacking effect



Buffer considerations

Velocity of migration (RF value) = V x M x P

V: Voltage gradient
M: Mobility of charged species
P: Portion of charged ions

Choice of pH

In practice pH limits are at 3 – 10 due to hydrolytic reactions
(SDS complexes are not critical to pH)
(non-dissociating systems are e.g. UREA)

For native PAGE

The further the pH is of the pI, the higher the charge of the protein

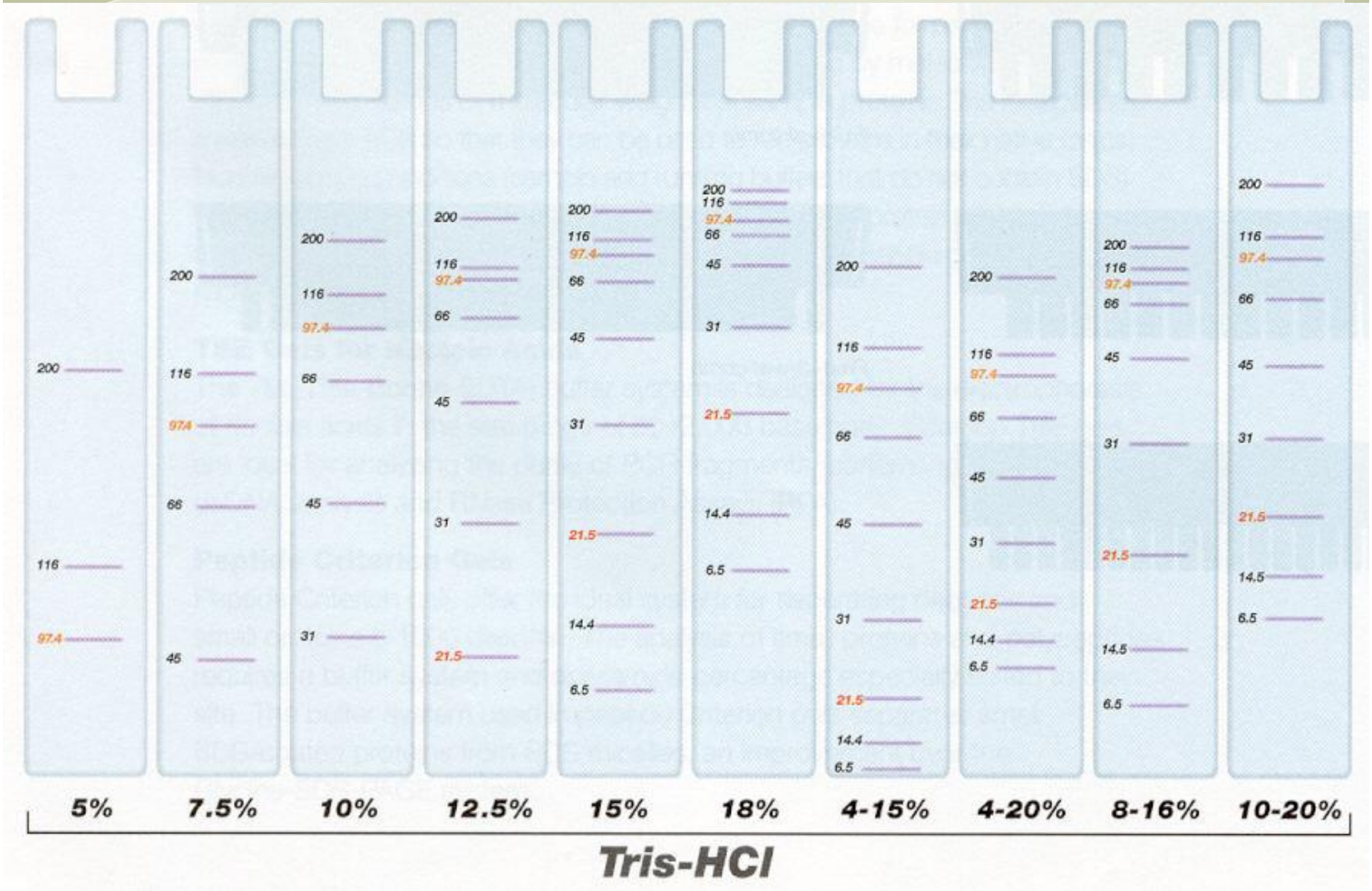
→ Shorter times for separation and less band spreading due to diffusion.

However: The closer the pH is to the pI, the greater the charge differencies between proteins. This will lead to greater change of separation.

Choice of the gel concentration?

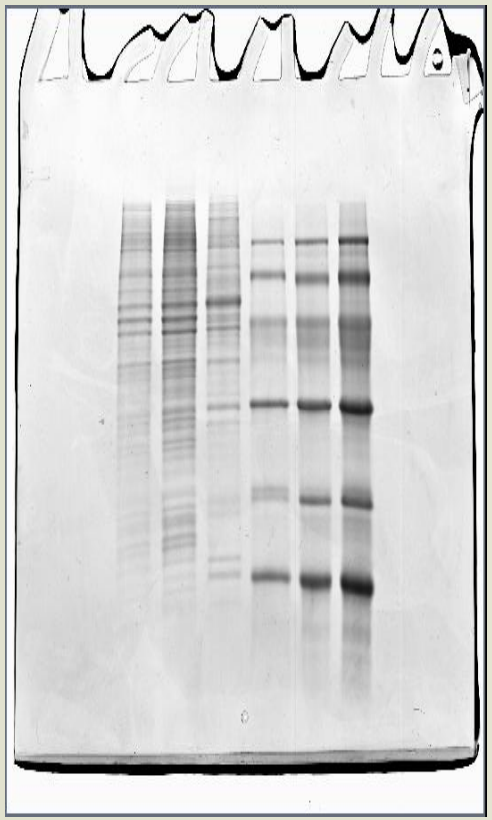
Check for the optimal concentration

→ Run a gradient gel

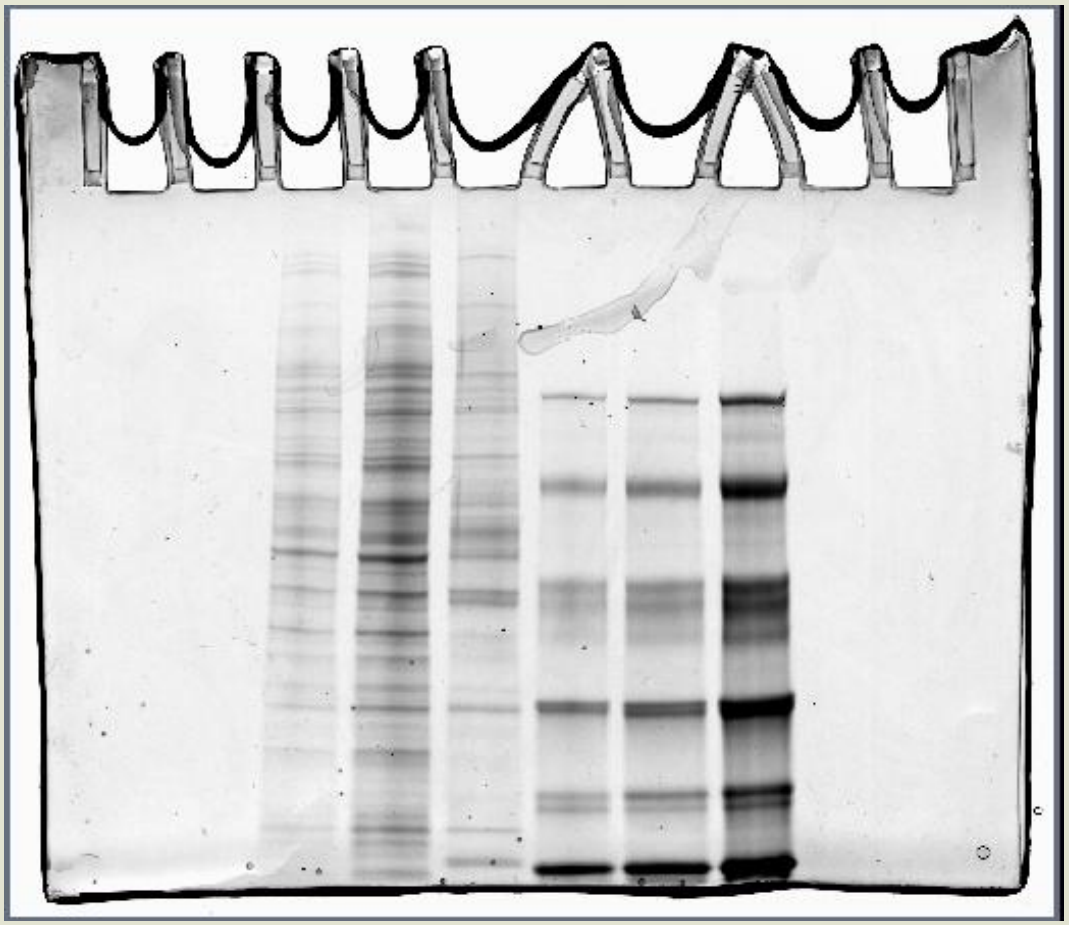

200
116
97.4
66
45
31
21.5
14.4
6.5
14.5
5%
7.5%
10%
12.5%
15%
18%
4-15%
4-20%
8-16%
10-20%
Tris-HCl

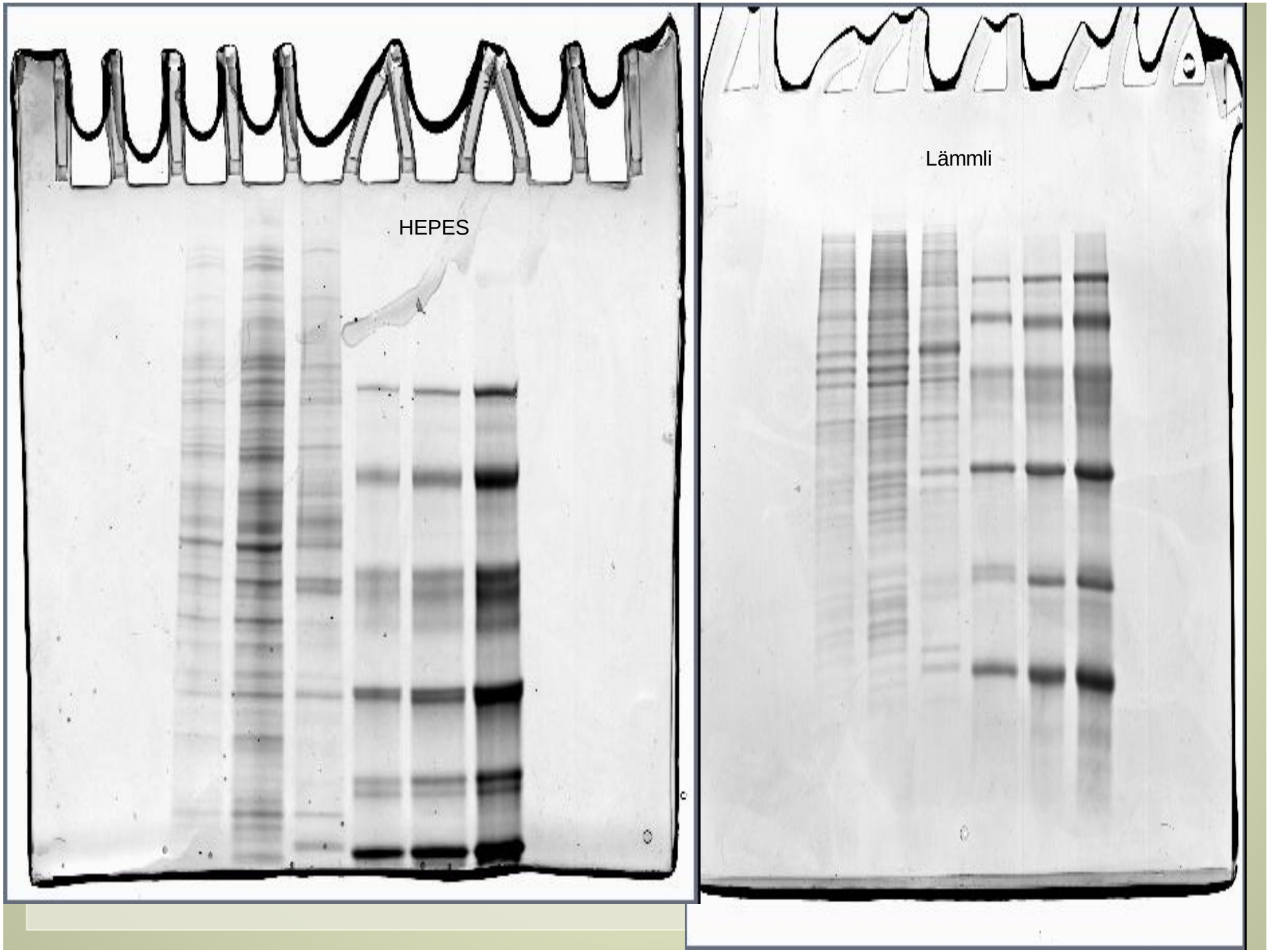
HEPES Fast Gels

Lämmli



HEPES

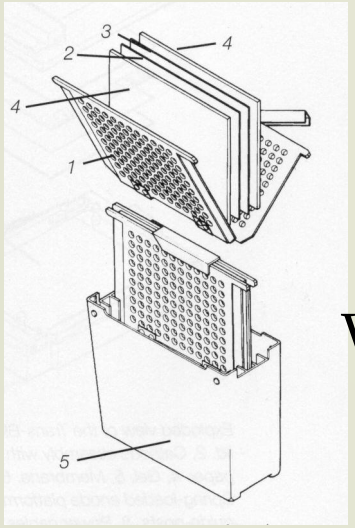



HEPES
Lämmli

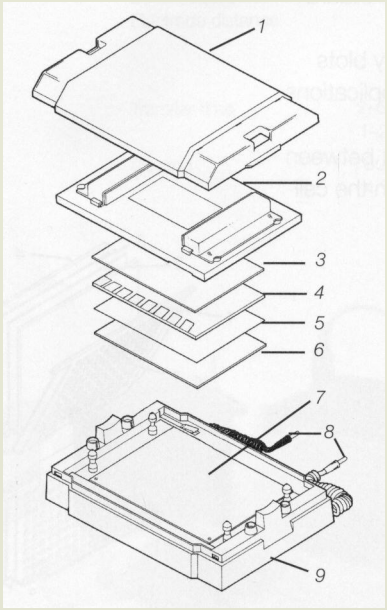
Applications

ELECTROBLOTTING:

Moving proteins onto a suitable membrane by electrophoresis



Wet blotting



Semi-Dry blotting

Nitrocellulose (NC)
Polyvinyldifluoride (PVDF)

- Immunostaining (immunological detection)(NC, PVDF)
- N-terminal sequence analysis (PVDF)

→ It is possible to cut out a band from the membrane (NC) and do an enzymatic digestion on the membrane (Mass spectrometry)

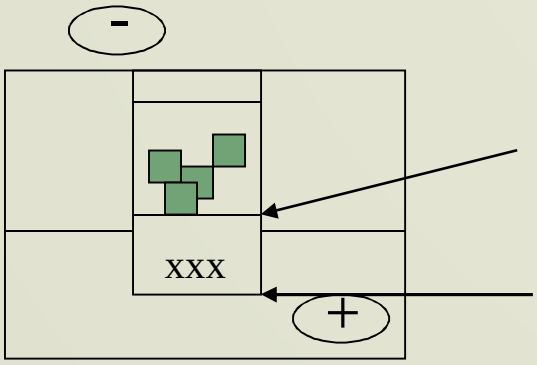
You may save a protein on the membrane for several years!

