

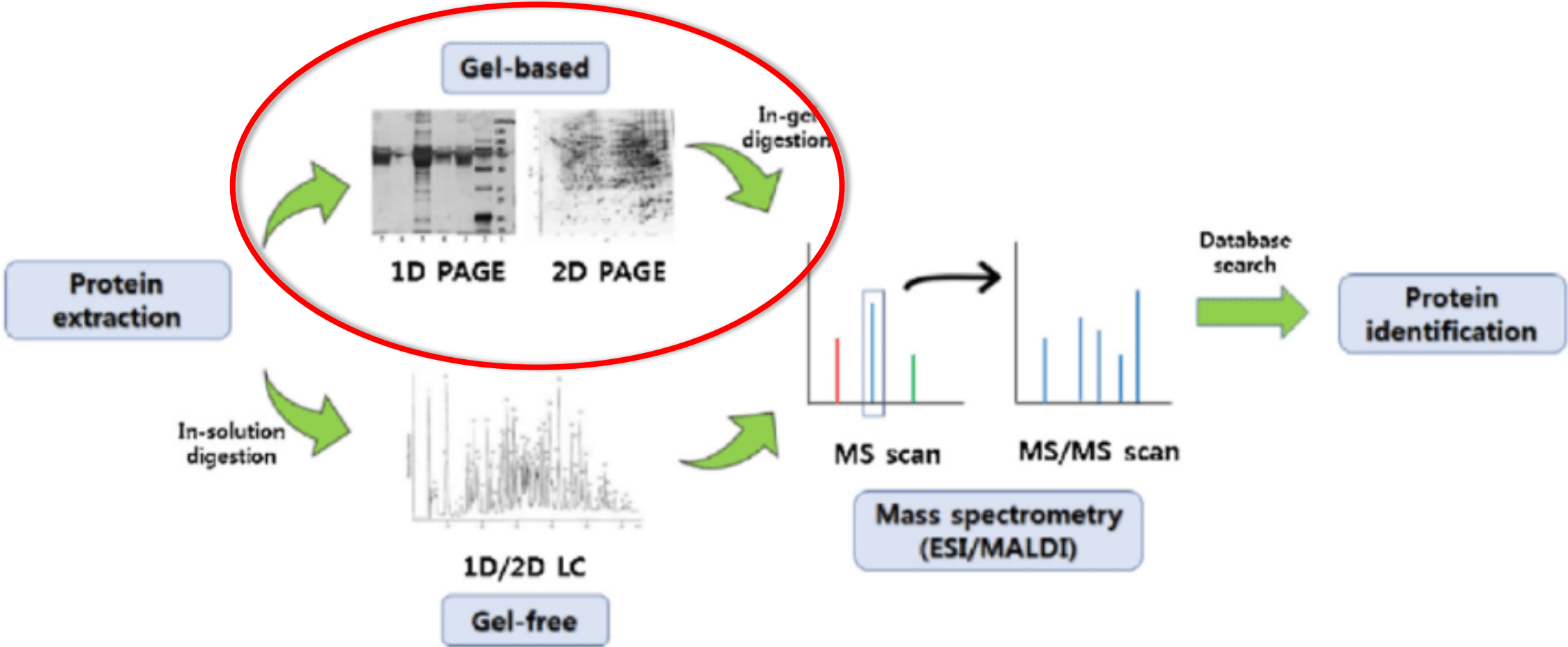
If I want to analyze by MS a complex mixture of intact proteins or protein fragments

I MUST FIRSTLY SEPARATE THEM

The MS must be coupled to a:

- chromatographic (“GEL-FREE”)
- electrophoretic (“GEL-BASED”)

THE TWO MAIN PROTEOMICS APPROCHES

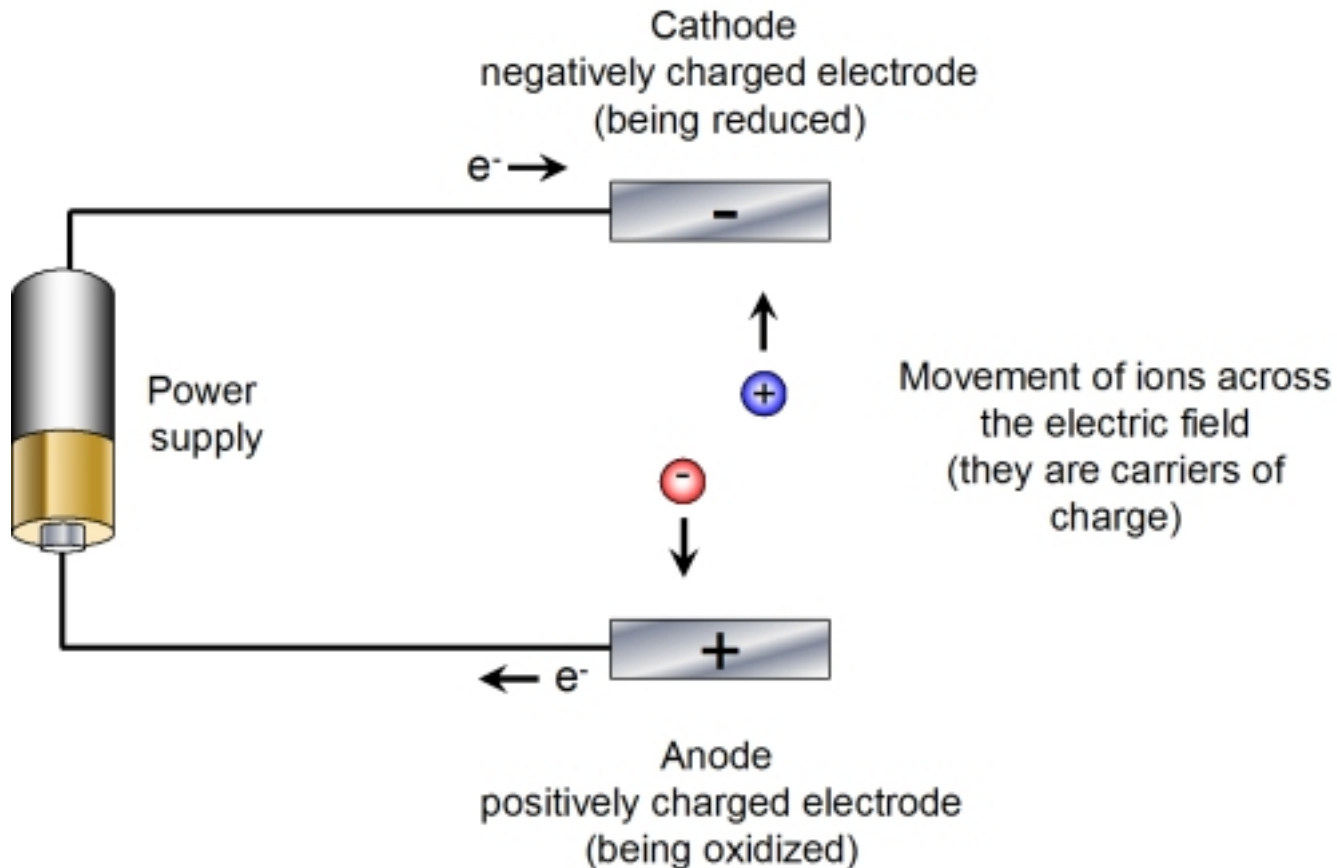




# Electrophoresis

Separation, analytical or preparative, of charged molecules by migration through a matrix due to application of an electric field, with net movement towards electrode of opposite charge