The blots may be stained with several dyes (Silver, Coomassie, Ponceau etc.)

ELECTROELUTION:

Preparative recovery of protein molecules from gels

Place gel pieces
in a tube and cover
with buffer solution



membrane 1

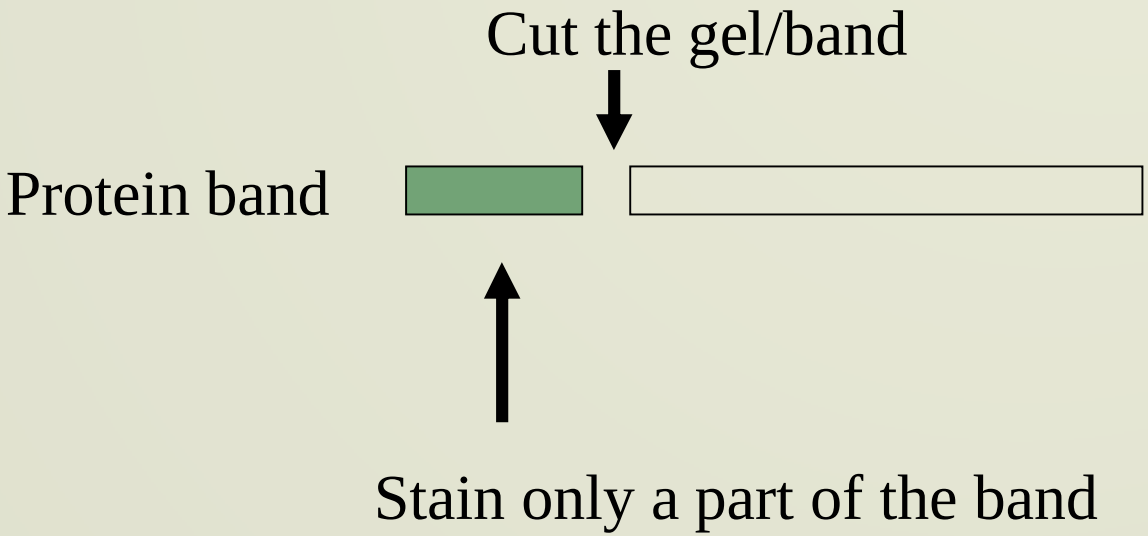
membrane 2

PASSIVE ELUTION:

Cut out the desired band from the gel and place it over night in 0.1 M sodium acetate, pH 8.5, 0.05% SDS.

If possible: Do not stain the band prior elution!!

Attn: Often only water is enough for a protein to be eluted!!



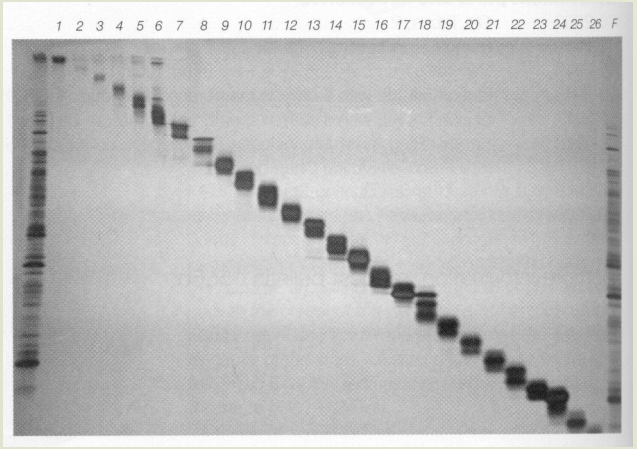
- large capacity
- easy to handle
- suitable for native proteins = recovery of enzyme activity

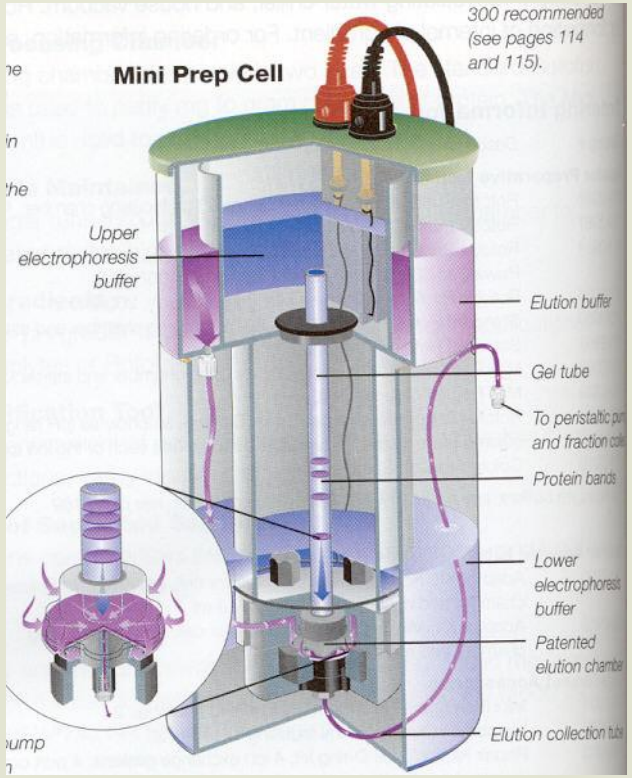
PREPARATIVE GEL ELECTROPHORESIS:

Normal electrophoresis

with on-line collection

and analysis.

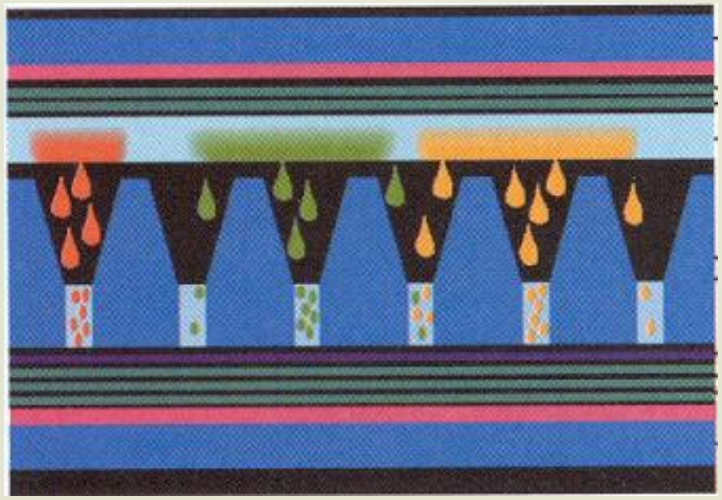




Special equipment in preparative gel electrophoresis:

- Rotofor Cell fractionates complex protein samples in free solution using preparative isolelectric focusing

- Whole Gel Eluter simultaneously elute and collect multiple bands of biomoleclues from whole gels



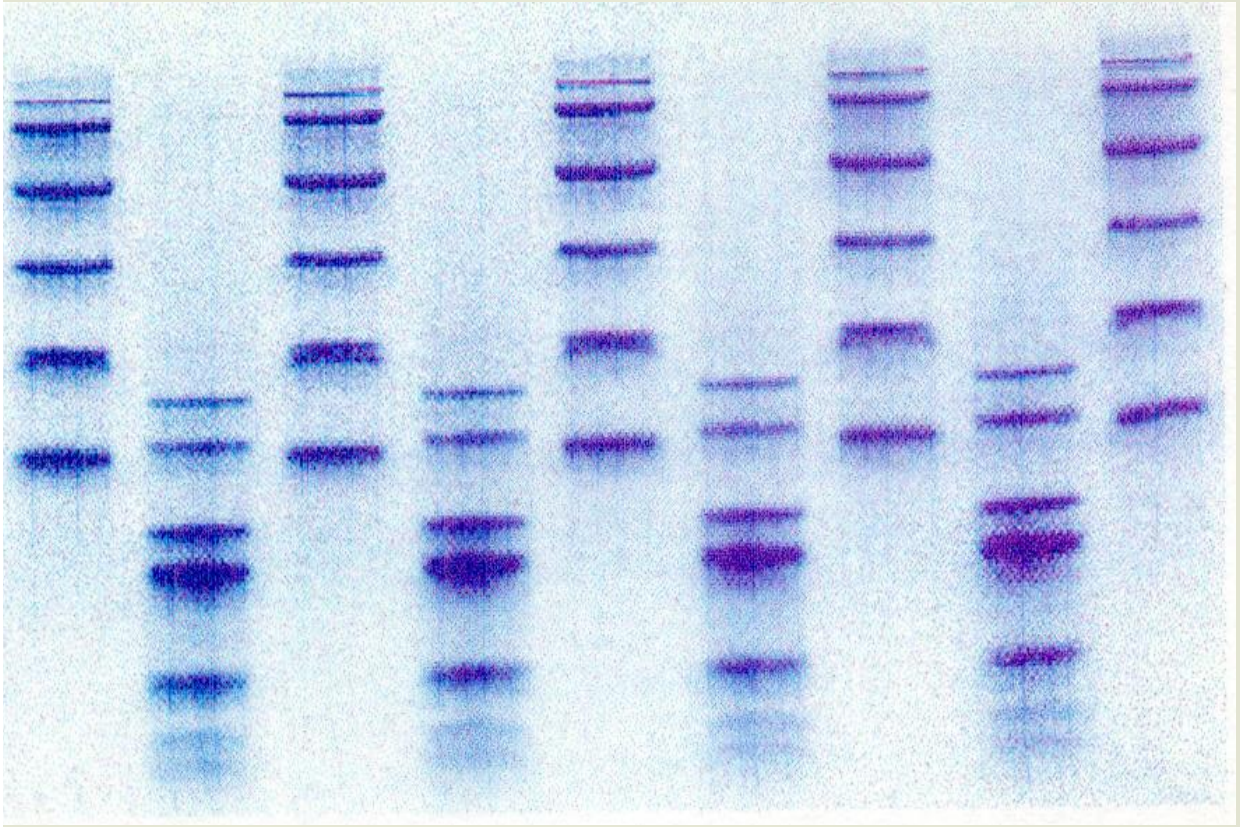
Staining methods

Stain	Sensitivity (ng)		Time	Comments
SILVER STAINS				
Silver Stain Plus (Gottlieb and Chavco)	0.6-1.2	3	90 min	Simple, robust; mass spectrometry compatible
Silver stain (Merril)	0.6-1.2	7	2 hr	Stains glycoproteins, lipoproteins, lipopolysaccharides, nucleic acids
NEGATIVE STAINS				
Zinc stain	6-12	3	15 min	High-contrast results, simple, fast, reversible; compatible with elution or blotting, as well as mass spectrometry
Copper stain	6-12	3	10 min	Simple, fast, single reagent, reversible; compatible with elution or blotting, as well as mass spectrometry
FLUORESCENT STAINS				
RUBY GEL stain	1-10	2	3 hr	High-sensitivity fluorescent protein stain; simple, robust protocol; broad dynamic range; mass spectrometry compatible
SYPRO Orange stain	4-8	1	45 min	Moderately sensitive fluorescent protein stain, compatible with subsequent blotting, protein sequencing and mass spectrometry
COOMASSIE STAINS				
Coomassie (brilliant blue)	36-47	2	2.5 hr	Simple, consistent laboratory standard
Bio-Safe Coomassie	8-28	3	2.5 hr	Non-hazardous, user-friendly Coomassie stain with linear dynamic range
IEF STAINS				
RUBY IEF stain	2-8	2	Overnight + 2 hr	High-sensitivity fluorescent protein stain optimized for IEF gel, robust protocol, broad dynamic range, mass spectrometry compatible
IEF stain	40-50	2	3 hr	Coomassie R-250 and Crocein Scarlet stain, optimized for IEF gels
BLOTTING STAINS				
RUBY BLOT stain	2-8	3	50 min	Fluorescent membrane stain, compatible with mass spectrometry, Edman-based sequencing and standard immunological procedures
Colloidal Gold stain	1	3	2 hr	Sensitive, total protein, membrane stain
Enhanced Colloidal Gold stain	10-100 pg	4	2 hr, 45 min	Increases sensitivity of Colloidal Gold kit
Amido Black	100-1,000	1	15 min	Standard membrane stain, economical
Biotin-Blot total protein detection kit	50	6	3 hr	Total protein membrane stain, compatible with nylon membranes

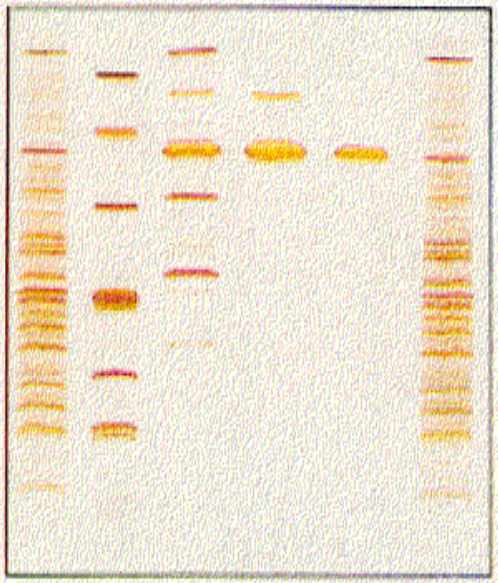
Coomassie staining of a SDS-PAGE

molecular size

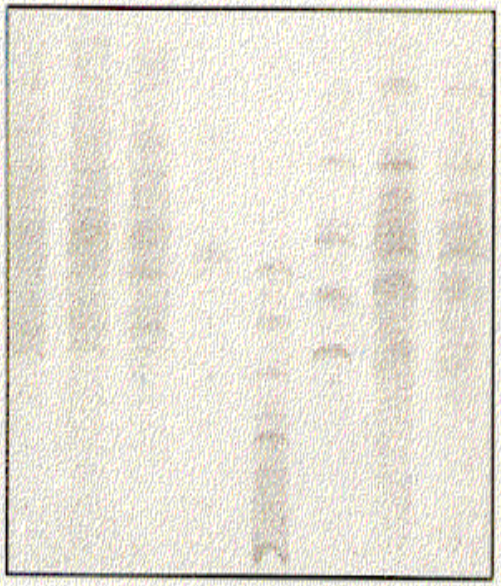
97 kDa →
64 kDa →
43 kDa →
30 kDa →
20 kDa →



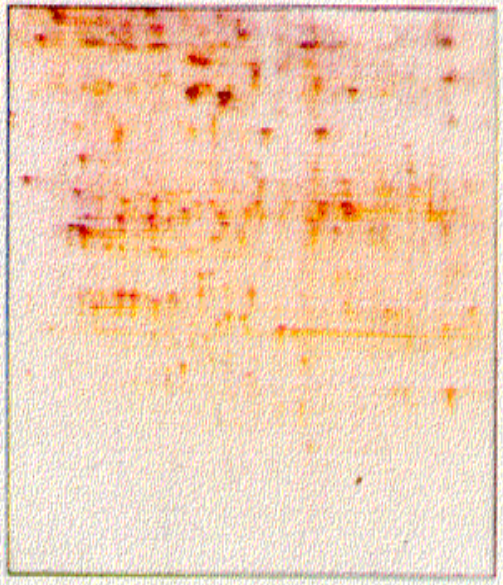
Two-dimensional gel electrophoresis (2-DE)



1-Dimensional



1-Dimensional



2-Dimensional

What is it all about???

2-DE sorts proteins according to two independent properties in two discrete steps:

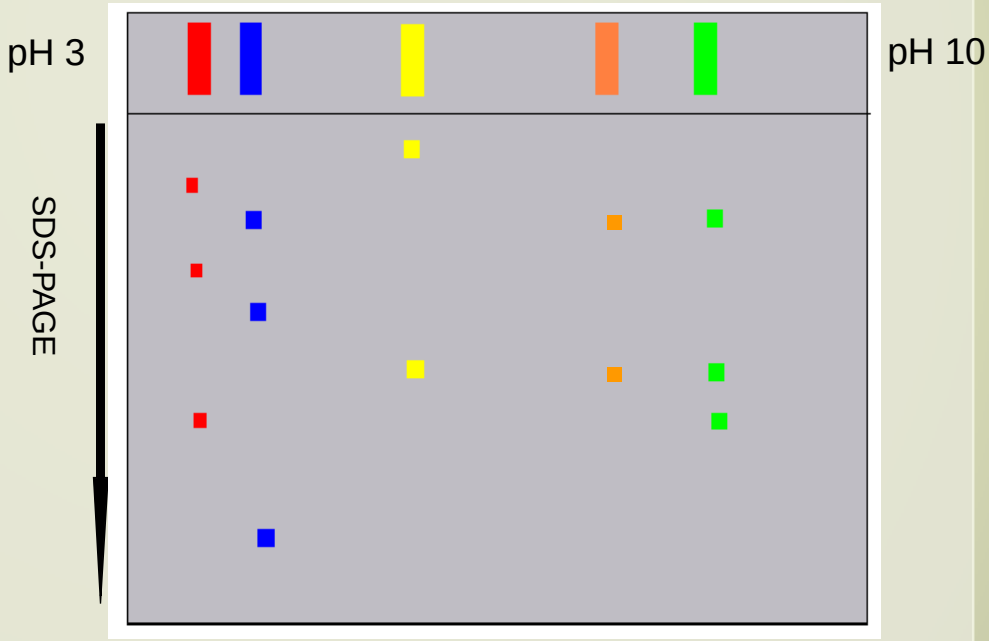
the first step, isolelectric focusing (IEF), separates proteins according to their isoelectric points (pI);
the second step, SDS-PAGE, separates proteins according to their molecular weight (MW).

Each spot of the resulting two-dimensional array corresponds to a single protein species in the sample.

Thus, thousands of different proteins can be separated, and information such as the protein pI, the apparent molecular weight, and often the amount of each protein can be obtained.

Two-dimensional gel electrophoresis (2D)

- 1st dimension, IEF, Proteins are separated
- according to their isoelectric point (IP)
- 2nd dimension, SDS-PAGE, Proteins are separated according to their molecular mass
- Efficient: More than a thousand proteins resolved in E-Coli cell lysates and ~8000 in brain lysates



First dimension:

Proteins are amphoteric molecules, the carry either positive, negative or zero net charge. The net charge of a protein is the sum of all the negative and positive charges of all amino acids.

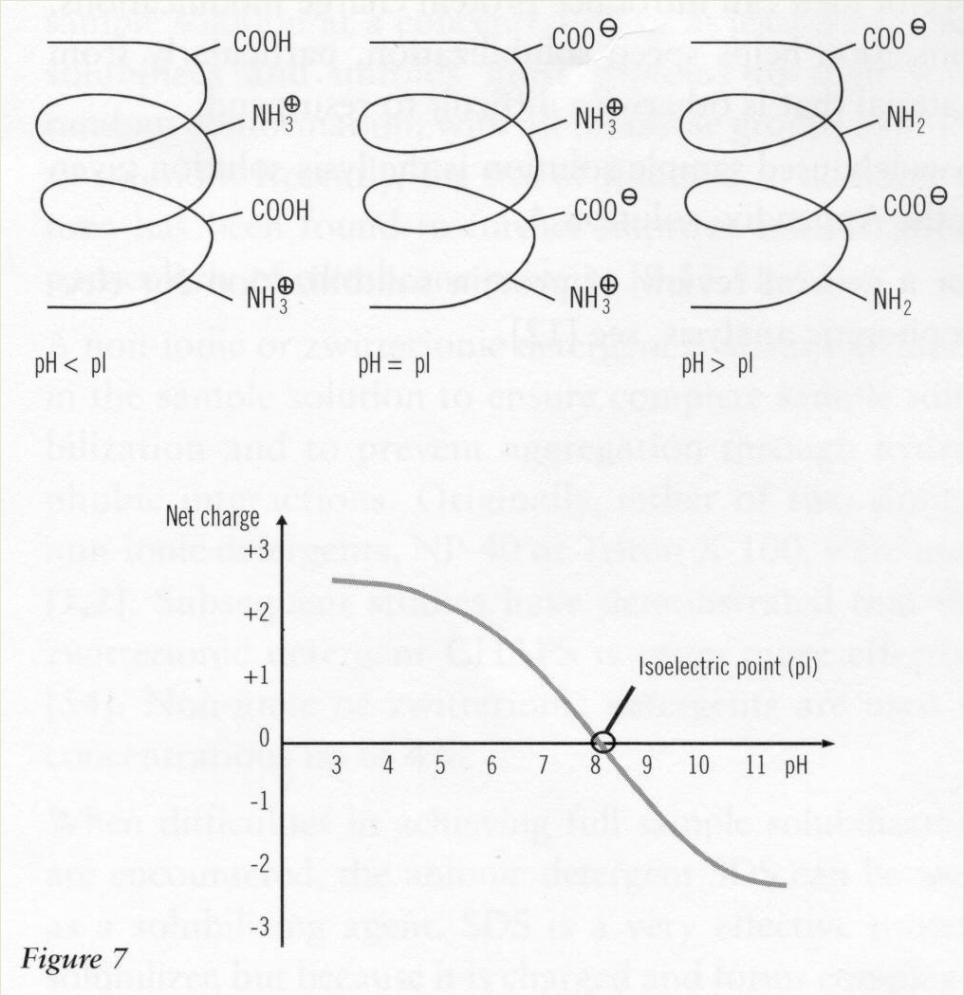
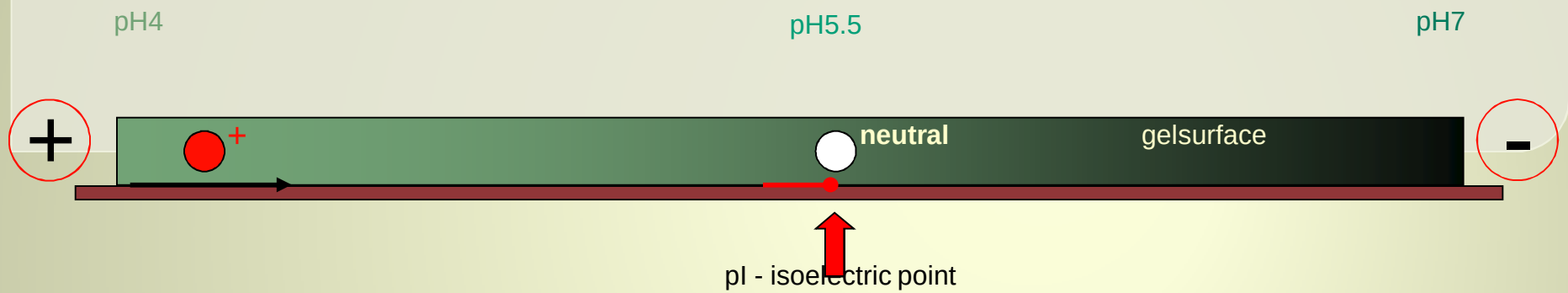