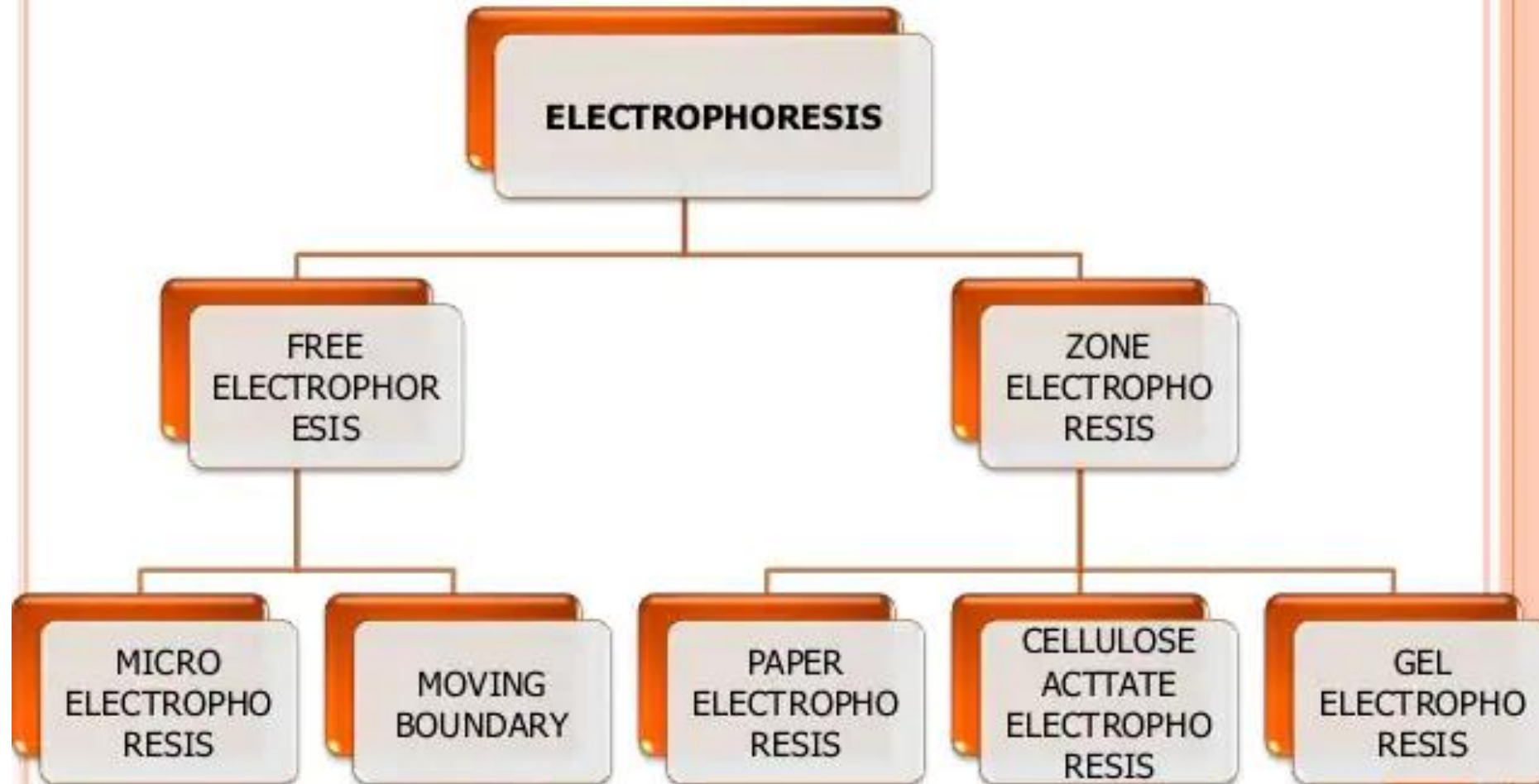
## General Principle



The term electrophoresis comes from a Greek word "Phoresis" which means "Being Carried Away". Hence literal meaning of the word electrophoresis means "to carry with electricity."



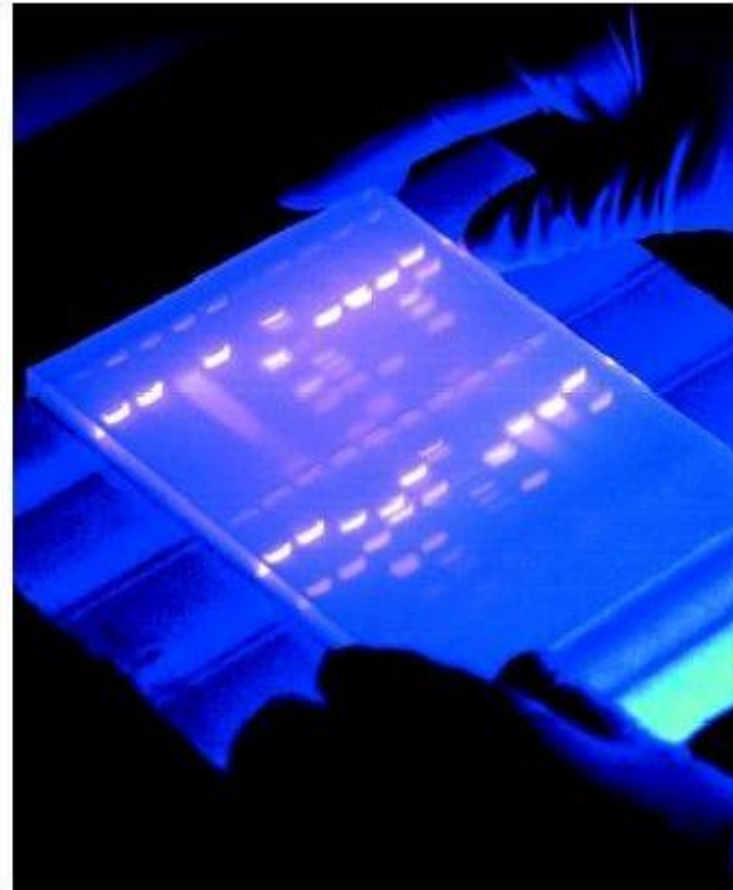
# TYPES OF ELECTROPHORESIS





# Zonal Electrophoresis

- Most common form of electrophoresis in biological studies
- Uses a support system, most commonly gel to separate proteins by their properties
- We will cover methods to separate by:
  - **Size (Through Frictional Properties)**
  - **Charge**
  - **Both**

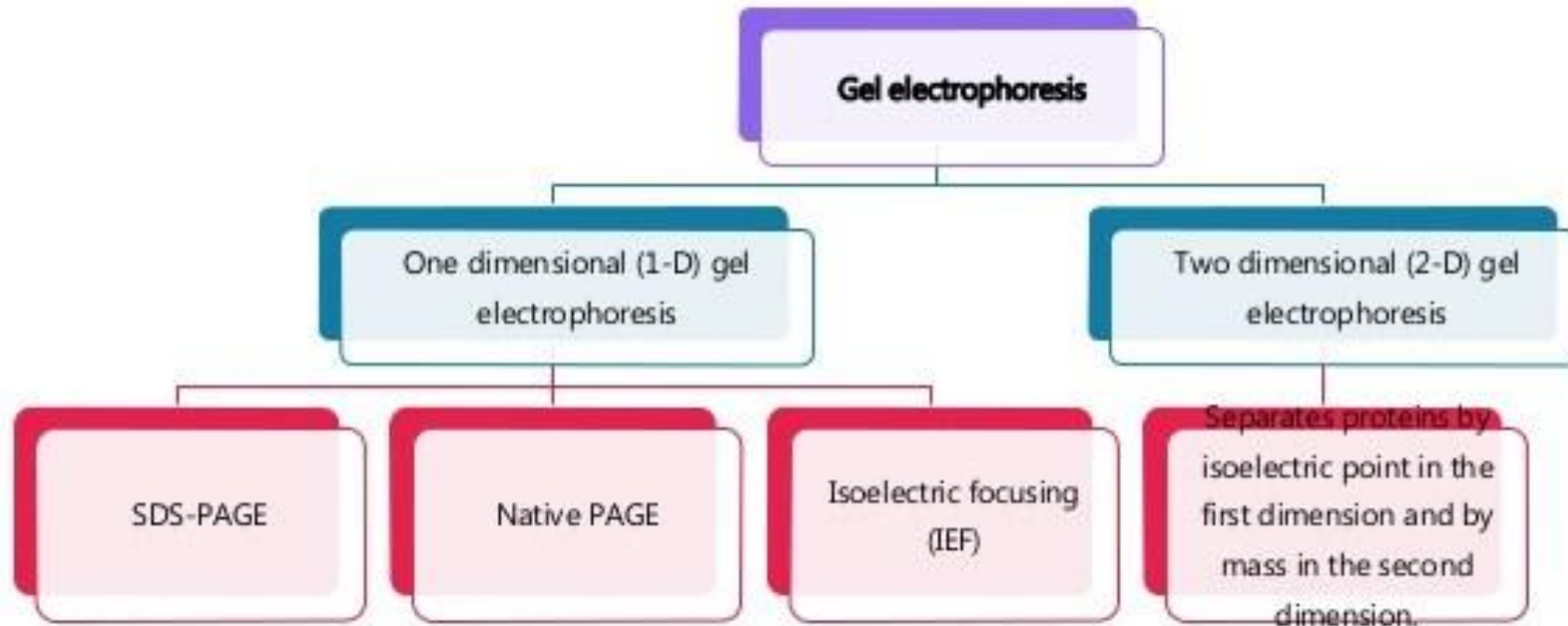


[http://www.biologyreference.com/images/biol\\_o2\\_img0140.jpg](http://www.biologyreference.com/images/biol_o2_img0140.jpg)

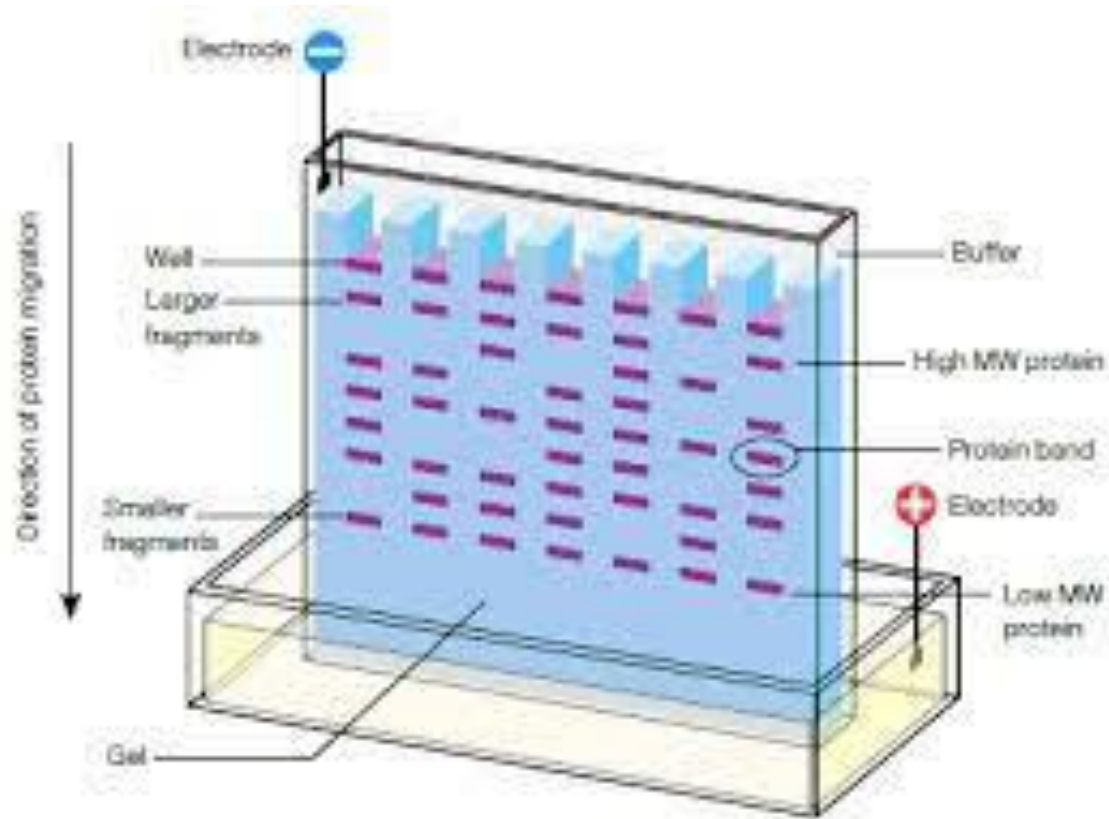


# GEL ELECTROPHORESIS

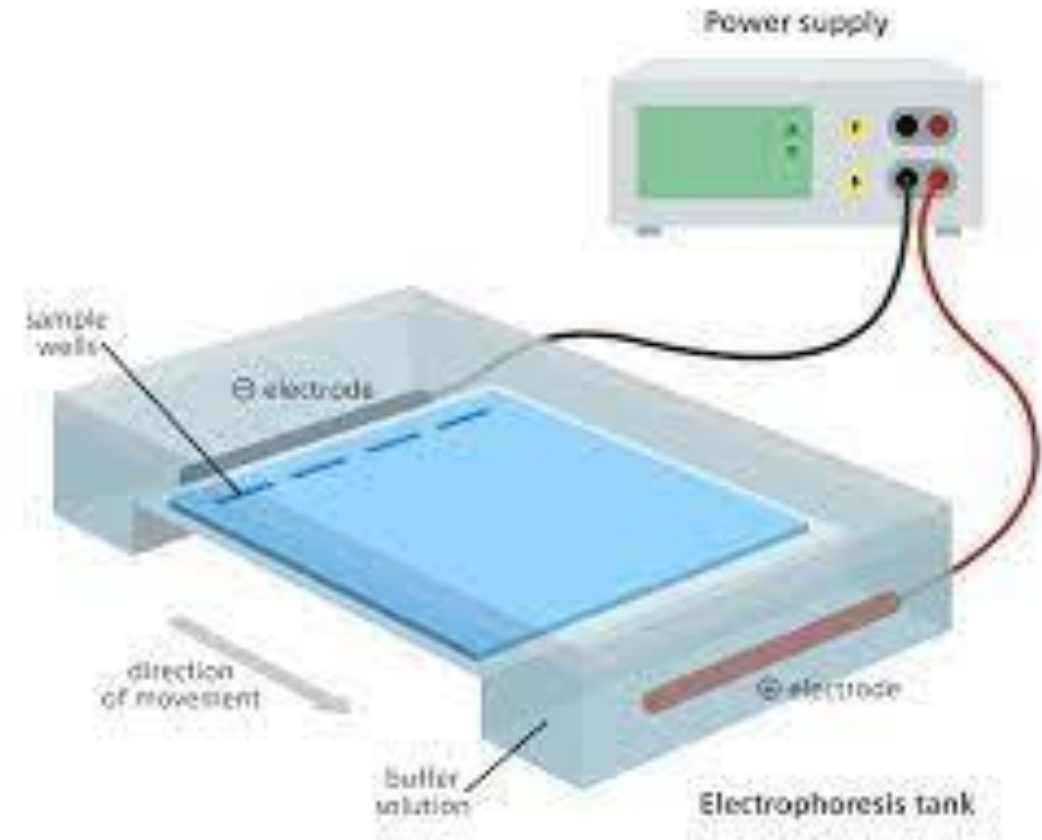
- It is a technique used for the separation of Deoxyribonucleic acid, Ribonucleic acid or protein molecules according to their size and electrical charge using an electric current applied to a gel matrix.
- **What is a gel?**
  - Gel is a cross linked polymer whose composition and porosity is chosen based on the specific weight and porosity of the target molecules.
- **Types of gels used:** Polyacrylamide gel, Agarose gel.
- **Types of gel electrophoresis:**



# Based on the Gel casting technique



**Vertical electrophoresis**  
(SDS-PAGE)

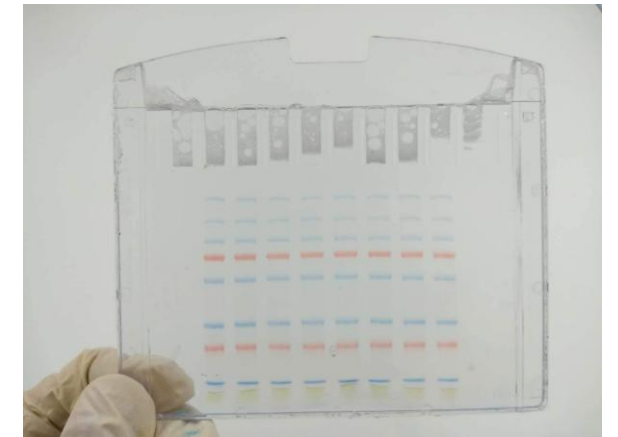
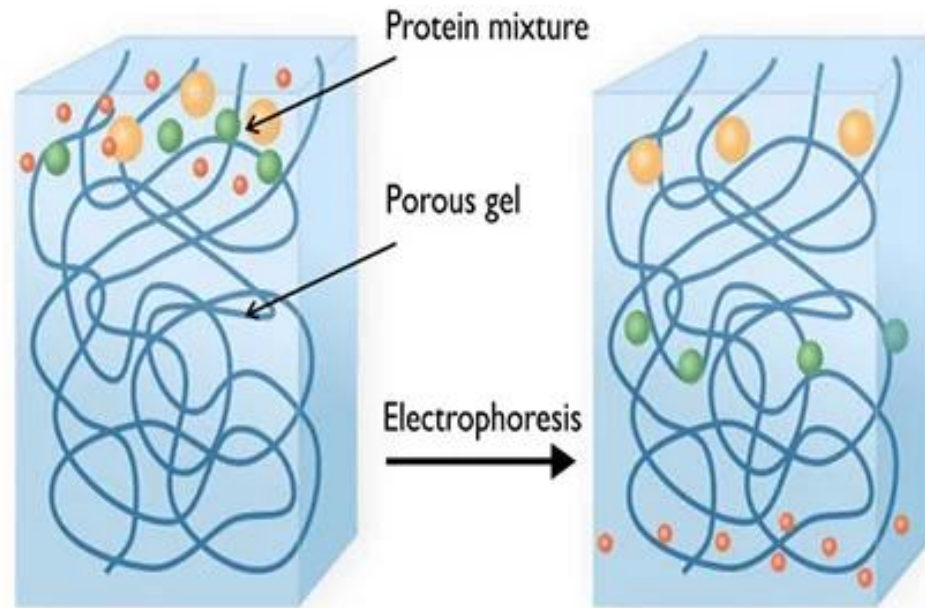
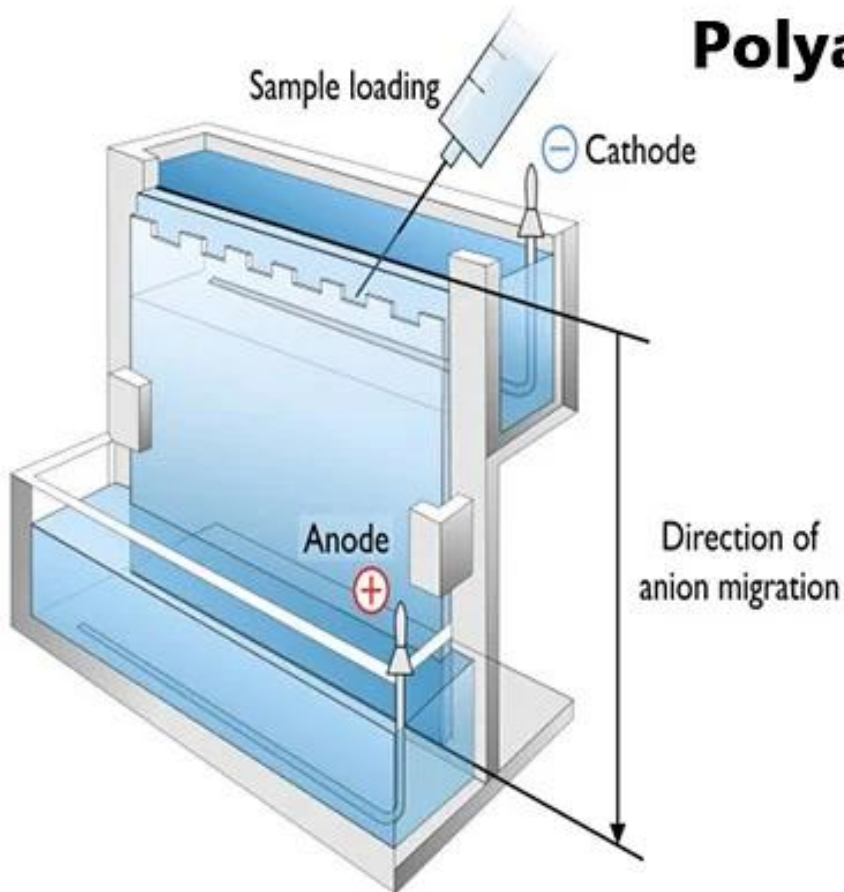