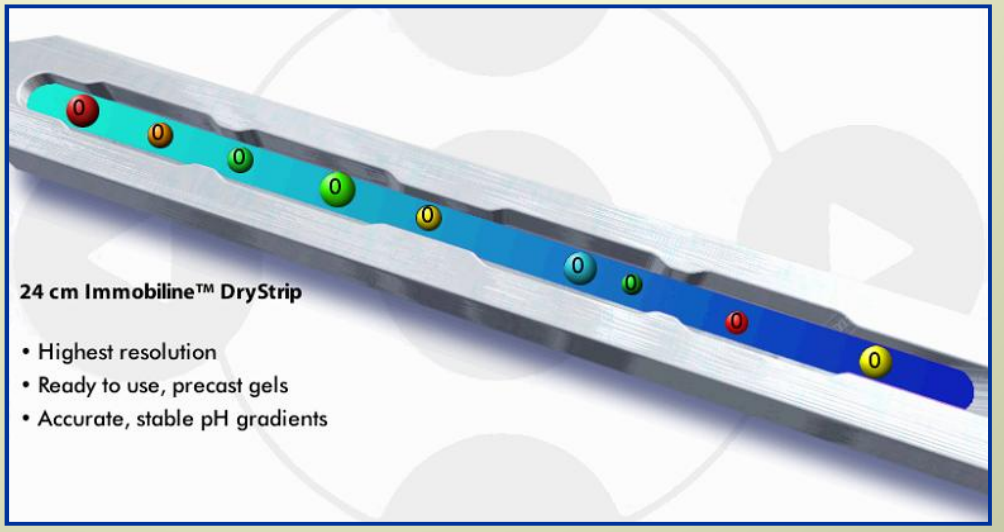
Figure 7

1st Dimension - Isoelectric Focusing



pI - isoelectric point

ready made Gel-strips



Immobilized ampholytes are incorporated into PAA

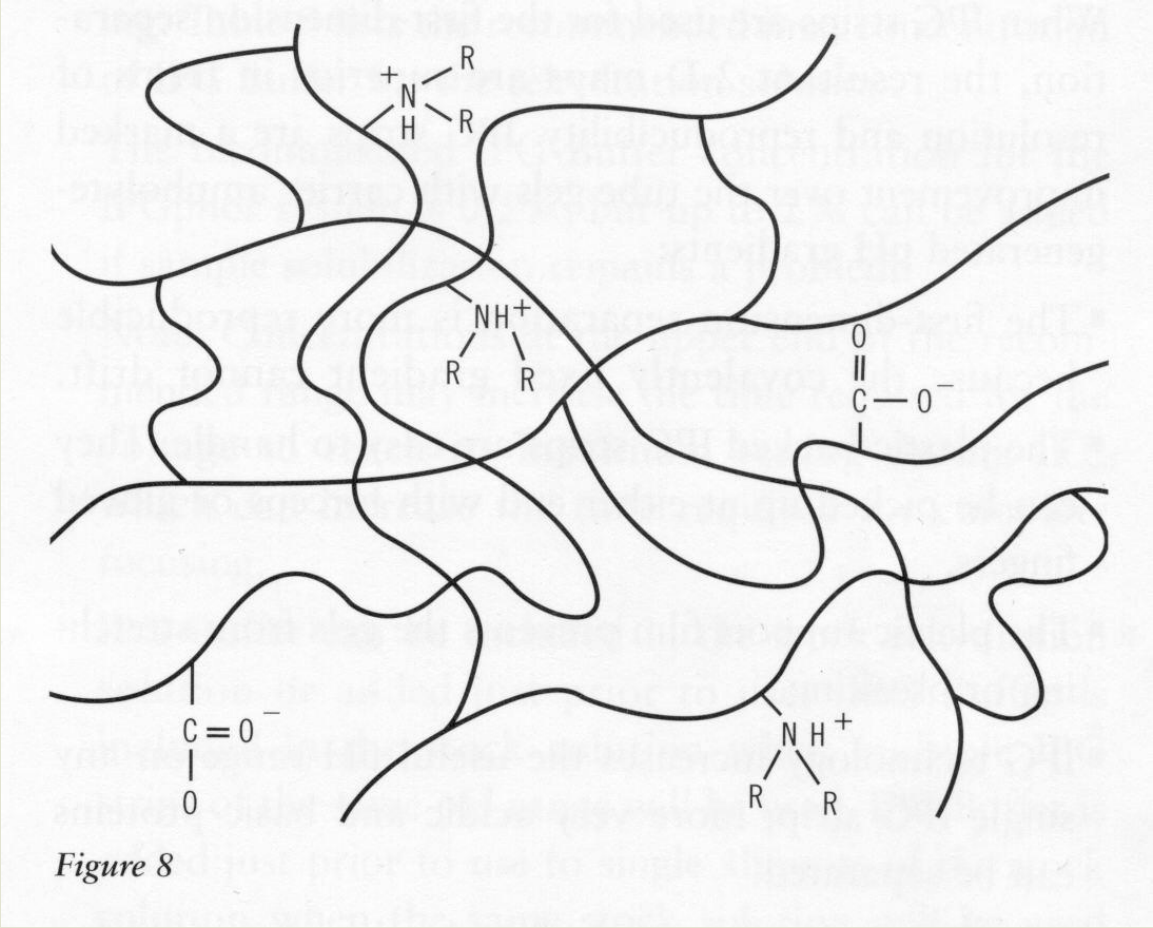
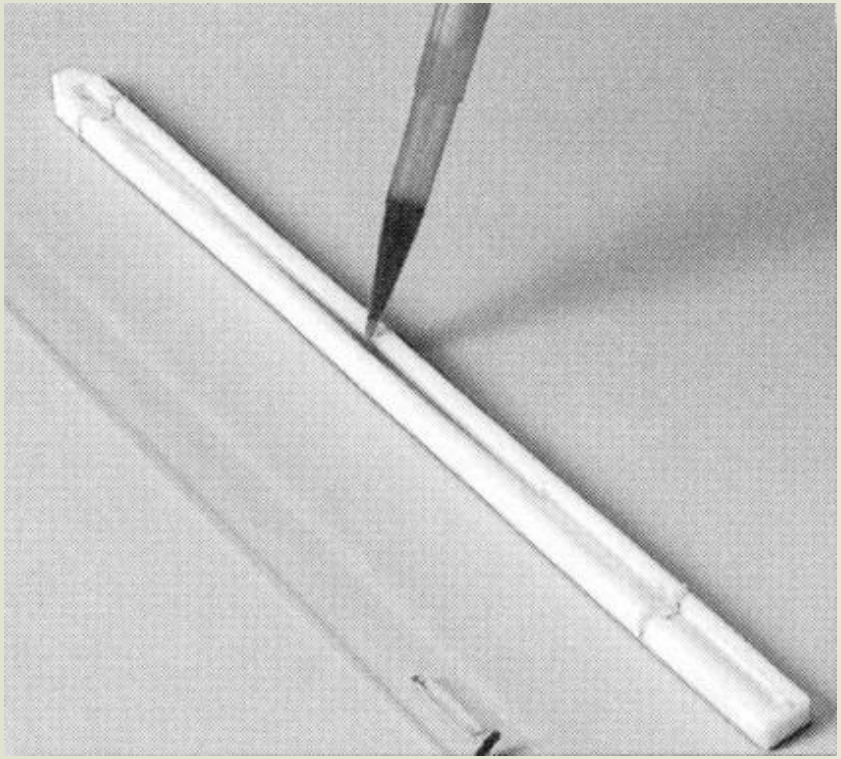


Figure 8



Gel strip with ampholytes
3-10 pH units or narrower range strips e.g. 5-6 pH
(linear or non-linear)

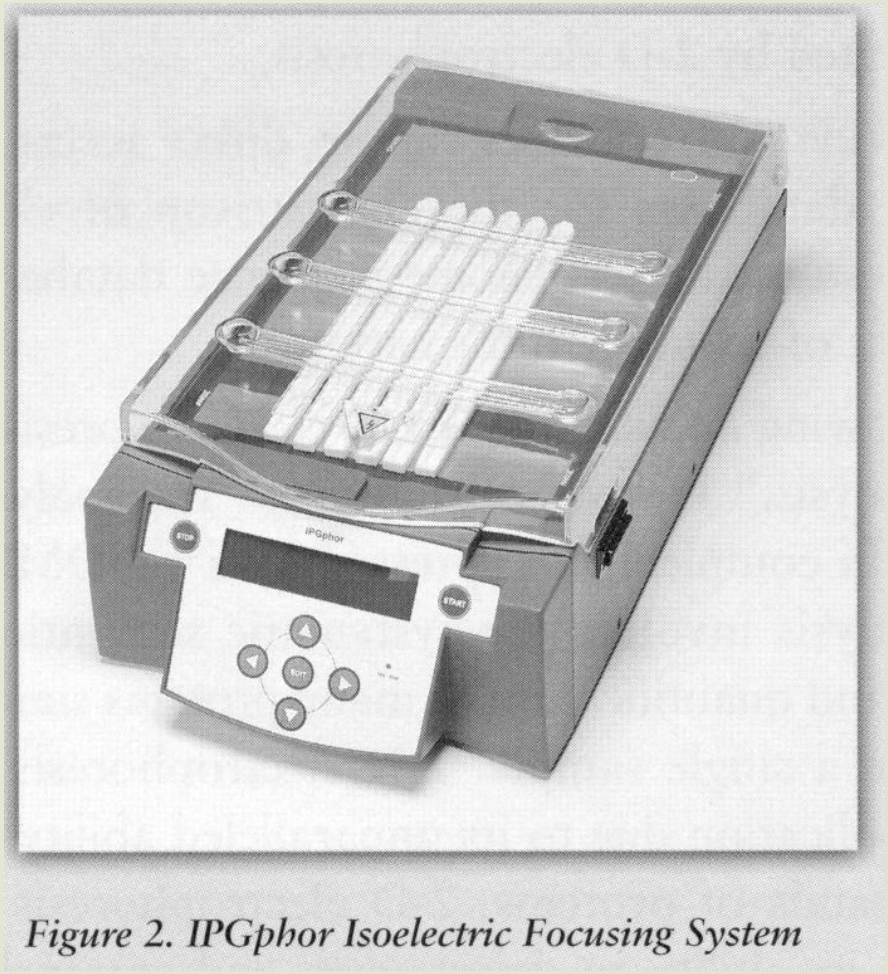


Figure 2. IPGphor Isoelectric Focusing System

Second dimension:

→ Regular SDS-PAGE with equilibrated IEF strip (equilibration in buffer with urea, glycerol, reductants, SDS, and dye).

Urea reduces the effect of electroendosmosis caused by ampholytes!

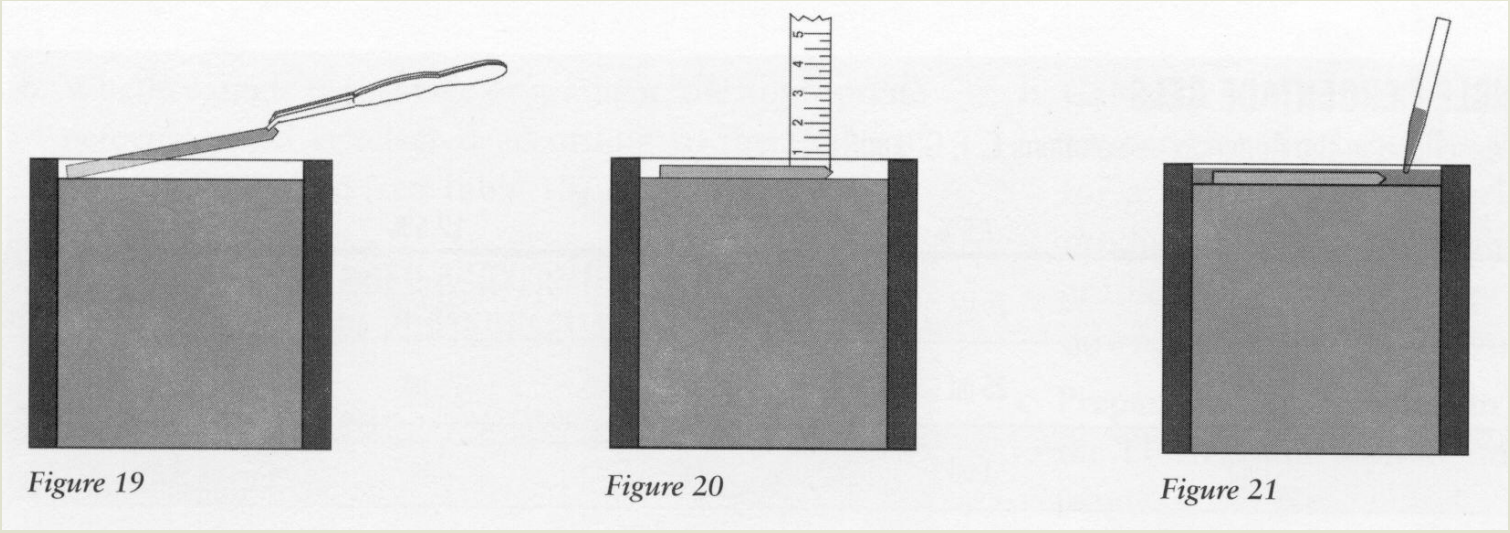
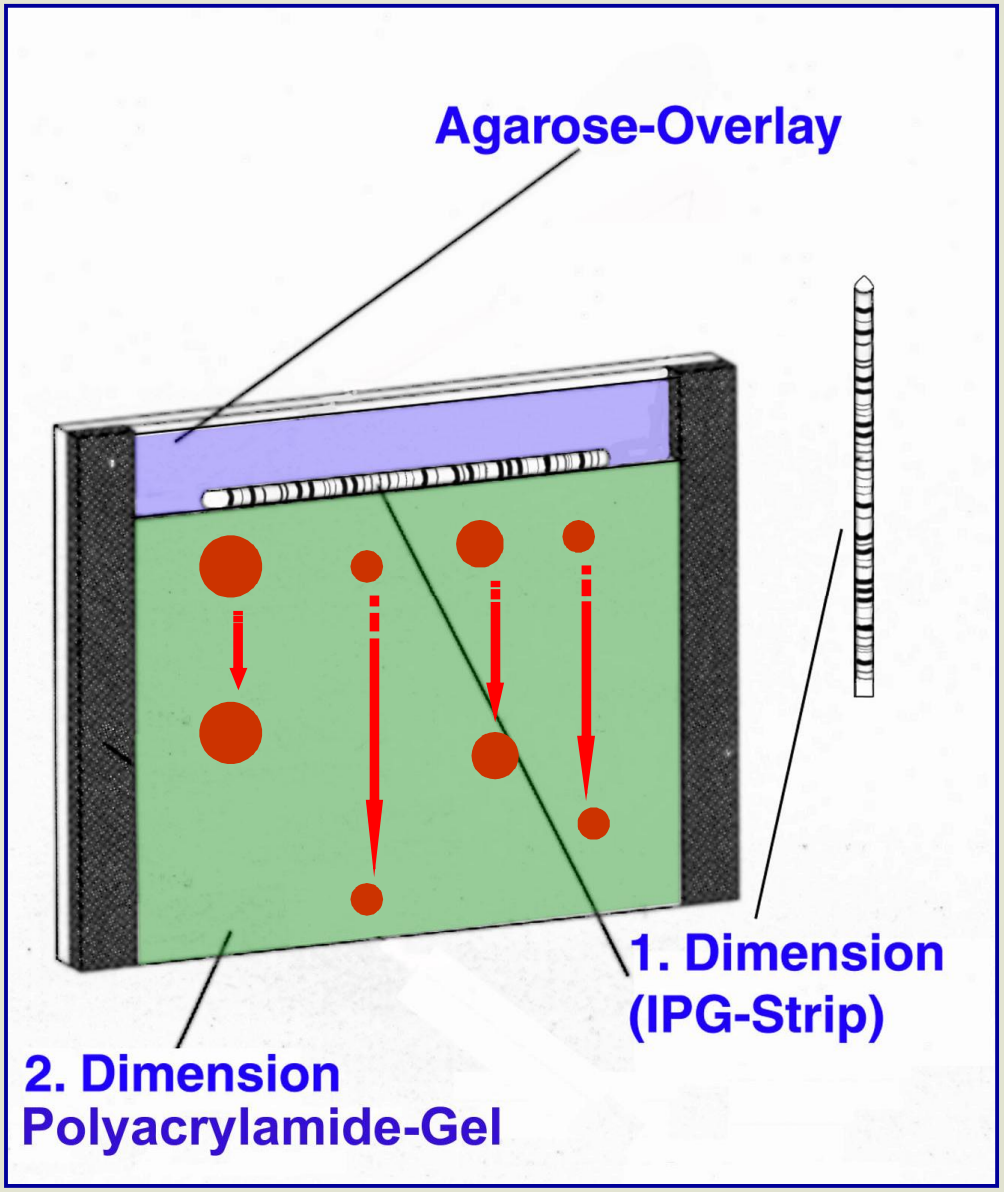