**Horizontal electrophoresis**  
(IEF, agarose)

# ZONAL ELECTROPHORESIS

- POLYACRYLAMIDE GEL ELECTROPHORESIS

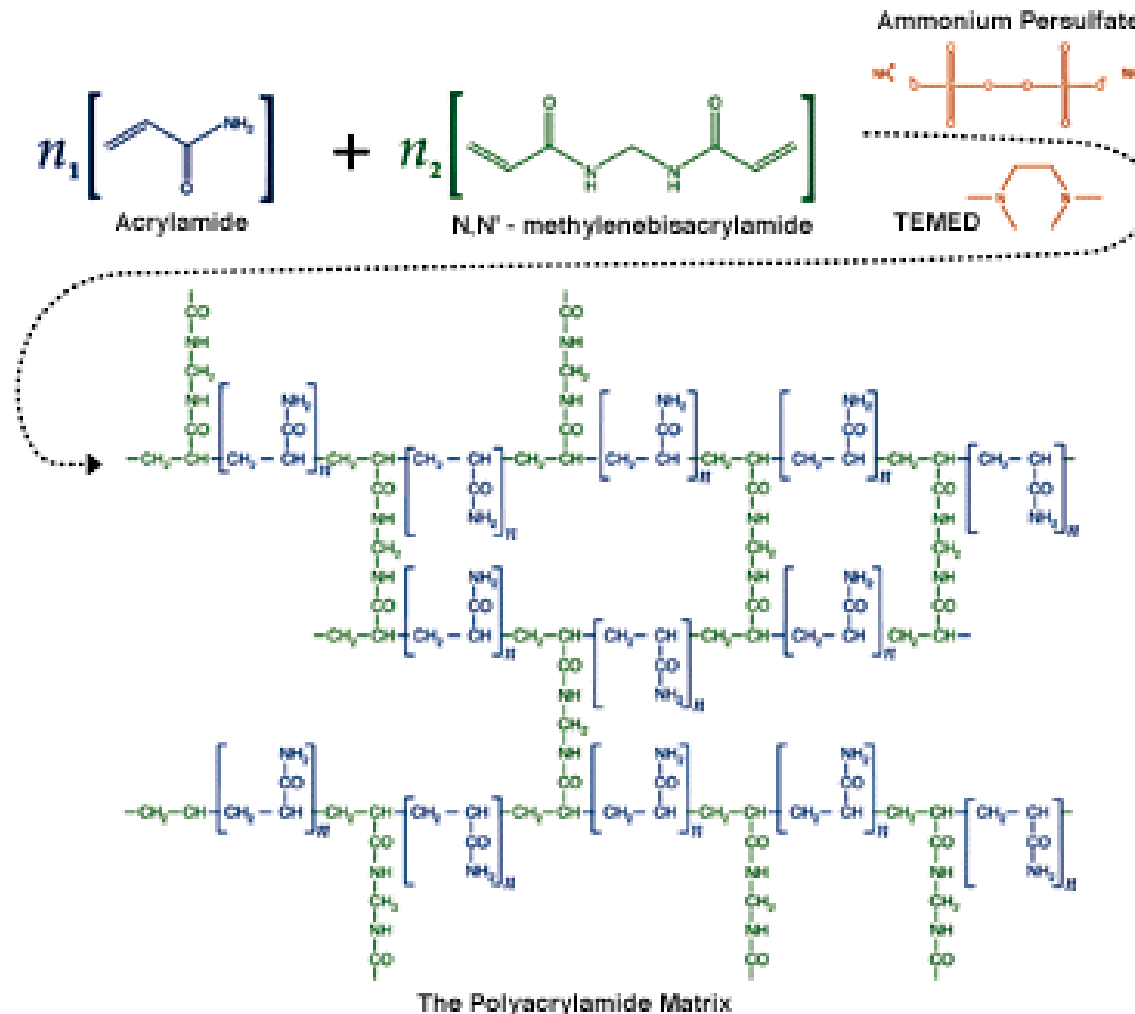
## Polyacrylamide Gel Electrophoresis (PAGE)





The gels typically consist of [acrylamide](#), [bisacrylamide](#), the optional [denaturant](#) (SDS or urea), and a [buffer](#) with an adjusted pH. A source of free radicals and a stabilizer, such as [ammonium persulfate](#) and [TEMED](#) are added to initiate polymerization. The polymerization reaction creates a gel because of the [bisacrylamide](#), which can form cross-links between two acrylamide molecules.

**Tetramethylethylenediamine (TEMED)** is an essential catalyst for polyacrylamide gel polymerization. TEMED is used with **ammonium persulfate (APS)** to catalyze acrylamide polymerization when preparing gels for electrophoresis.



radical polymerization.

Unreacted acrylamide (but not polyacrylamide!!) is a [neurotoxin](#) and [carcinogen](#).



Bigger protein have slowed down

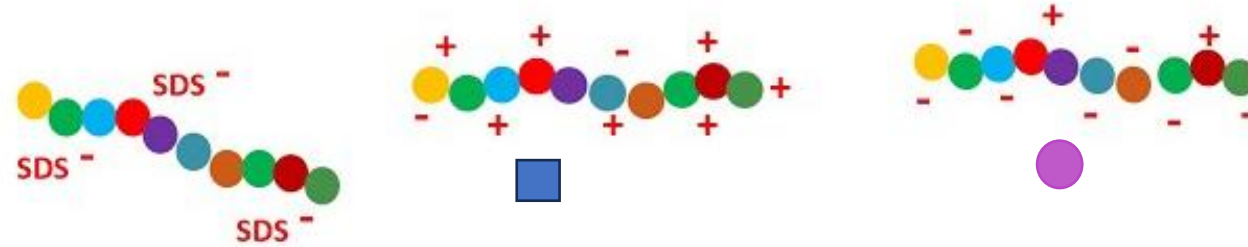
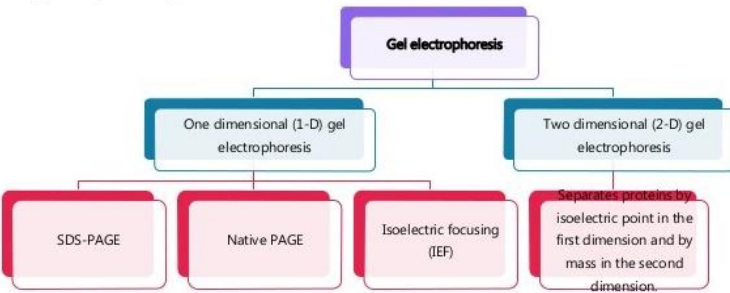
- ▶ Polyacrylamide gels are characterized by two parameters: total monomer concentration (%T, in g/100 ml) and weight percentage of cross linker (%C).
- ▶ **%T -the relative pore size of the resulting polyacrylamide gel**
- ▶ higher %T - smaller pores.
- ▶ The practical ranges for **monomer concentration** are stock solutions of 30-40%

Gel concentration		8%	10%	12%	15%
↓ % length of gel	10			170	170
	20	170	130	130	130
	30	130	100	100	100
	40	100	70	70	70
	50	70	55	55	55
	60	55	40	40	40
	70	40	35	35	35
	80	35	25	25	25
	90	25	15	15	15
	100	15	10	10	10

Migration pattern in PAGE

$$\%T = \frac{\text{Acrylamide (g)} + \text{Bisacrylamide (g)}}{\text{Volume (ml)}} \times 100$$

$$\%C = \frac{\text{Bisacrylamide (g)}}{\text{Acrylamide (g)} + \text{Bisacrylamide (g)}} \times 100$$

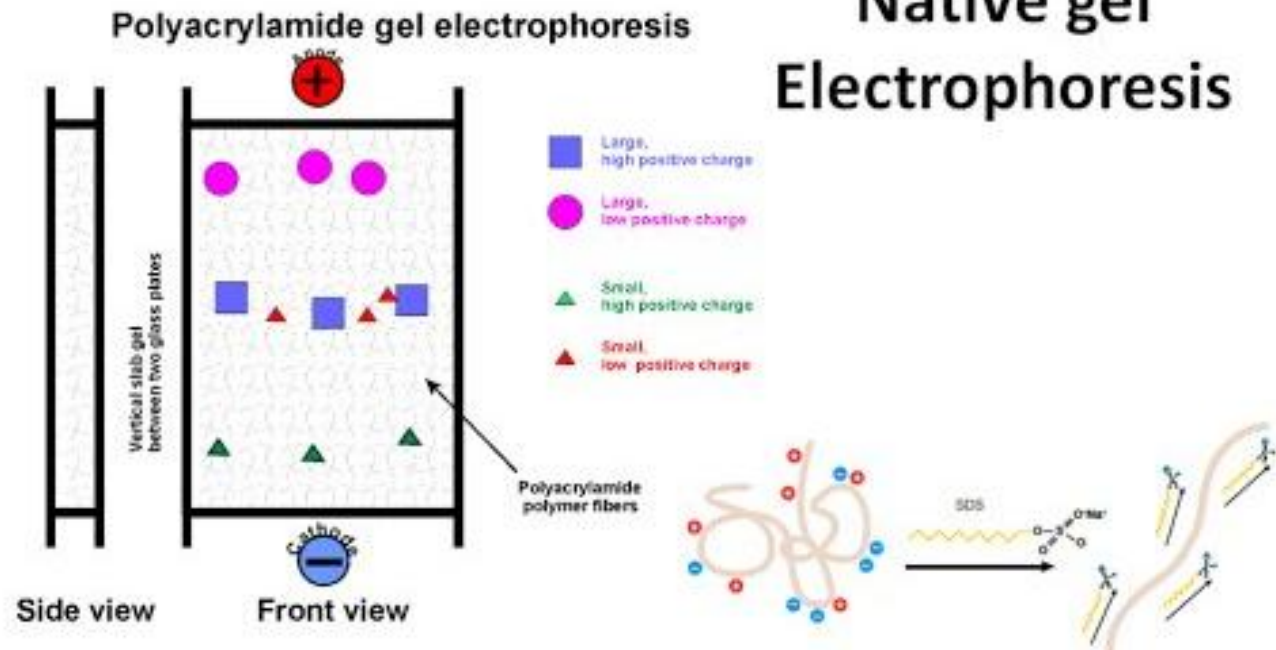


## SDS-PAGE vs Native PAGE

Standard use for protein separation based on size (MW)

Uses sodium dodecyl sulfate (SDS) to denature protein (disrupt 2D AND 3D structures) and provides negative charge for mobility

Without SDS, different proteins with similar molecular weights would migrate differently due to differences in charge/mass ratio and structure

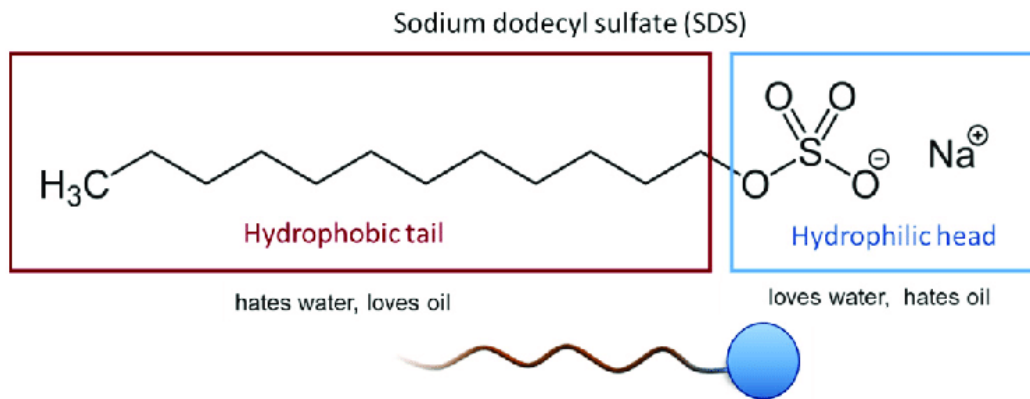


# Gel Electrophoresis-**SDS PAGE**



Standard use for protein separation based on size (MW)

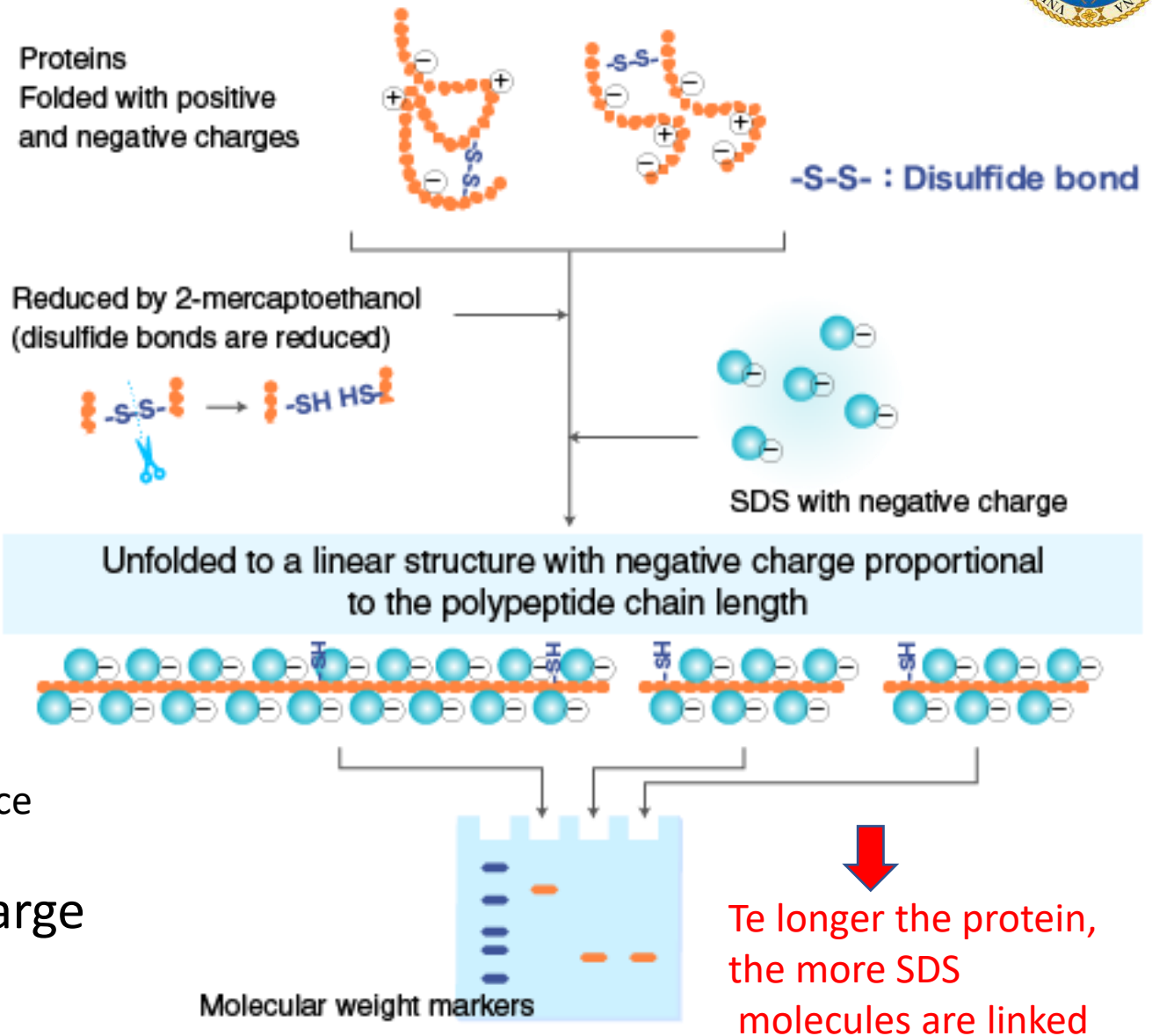
Uses sodium dodecyl sulfate (SDS) to denature protein (distrupt 2D AND 3D structures) and provides negative charge for mobility