Figure 19 *Figure 20* *Figure 21*

2nd Dimension - Isoelectric Focusing

2DE

Mw



SAMPLE PREPARATION

→ Sample preparation is absolutely essential for good 2-D results

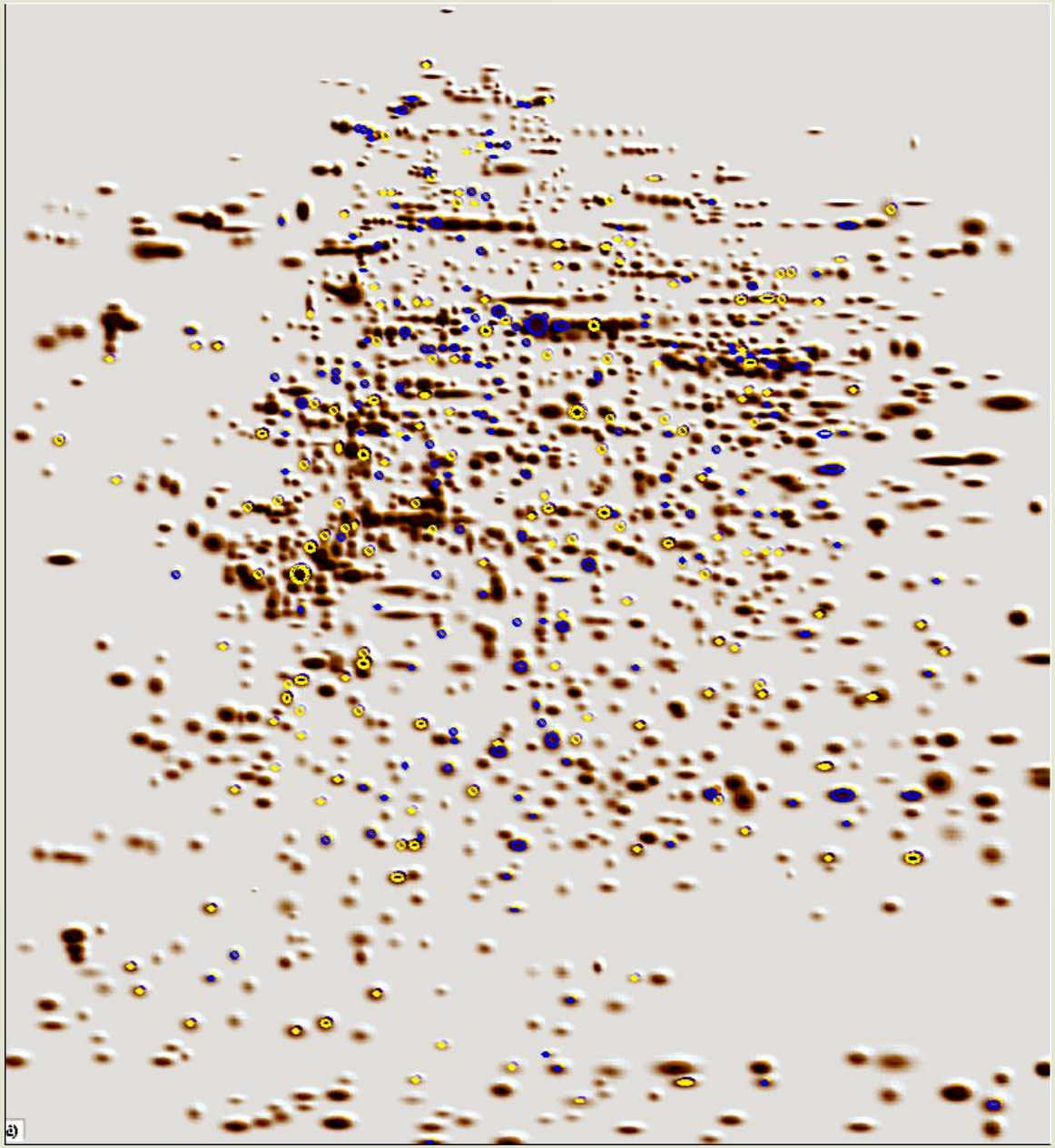
- what do you want to see??
  - all proteins or a spot area??

- soluble proteins or membrane protein fractions??

e.g. to analyze all intracellular proteins the cell must be effectively disrupted

- osmotic lysis
- freeze-thaw lysis
- detergent lysis (beware of interference with IEF)

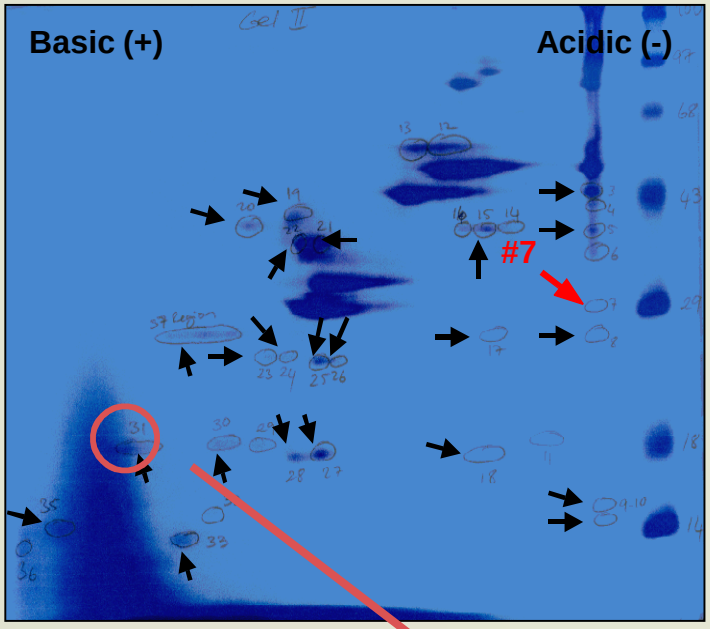
Protein Fingerprint: 2-DE



About 2000
proteins

2D-PAGE Direct In-situ Digest

To identify the separated proteins



100 pmol

running the gel

- staining each spot of interest (→)

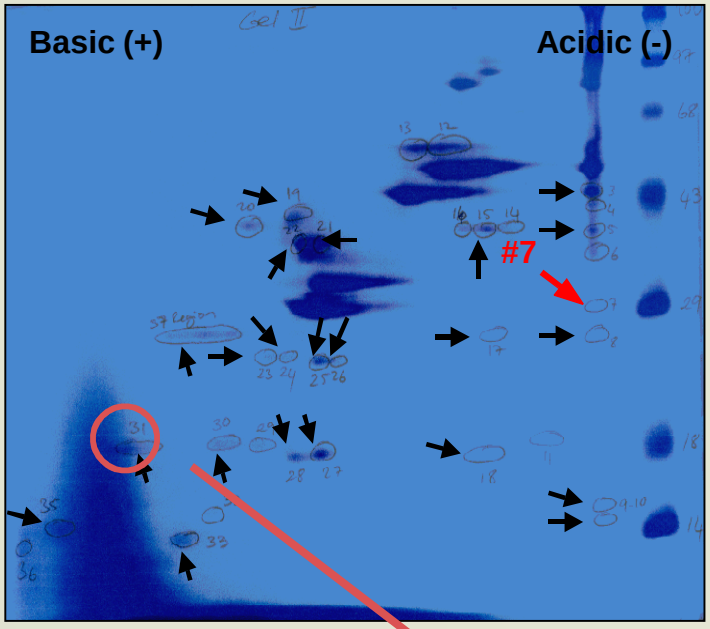
- excise

- *in-gel* digestion

Trypsin digest

→ **PEPTIDE ANALYSIS**

2D-PAGE Direct In-situ Digest



100 fmol

running the gel

- staining each spot of interest (→)

- excise

- *in-gel* digestion

Trypsin digest

→ **PEPTIDE ANALYSIS**

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BRUKER

map II

The MALDI AutoPrep Robot System

- click to enlarge -

Automated MALDI sample prepara
of robot systems - including the m
washing or purification with Millipo
8-channel liquid handler.

MAP II and MAP II/8 use disposab
Samples can be placed in capped
plate formats are supported and c
MALDI target.

- click to enlarge -

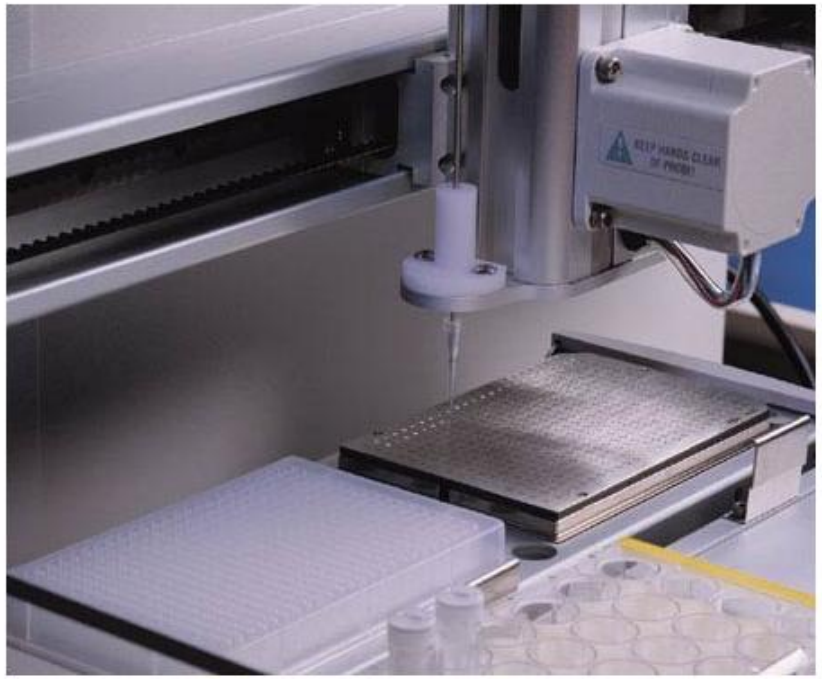
One single sample spreadsheet cc
sample preparation, measurement
Control and the FLEX III MALDI-T
guiding the path from sample puri

- Corporate Info
- Products
- Applications
- Sales + Service
- News + Events
- Job Offers

BRUKER DALTONICS

Enabling Life Science Tools Based On Mass Spectrometry™

http://www.bruker-daltonik.de/images/mapii5.jpg - Microsoft Intern...



- click to enlarge -

Internet

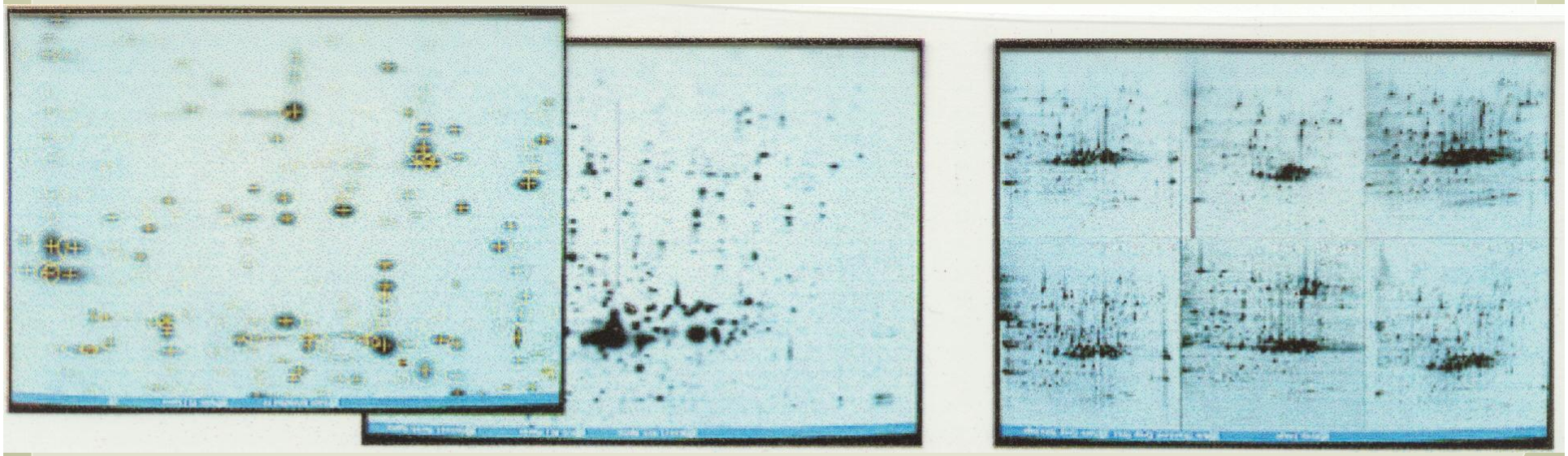
Start | Bruker Daltonik GmbH - Mi... | USB Bridge Cable Applicat... | Microsoft PowerPoint - [P... | http://www.bruker-da... | 5:17 PM

MALDI TOF/TOF

What can you do with 2-DE? (2-DE in Proteomics)

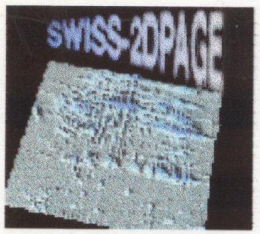
- Separate thousands of proteins in one analysis
- Quantitate gene expression on protein level
- Identify proteins from gel by mass spectrometry
- etc....

You may create your own data bases:



Or you may compare results with other existing data bases:

ExPASy Home page | Site Map | Search ExPASy | Contact us



SWISS-2DPAGE
Two-dimensional polyacrylamide gel electrophoresis database

Important announcement

SWISS-2DPAGE contains data on proteins identified on various 2-D PAGE reference maps. You can locate these proteins on the 2-D PAGE maps or display the region of a 2-D PAGE map where one might expect to find a protein from SWISS-PROT [More details / References / Disclaimer].

Release 9.0, January 1999 and updates up to 28-Jan-1999 (contains 544 entries in 22 reference maps from human, mouse, *Saccharomyces cerevisiae*, *Escherichia coli* and *Dictyostellium discoideum*).

[Search][Documents][Services][Software][Related servers][Other databases][Job openings]

Access to SWISS-2DPAGE

- by description line (DE) or by ID
- by accession number (AC lines)
- by clicking on a spot: select one of our 2-D PAGE reference maps, click on a spot and then get the corresponding information from the SWISS-2DPAGE database.
- by author (RA lines)
- by full text search

- SRS, searching in SWISS-2DPAGE using the Sequence Retrieval System

- compute estimated location on reference maps for a user-entered sequence new

SWISS-2DPAGE documents

- User manual
- Release notes (January 14, 1999)
- **Protocols:**
  - Technical information about 2-D PAGE (IPG's, silver staining, protocols, etc)
  - High performance 2-D gel comparison
- **2-D PAGE maps published:**
  - Human CSF, ELC, HEPG2, HEPG2SP, LIVER, LYMPHOMA, PLASMA, PLATELET, RBC, U937, CEC, KIDNEY.
  - *Dictyostelium discoideum*, *Escherichia coli*, *Saccharomyces cerevisiae*.

Services

- Downloading SWISS-2DPAGE by FTP

- SWISS-2DSERVICE - Get your 2-D Gels performed according to Swiss standards
- 2-D PAGE training - attend a one week course in Geneva
- 2-D PAGE museum - gels run by trainees during the 2-D PAGE courses

Software

- Melanie - Software package for 2-D PAGE analysis
- Make2ddb package - A package preparing the data and the programs necessary to build a federated 2-DE database on one's own web site.