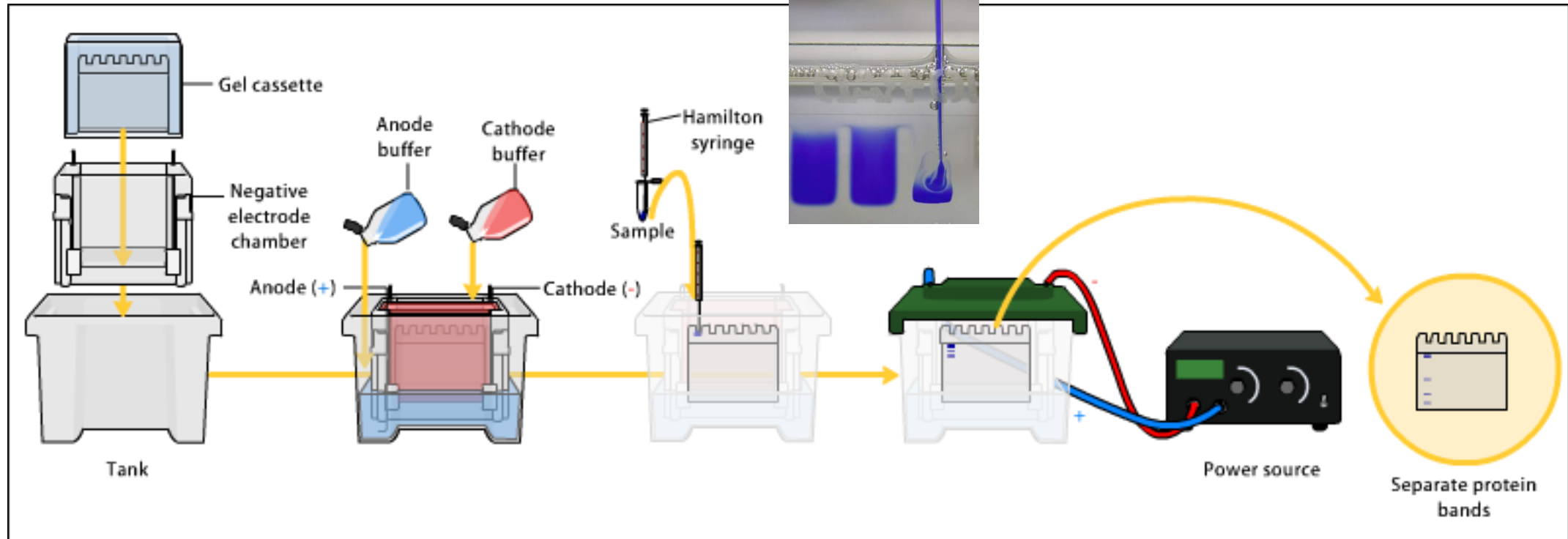
Interact very strong with the peptide backbone in a sequence independent constant ratio of about 1 SDS to 1.4 AA:

Protein denaturation and strong negative charge

Proteins acquire the same charge/mass ratio







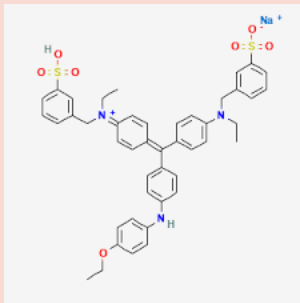
## How to Run an SDS-PAGE gel

<https://www.youtube.com/watch?v=XUjLO-ek2C8>

# Staining Polyacrylamide Gels

**Coomassie Blue Stain-** can usually detect a 10-50 ng protein per band

- Requires an acidic water/alcohol fixing solution
- Binds preferentially tyrosins
- Compatible with MS
- Requires destaining to remove aspecific binding of the dye from the gel



**Blue Safe Stains** -Similar to Coomassie. Destaining optional or water rinse. (8 ng/band)

- BioSafe Blue
- SimplyBlue
- InstantBlue-destaining not recommended

**Silver Staining-** 50 times more sensitive than Coomassie Blue. (0.3ng/BAND)

- Fixation [Acetic acid-methanol]
- Stain with silver solution and rinse with water
- Develop with formaldehyde and carbonate followed by stopping with Glacial acetic
- Not compatible whit MS

**Fluorescent Stains** –almost as sensitive as Silver but requires excitation source

- Flamingo Fluorescent Gel Stain
- Deep Purple\* Total Protein Stain
- SYPRO\* Ruby Protein Gel Stain
- Krypton Protein Stain
- IR stains
- Stain-free: allow immediate visualization of proteins without the time, mess, and cost associated with traditional gel staining and destaining.



# Destaining Gels

Most gels require destaining to see banding and to eliminate background stain for high resolution.

Gels with abundant protein need not be destained when using certain SafeBlue stains such as InstantBlue

## Coomassie blue destaining

Usually requires acetic acid , methanol, and water

## Safe Blue destaining

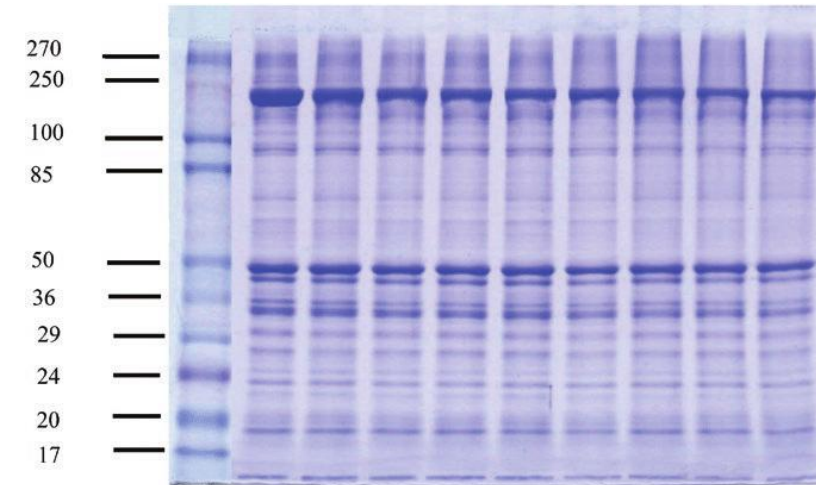
Usually requires water rinse

## Silver Stains

Some methods use Potassium Ferricyanide -Sodium Thiosulfate solutions

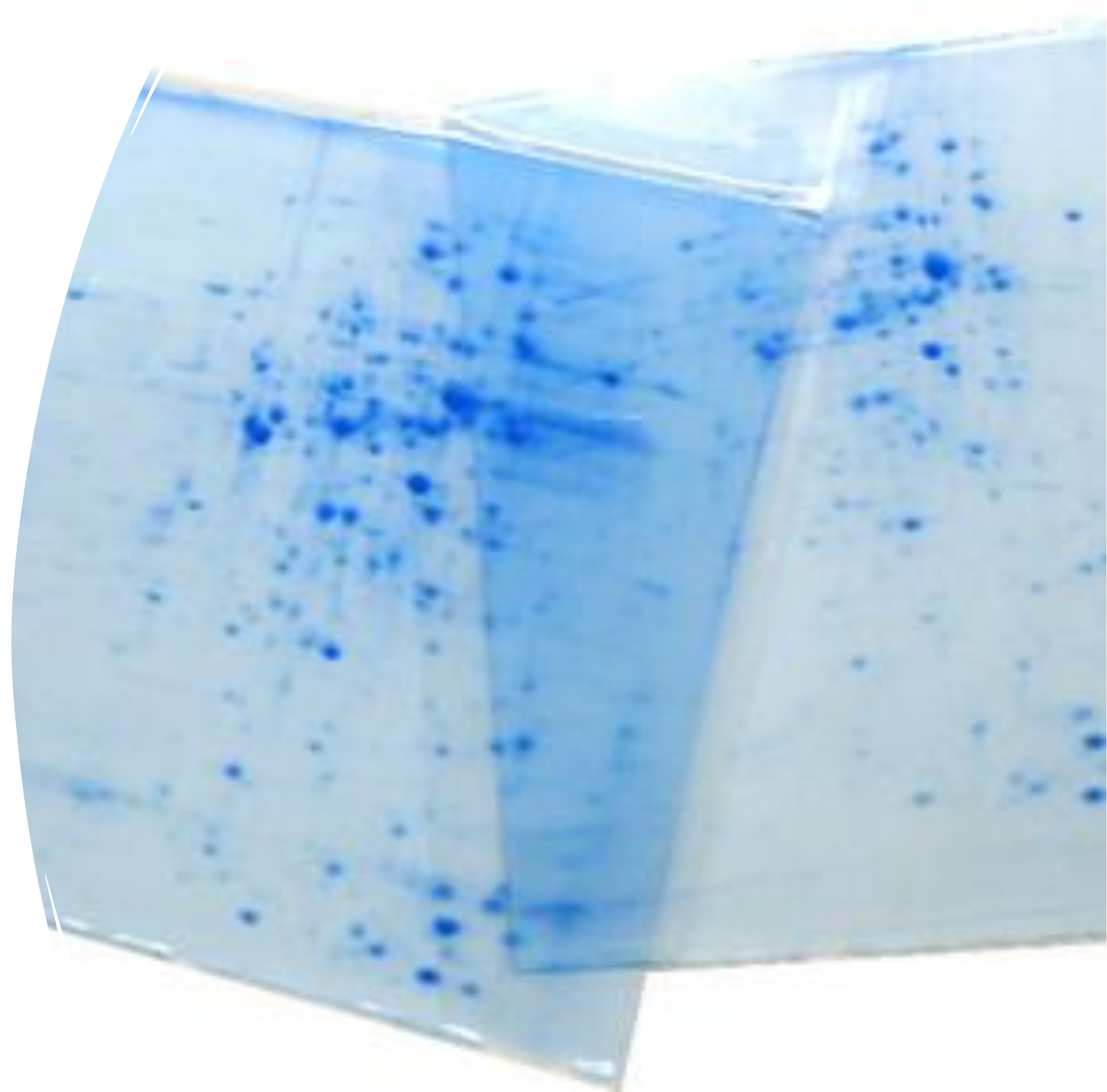
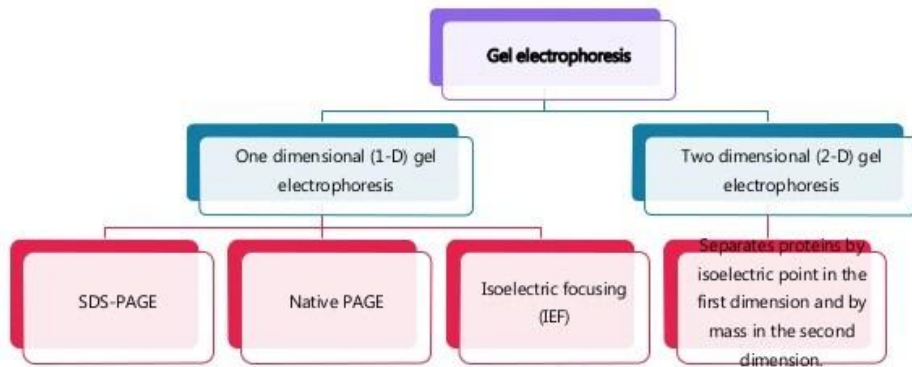
Some methods use Sodium chloride -Cupric sulfate -Sodium thiosulfate pentahydrate.

Some destaining may require a stop solution including 10% Acetic acid



# 2D Gel Electrophoresis

- Separation of proteins based on
  - -pI (ISOELECTRIC FOCUSING)
  - -MW (SDS)
- Up to 10,000 proteins can be seen using optimized protocols



# Why 2D Gels?

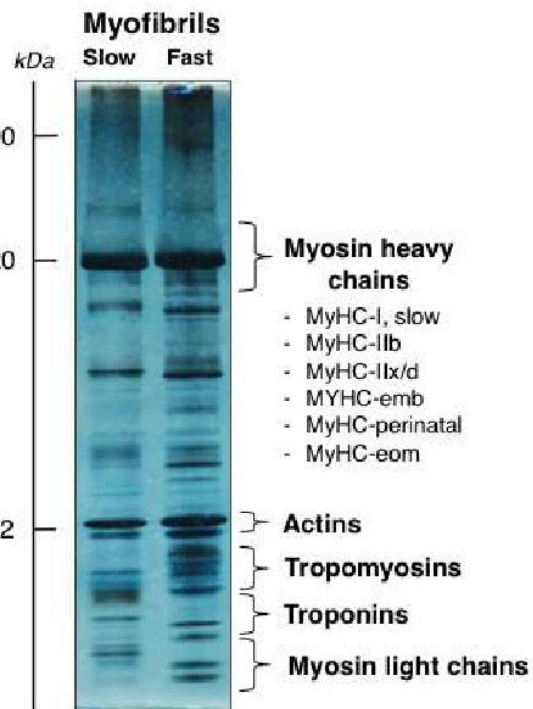
Method for protein display and do proteomics- one spot at a time

Can be used in conjunction with Mass Spec

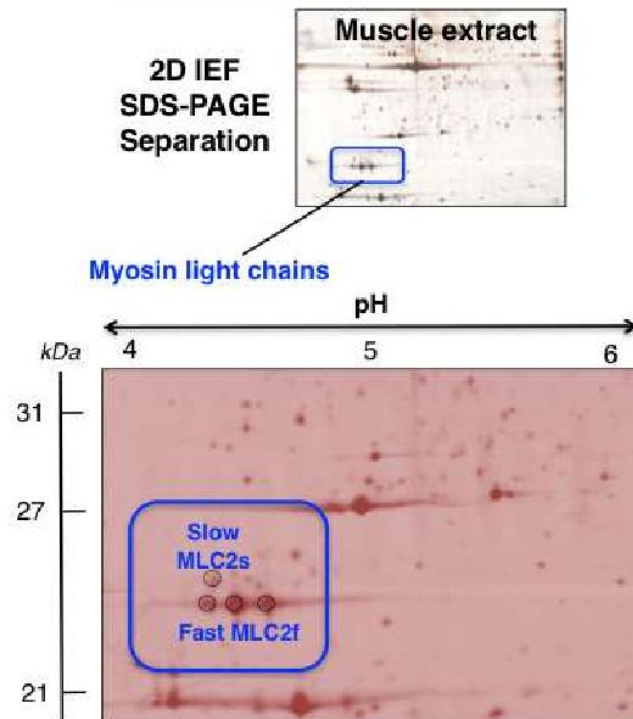
Permits simultaneous detection, display, purification, identification, quantification, pI, and MW.

Robust, reproducible, simple, cost effective, scalable

## 1D Gel Electrophoresis



## 2D Gel Electrophoresis







# Isoelectric Focusing (IEF):

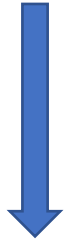
## "First Dimension" in 2-D gel

Proteins separate according to their isoelectric point (pI)

SUPPORT: Utilizes a special **pH gradient gels/strips**

**high voltage** electrophoretic equipment

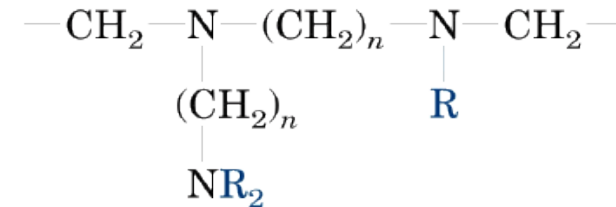
Variable pH **Ampholytes** embedded in Acrylamide



**Ampholytes** are compounds that when dissolved in water (which is itself an amphoteric compound) can act either as acid or as a base.



*IEF apparatus from Bio-Rad*



$$n = 2 \text{ or } 3$$

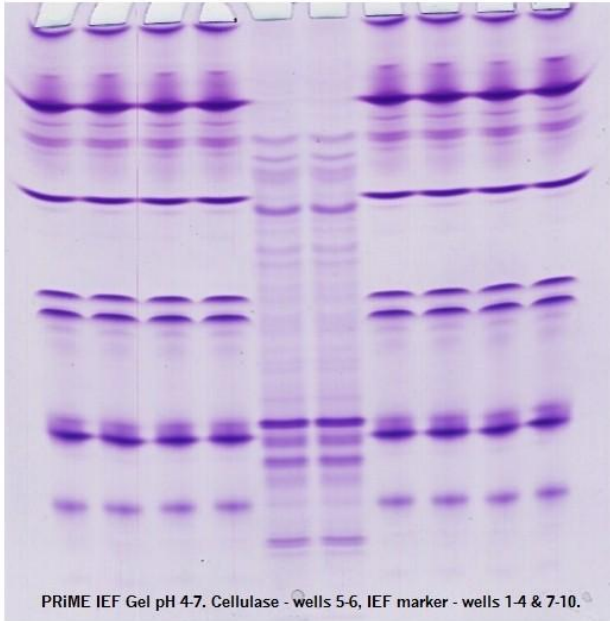
$$\text{R} = \text{H or } \text{---(CH}_2\text{)}_n\text{---COOH}$$

General formula of the ampholytes used in isoelectric focusing

# Two types of support:

**Gel:** separates many samples

**STRIP:** separates one sample for each strip



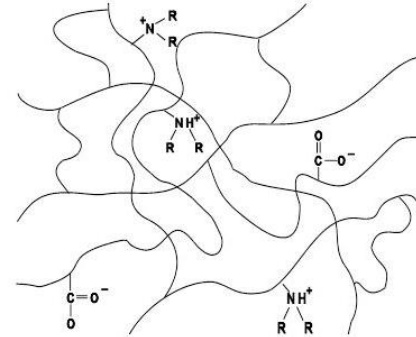
# IEF and IPG (immobilized pH Gradient)