Gateways to other 2-D PAGE related servers and services

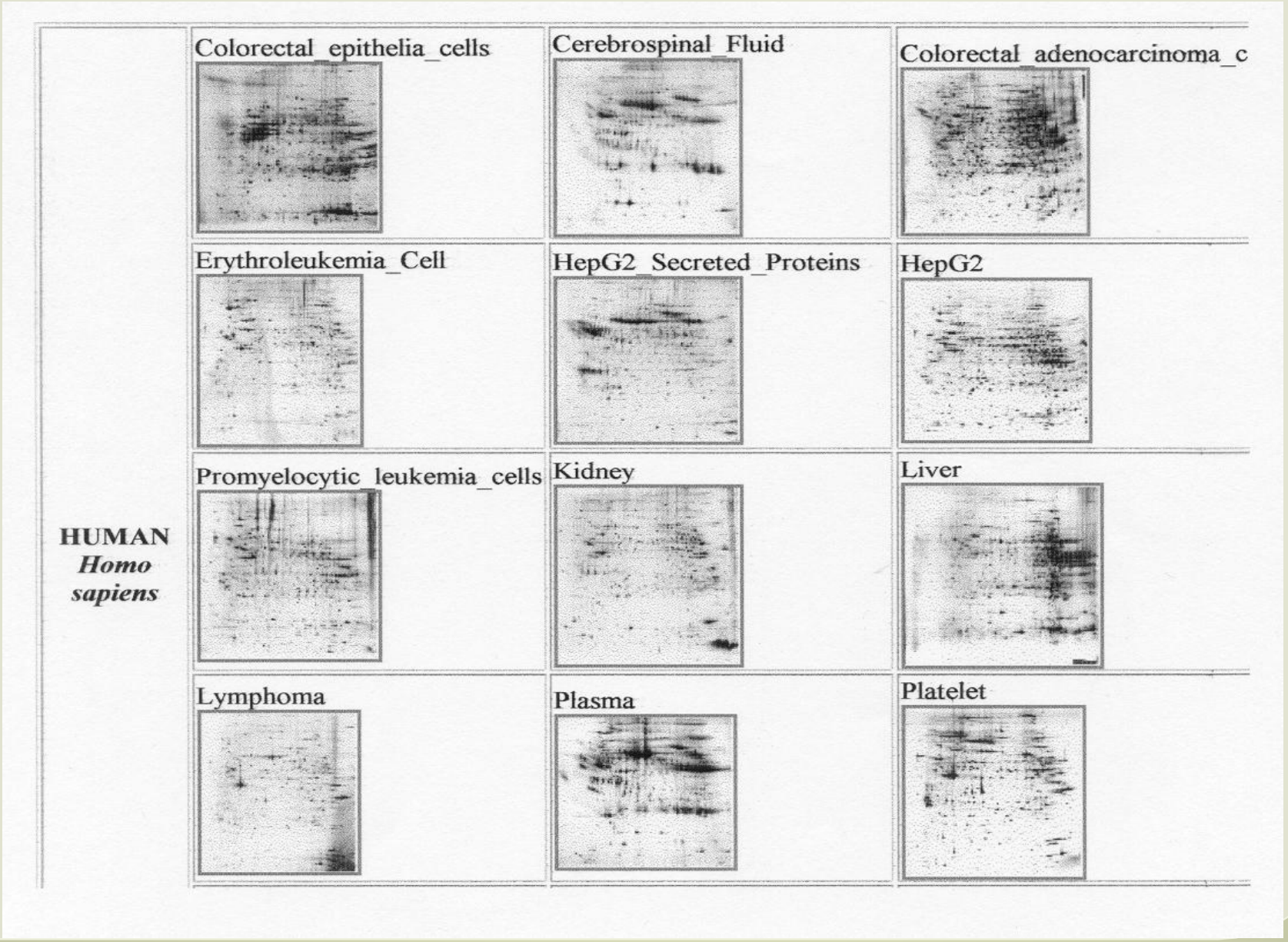
- 2D Hunt - 2-D electrophoresis web site finder
- WORLD-2DPAGE - Index to other Federated 2-D PAGE databases

Access to other databases and tools on ExPASy

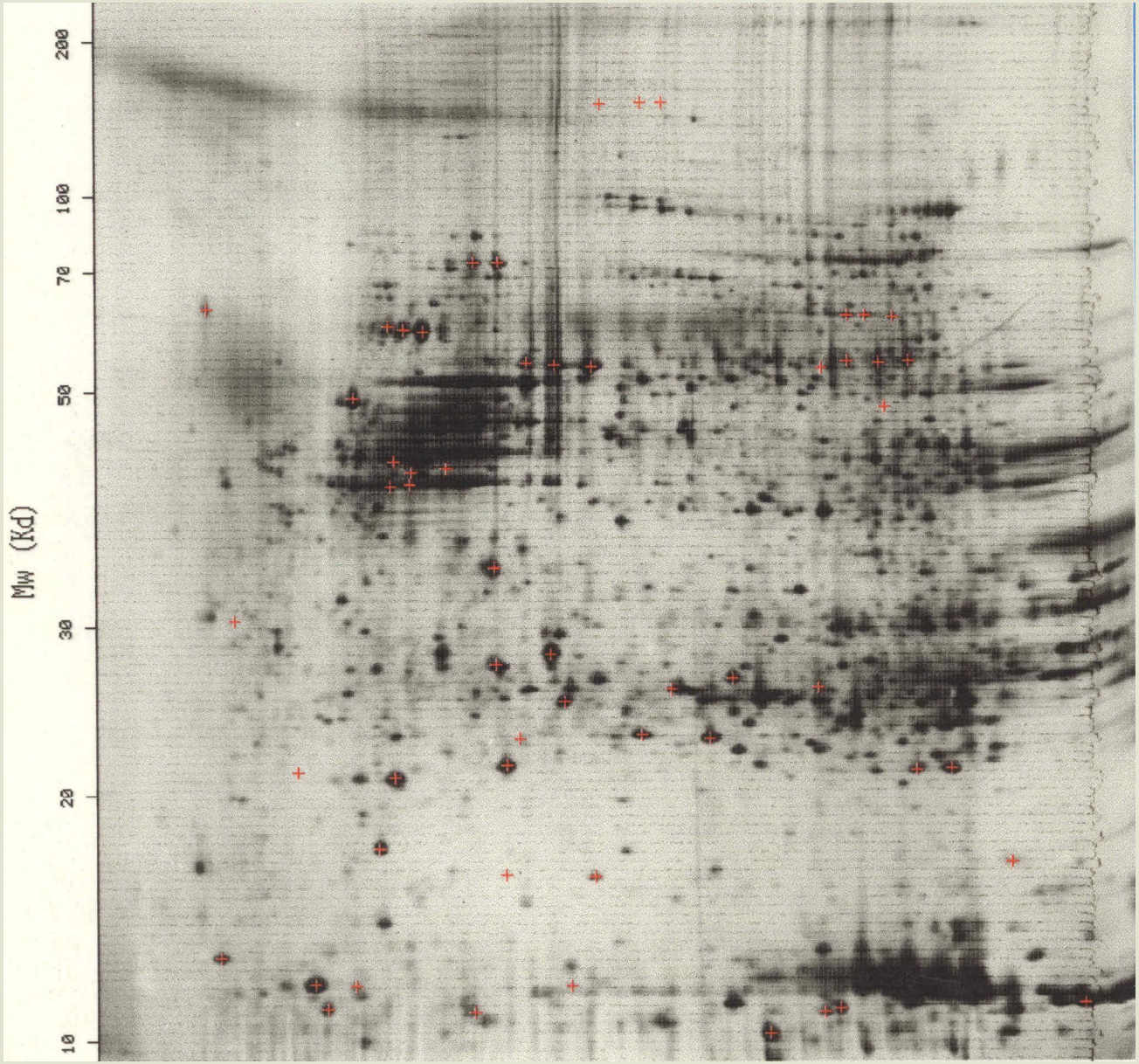
- SWISS-PROT
- PROSITE
- ENZYME
- SWISS-3DIMAGE
- SWISS-MODEL Repository
- SeqAnalRef
- CD40Lbase

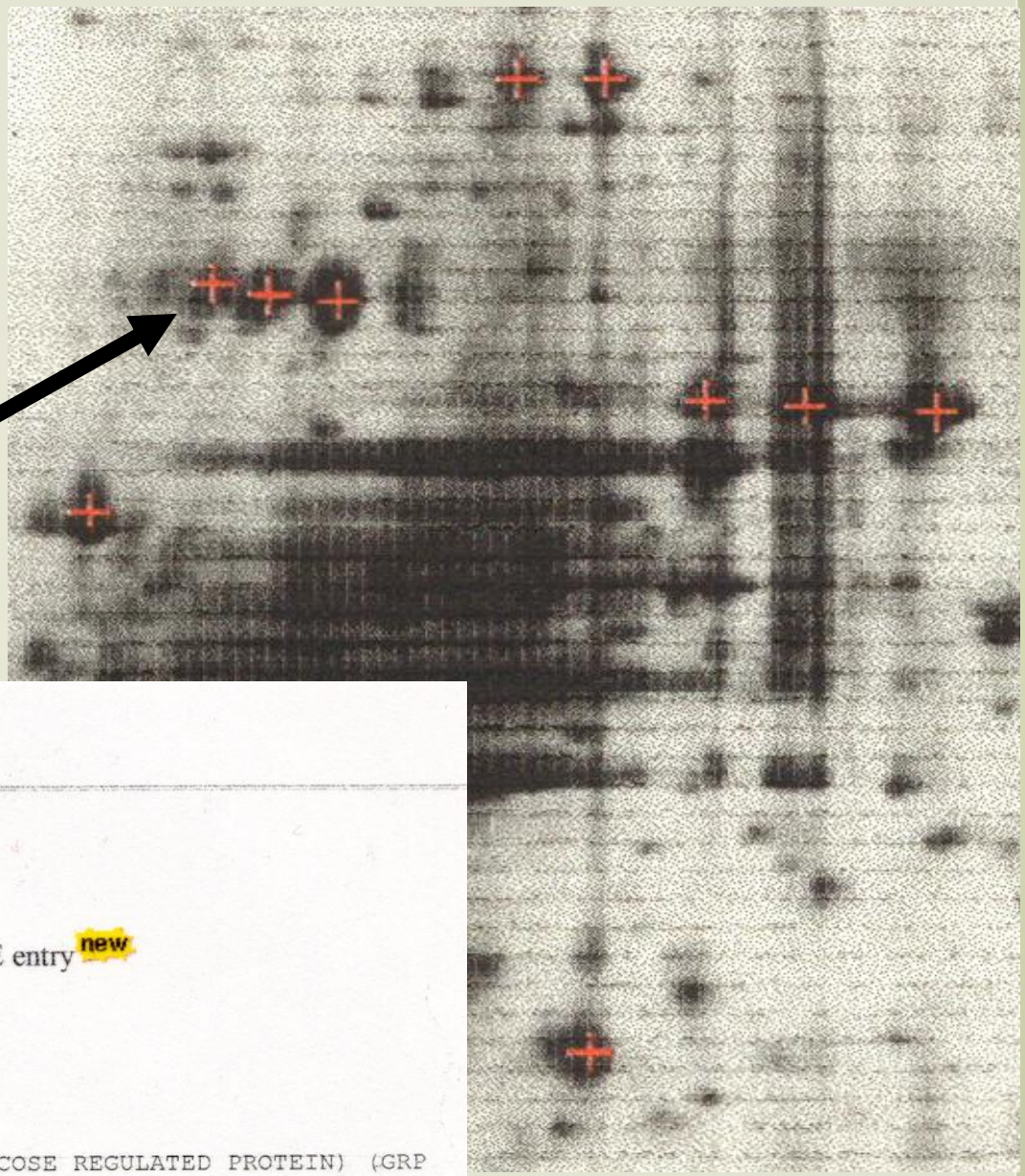
- Proteomics tools

Also 2D Databases exist!



Map Selection: CEC-Human





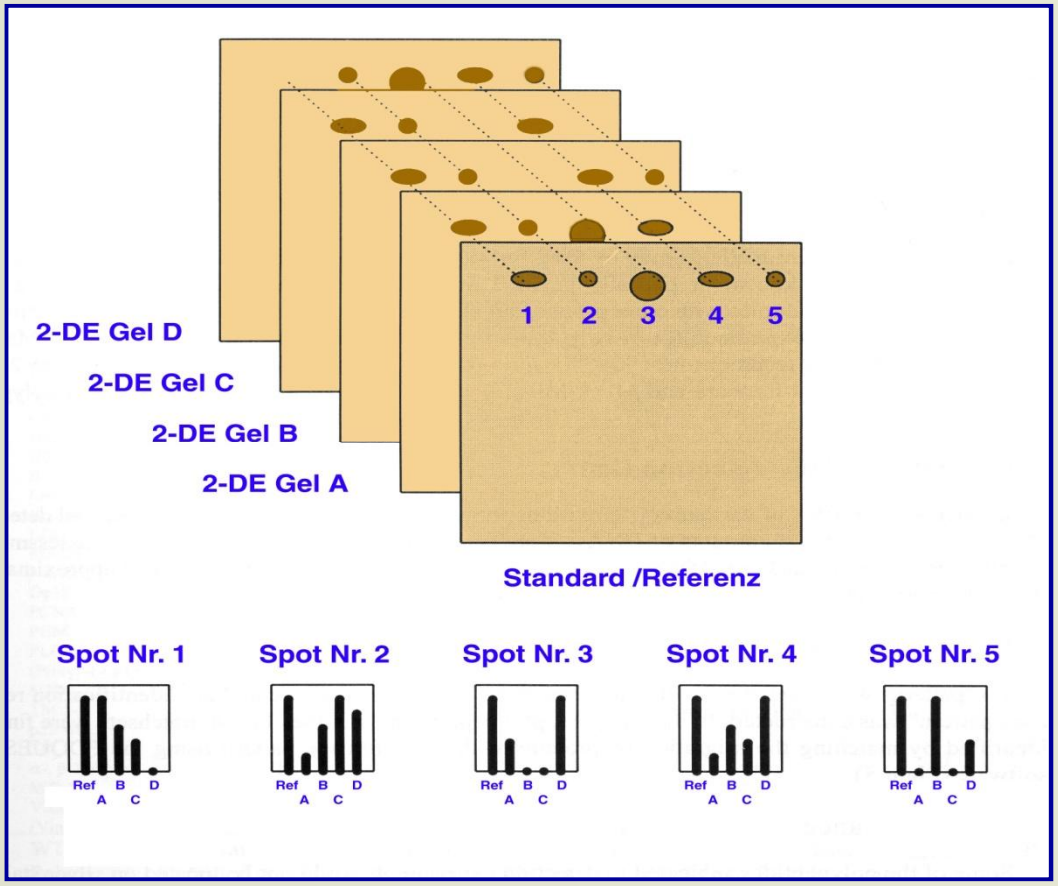
1 protein has been found:

P38646:

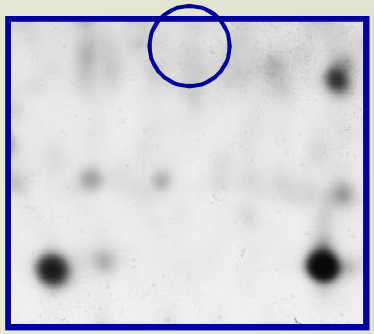
Nice2DPage - a user-friendly view of this SWISS-2DPAGE entry new
Compute the theoretical pI/Mw

```
ID   GR75_HUMAN; STANDARD; 2DG.
AC   P38646; P30036;
DT   01-AUG-1993 (REL. 00, CREATED)
DT   15-JAN-1999 (REL. 09, LAST UPDATE)
DE   MITOCHONDRIAL STRESS-70 PROTEIN (75 KD GLUCOSE REGULATED PROTEIN) (GRP
DE   75) (PEPTIDE-BINDING PROTEIN 74) (PBP74) (MORTALIN) (MQT).
```

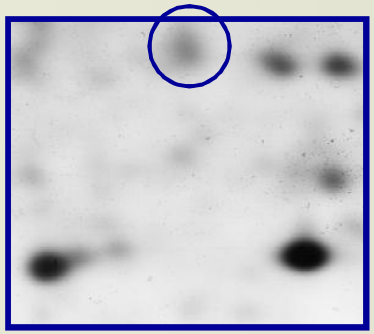
Scanning/ Gel-Matching /Imageanalysis



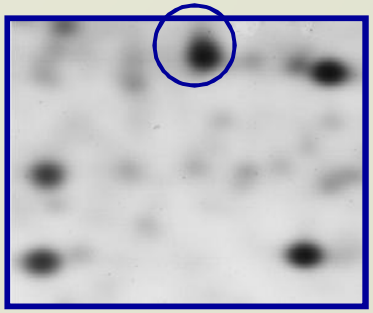
Normal

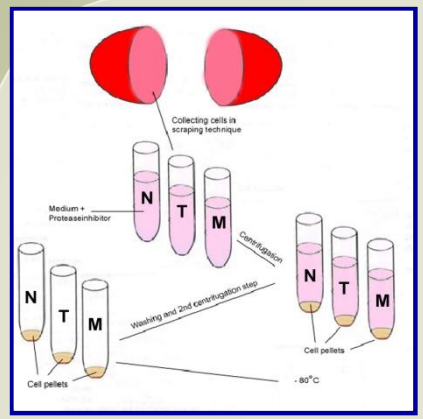


Polyp

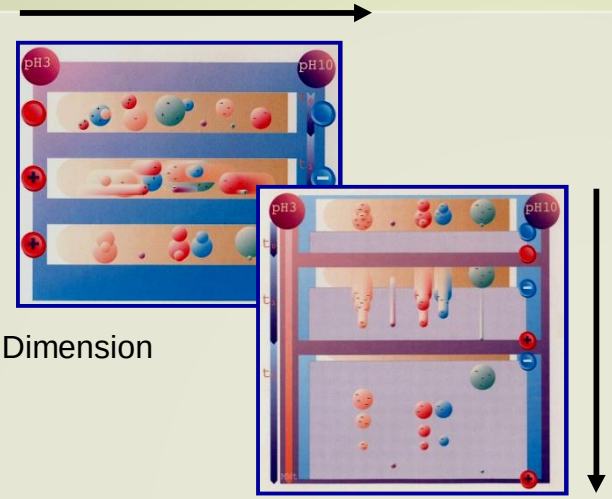


Tumor



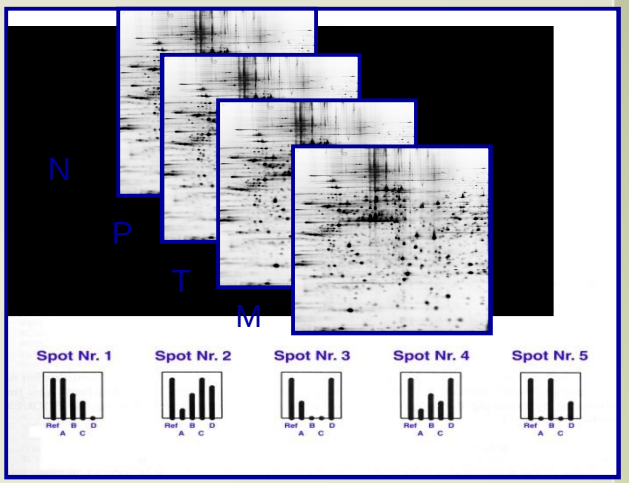


Sample enrichment/ preparation



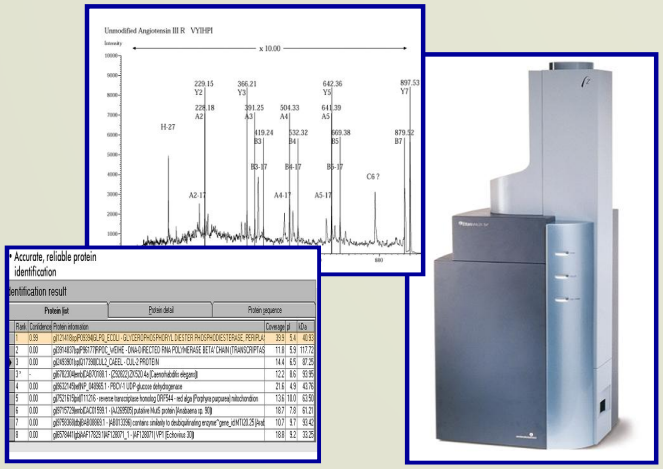
1st Dimension

2nd Dimension

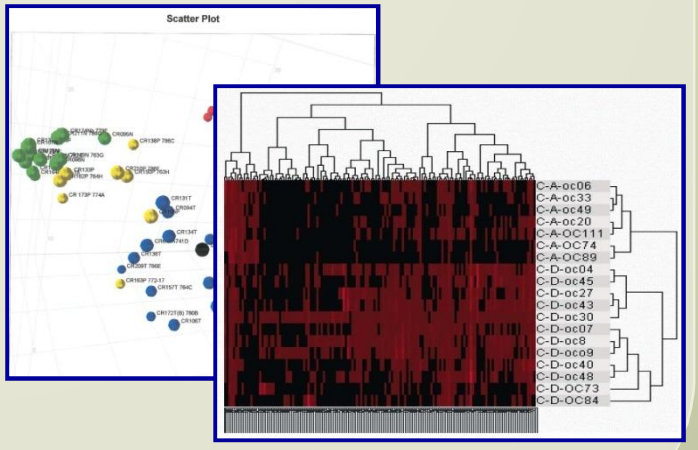


PC based matching

2-DE Algorithm

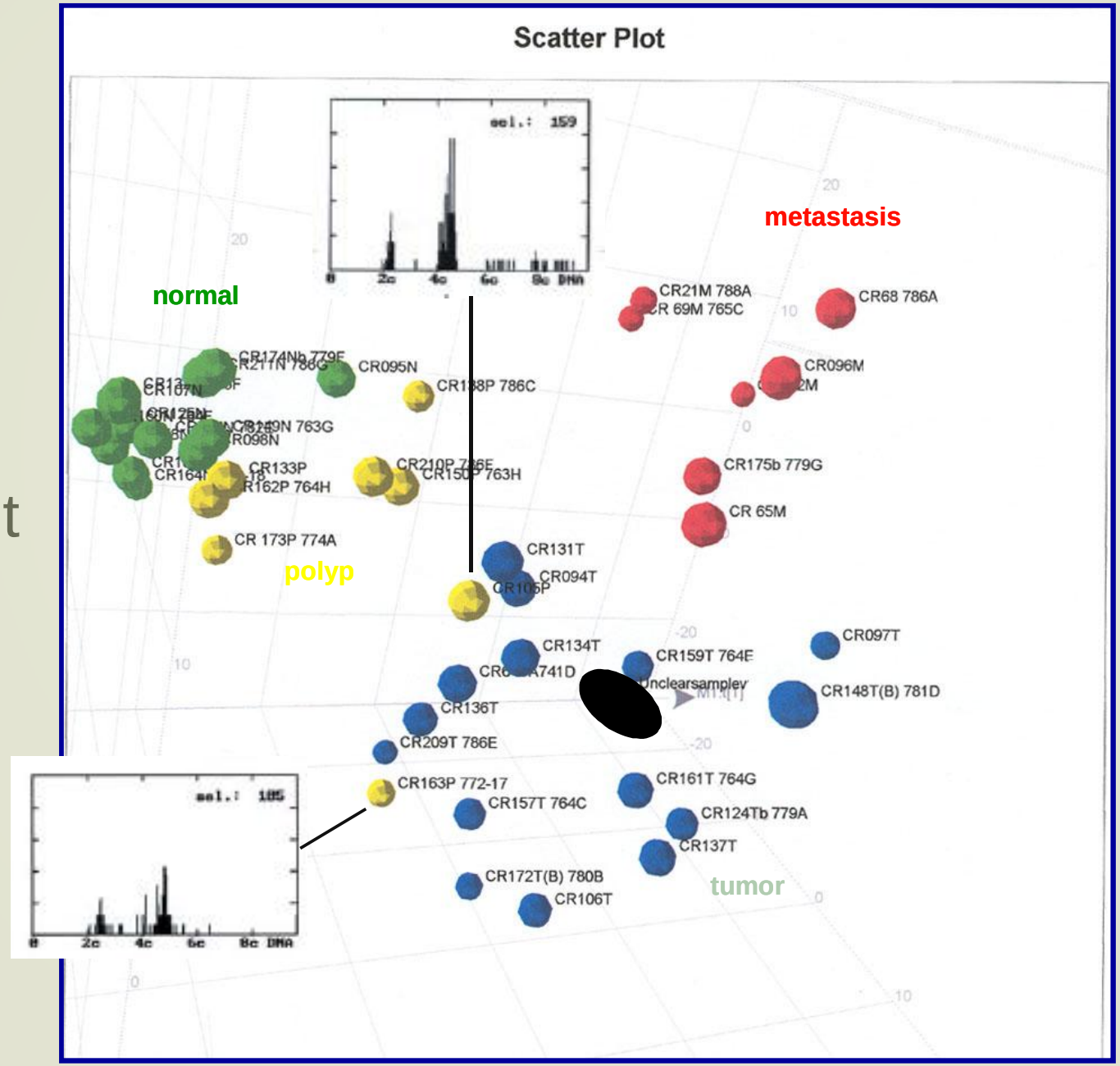


Mass spectrometrical identification



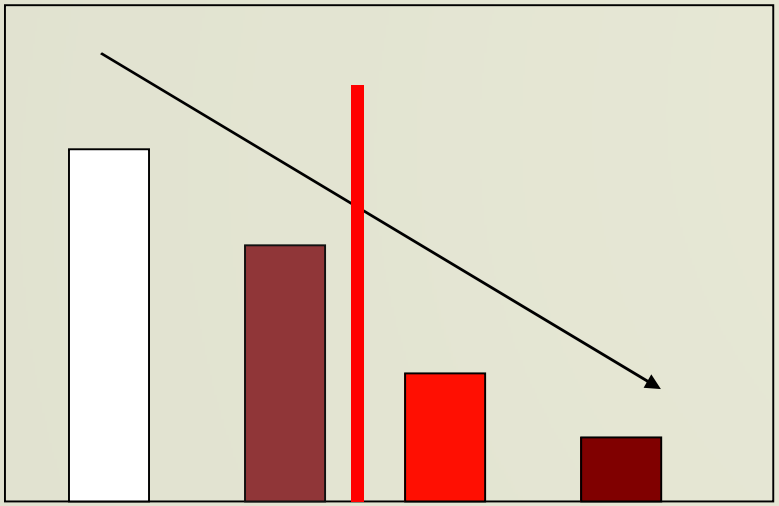
Statistics

Principal Component Analysis (t1,t2,t3)



Deviating Proteins (n=112)
Identification of 72

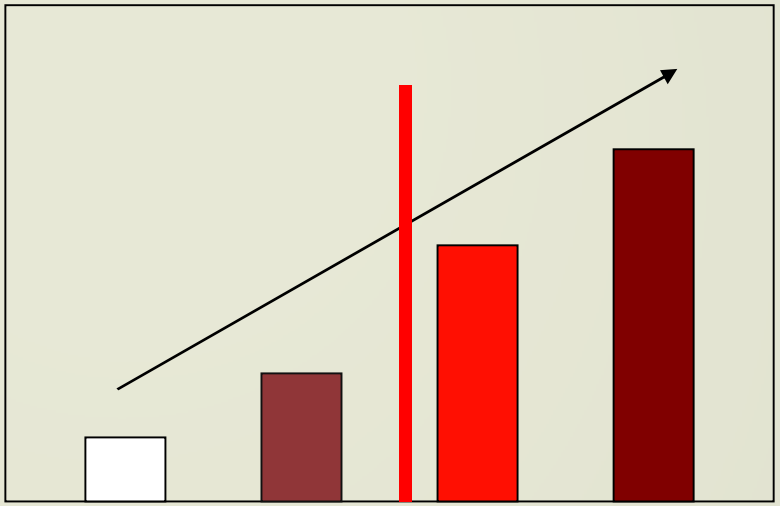
N=26



N **P** **T** **M**

benign maligne

N=46

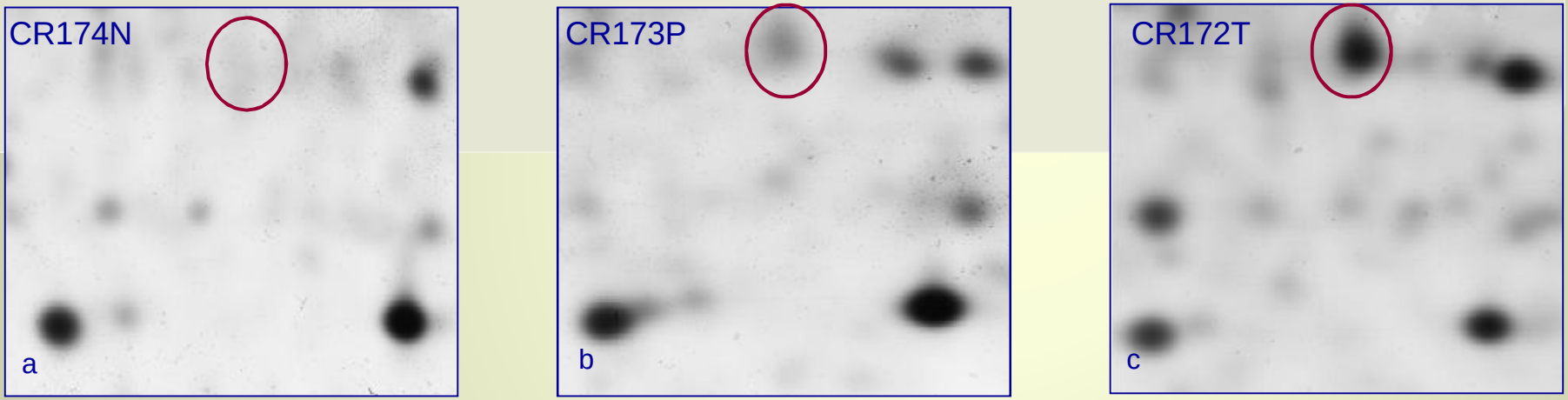


N **P** **T** **M**

benign maligne

Controls: normal liver tissue
HCT116 and Lovo cell lines

Intra-individual expression differences
of Cytokeratin 20 in patient 14.



The left gel segment (a) is zoomed from the normal mucosa,

gel b represents the patients polyp and

gel c is the corresponding segment of the same patients adenocarcinoma

Ettan™ DiGE

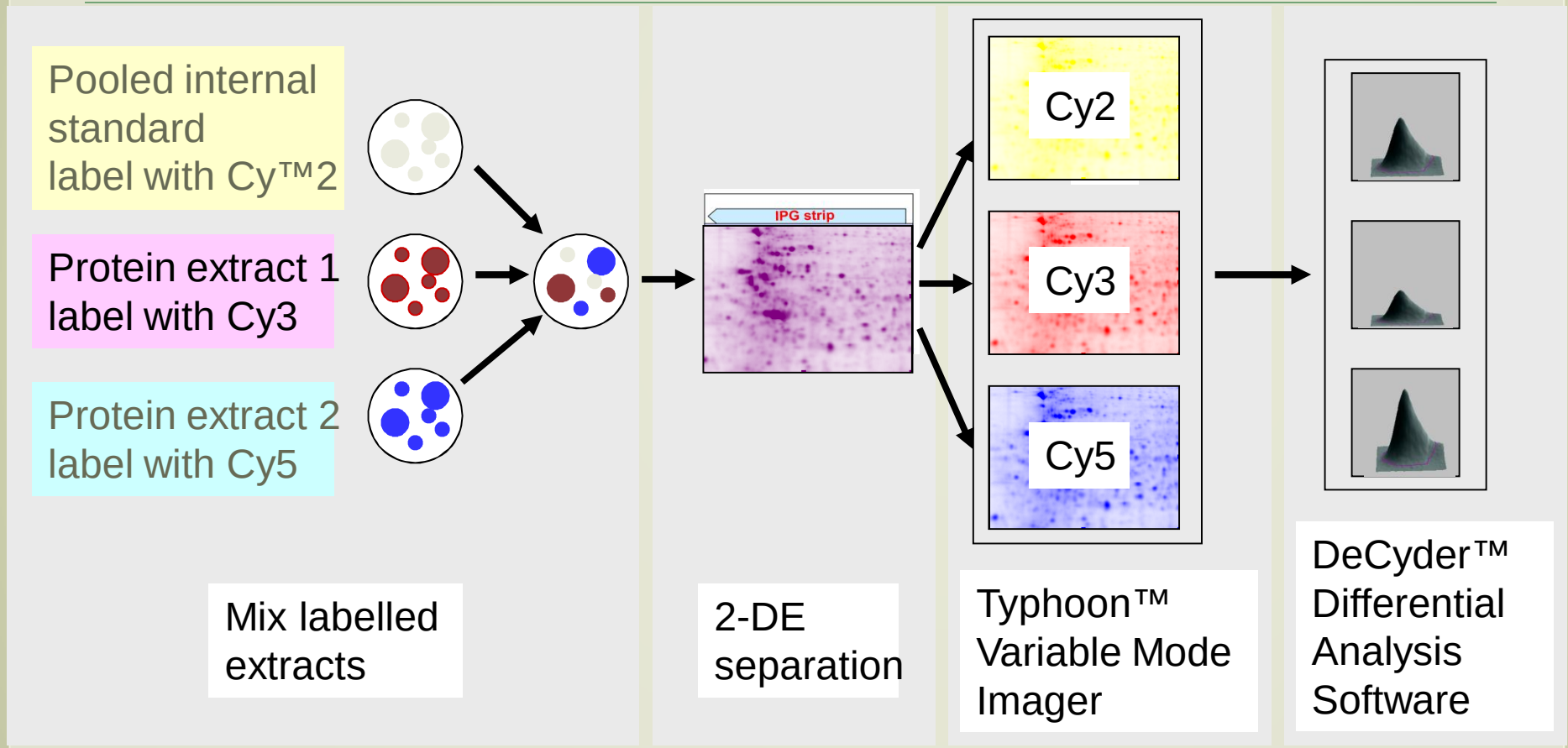
the quantitative approach to do Proteomics

CyDye DIGE Fluor dyes

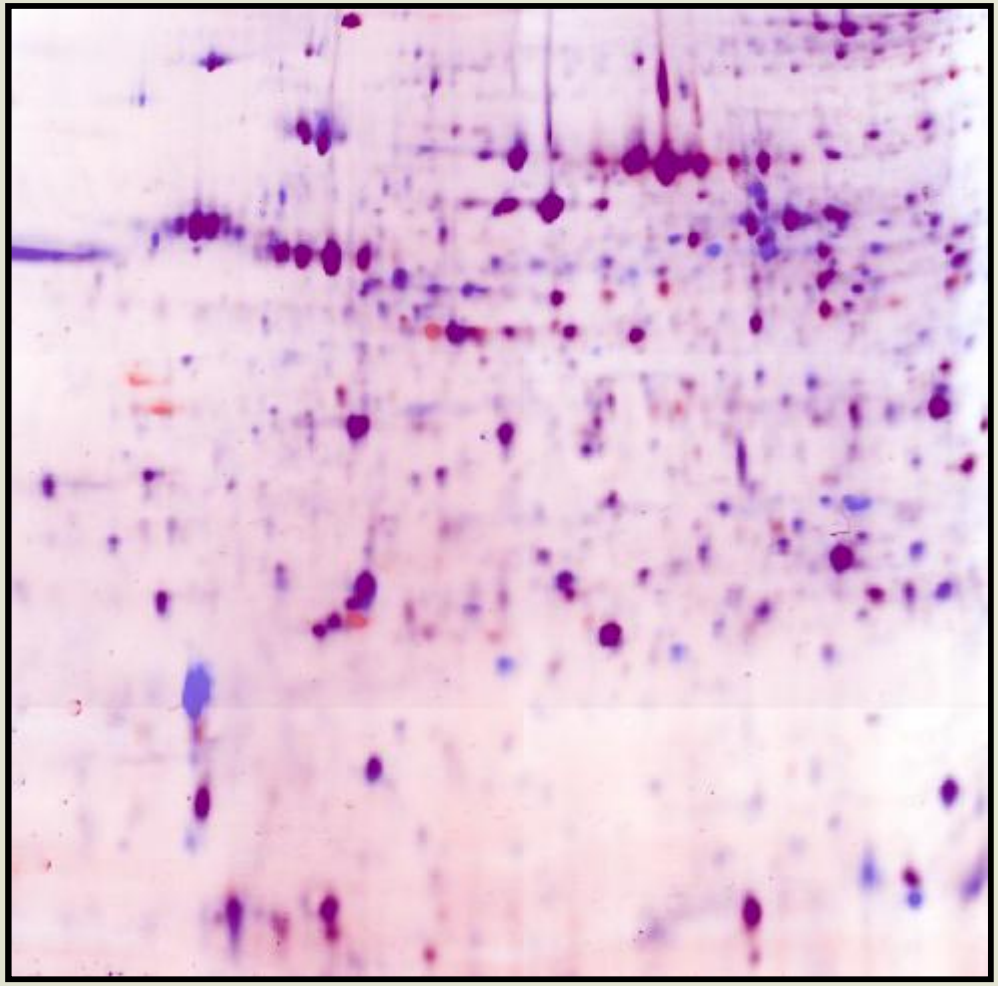
Minimal labelling dyes

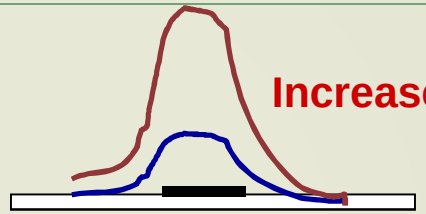
- Label 50 µg of protein
- 3 colors: Cy™2, Cy3, Cy5
- MW matched (~450Da)
- Charge matched (positive)
- Label ε-amino group of lysine
- Sensitivity - 0.025 ng
- Linear dynamic range over 4 orders of magnitude

Ettan™ DIGE system

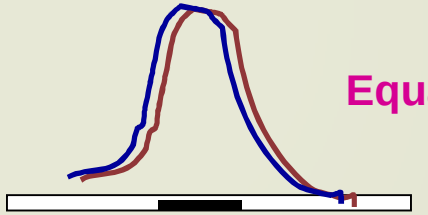


Overlay of normal and patient protein samples

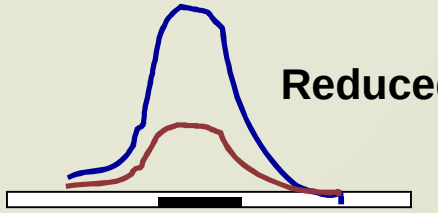




Increased abundance



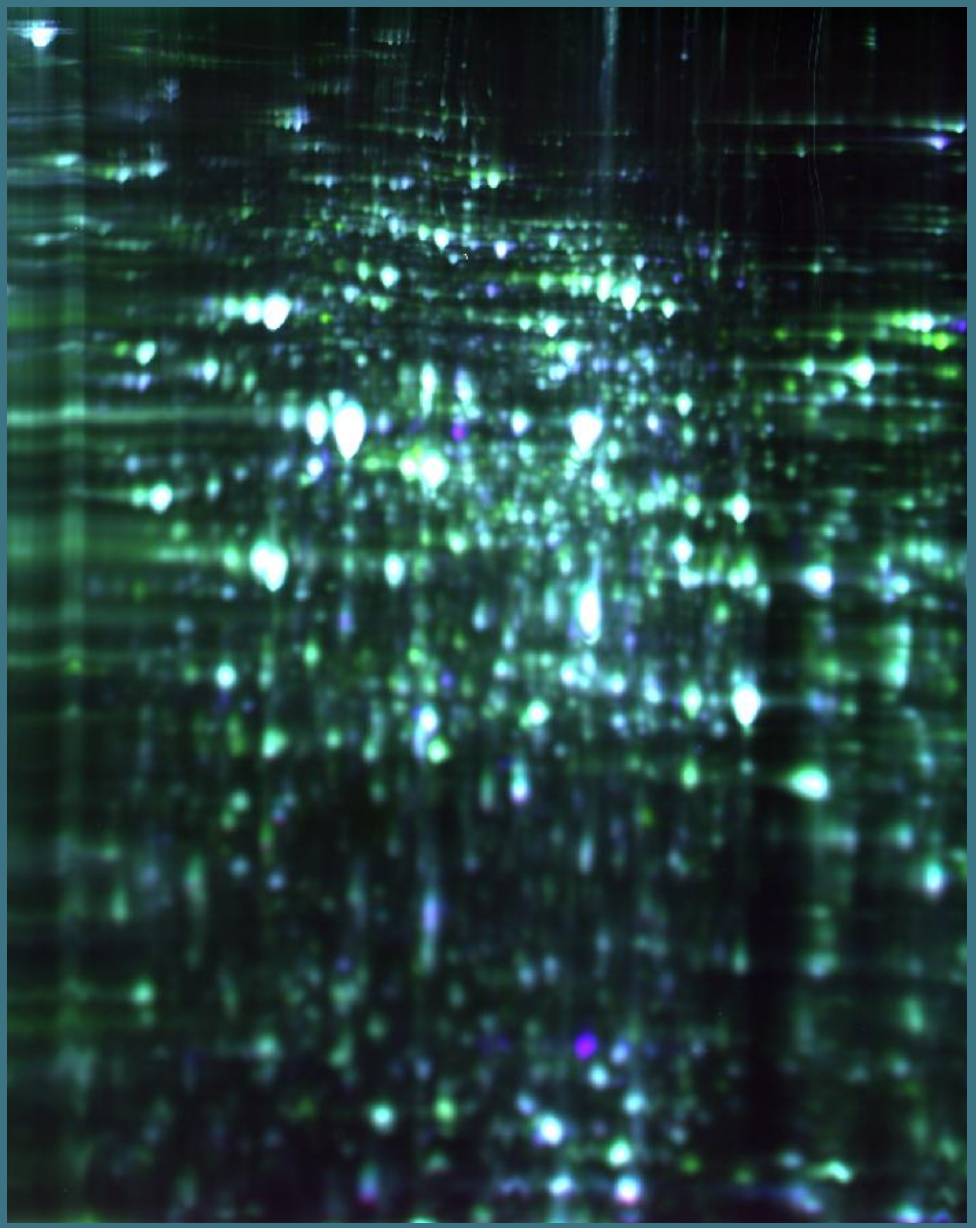
Equal abundance



Reduced abundance

Normal control = Cy™3 labelled - Blue

Patient A sample = Cy5 Labelled - Red



Science 1997

Association of Mutations in a Lysosomal Protein with Classical Late-Infantile Neuronal Ceroid Lipofuscinosis

David E. Sleat, Robert J. Donnelly, Henry Lackland, Chang-Gong Liu, Istvan Sohar, Raju K. Pullarkat, Peter Lobel*

Classical late-infantile neuronal ceroid lipofuscinosis (LINCL) is a fatal neurodegenerative disease whose defective gene has remained elusive. A molecular basis for LINCL was determined with an approach applicable to other lysosomal storage diseases. When the mannose 6-phosphate modification of newly synthesized lysosomal enzymes was used as an affinity marker, a single protein was identified that is absent in LINCL. Sequence comparisons suggest that this protein is a pepstatin-insensitive lysosomal peptidase, and a corresponding enzymatic activity was deficient in LINCL autopsy specimens. Mutations in the gene encoding this protein were identified in LINCL patients but not in normal controls.

Extracted brain proteins (50ug)

→ stained for Man-6-phosphate

→ missing spot isolated and identified by MS.