Strip of PA made by covalently integrating acrylamide and variable pH ampholytes

Available in different pH ranges

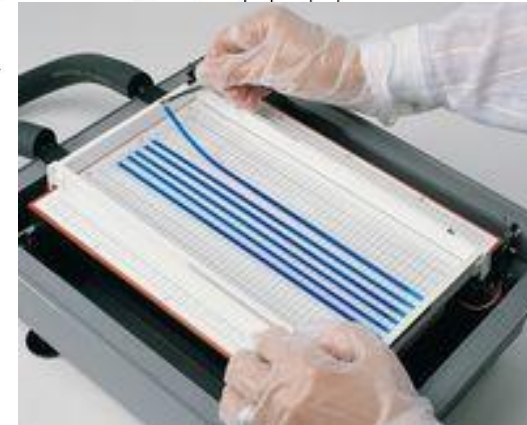
- 3-10
- 4-8
- 5-7

## Immobilized pH gradients (IPG)



Acrylamido buffers: Immobiline®  
 $\text{CH}_2=\text{CH}-\text{CO}-\text{NH}-\text{R}$ ,  
 R contains a carboxylic or a tertiary amino group

**Immobiline Gels**  
 (0.5 mm gel layers on film supports)

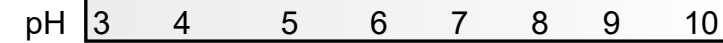


Guidelines for choosing Immobiline DryStrip gels

pH range	Strip length				
	24 cm	18 cm	13 cm	11 cm	7 cm
3-5.6 NL	•	•	•	•	•
5.3-6.5	•	•	•	•	•
6.2-7.5	•	•	•	•	•
7-11 NL	•	•	•	•	•
3-11 NL	•	•	•	•	•
3.5-4.5	•	•	•	•	•
4.0-5.0	•	•	•	•	•
4.5-5.5	•	•	•	•	•
5.0-6.0	•	•	•	•	•
5.5-6.7	•	•	•	•	•
3-7 NL	•	•	•	•	•
4-7	•	•	•	•	•
6-9	•	•	•	•	•
6-11	•	•	•	•	•
3-10	•	•	•	•	•
3-10 NL	•	•	•	•	•

Wide pH range

Narrow pH range





Sample Preparation



First-Dimension IEF



Second-Dimension SDS-PAGE



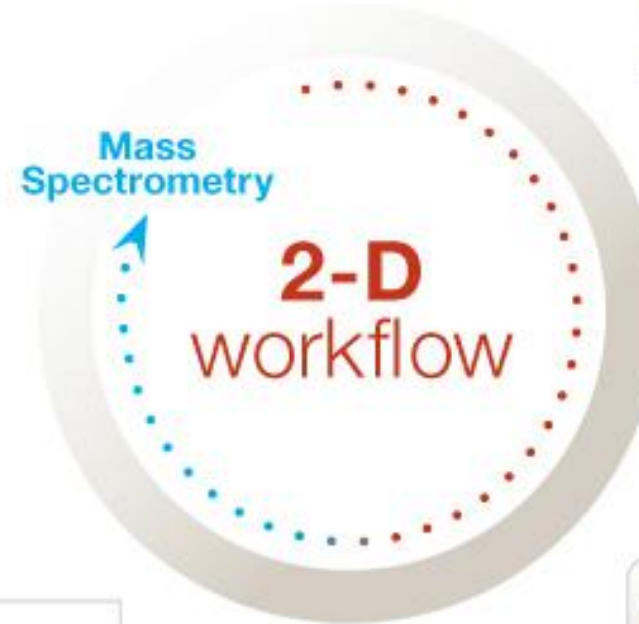
Gel Staining



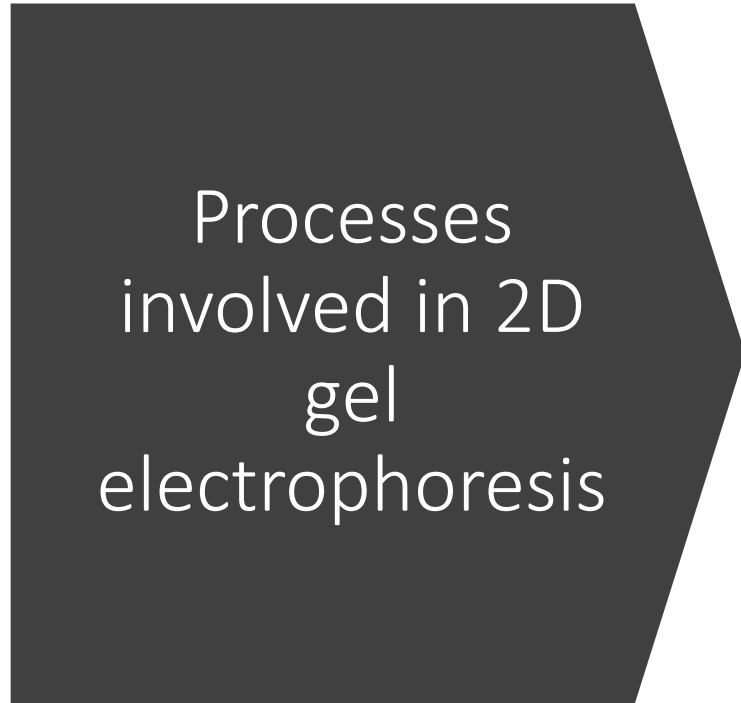
Imaging and Analysis



Blotting



Processes  
involved in 2D  
gel  
electrophoresis



# Sample Preparation



Sample preparation is key to successful 2D gel experiments

Must select appropriate method to get selected proteins from cellular compartment of interest

Membrane proteins, nuclear proteins, and mitochondrial proteins require special steps

Must break all non-covalent protein-protein, protein-DNA, protein-lipid interactions, disrupt S-S bonds

Must prevent proteolysis, accidental phosphorylation, oxidation, cleavage, ect..

Must remove substances that might interfere with separation process such as salts, polar detergents (SDS), lipids, polysaccharides, nucleic acids

Must try to keep proteins soluble during both phases of electrophoresis process

Must be compatible with quantification methods of protein



# The key step of sample preparation is the Protein Solubilization:

## 2D electrophoresis rehydration buffer components

protein sample

2-20 mM Tris base (Carrier ampholytic buffer)

5-20 mM DTT (to reduce disulfide bonds)

8 M Urea (neutral chaotrope)

- Increases the solubility of some proteins
- Chaotropic agents interfere with stabilizing non-covalent forces (hydrogen bonds, van der Waals forces, and hydrophobic)

4% CHAPS Detergent (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate)

- pH of 5-7
- Zwitterionic detergent (electronically neutral-has a both Neg and Pos useful for variable charged peptides )
- Protects the native state of proteins
- no affects on pH gradients

Bromophenol blue

## Rehydration steps with a IPG STRIP

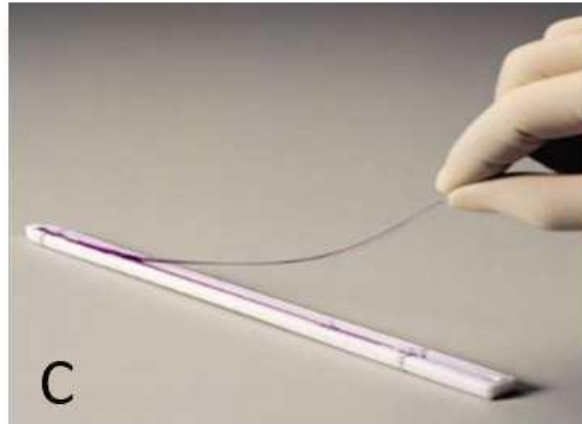


Peel-off the IPG strip

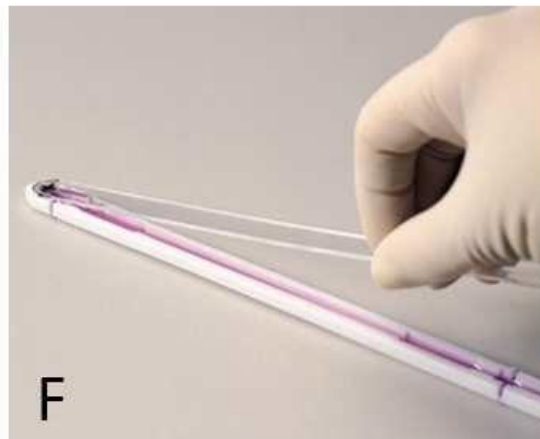


Apply rehydration solution into the tray

Lay strip gel side facing down



Apply oil



Place cover

Rehydration lasts all night

## IEF APPARATUS

IEF system: a fully integrated isoelectric focusing system optimized to deliver speed and reliability in the first dimension of 2-D electrophoresis



**2D Gel Electrophoresis 1st Dimension**

<https://www.youtube.com/watch?v=mkMPx49QZtw>

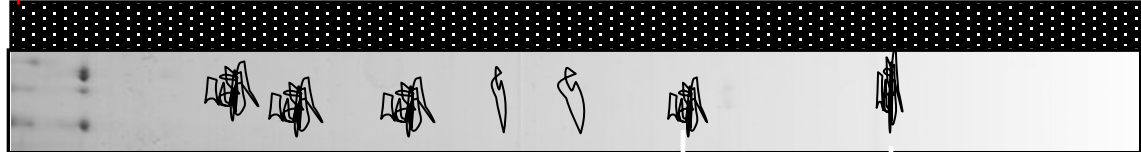




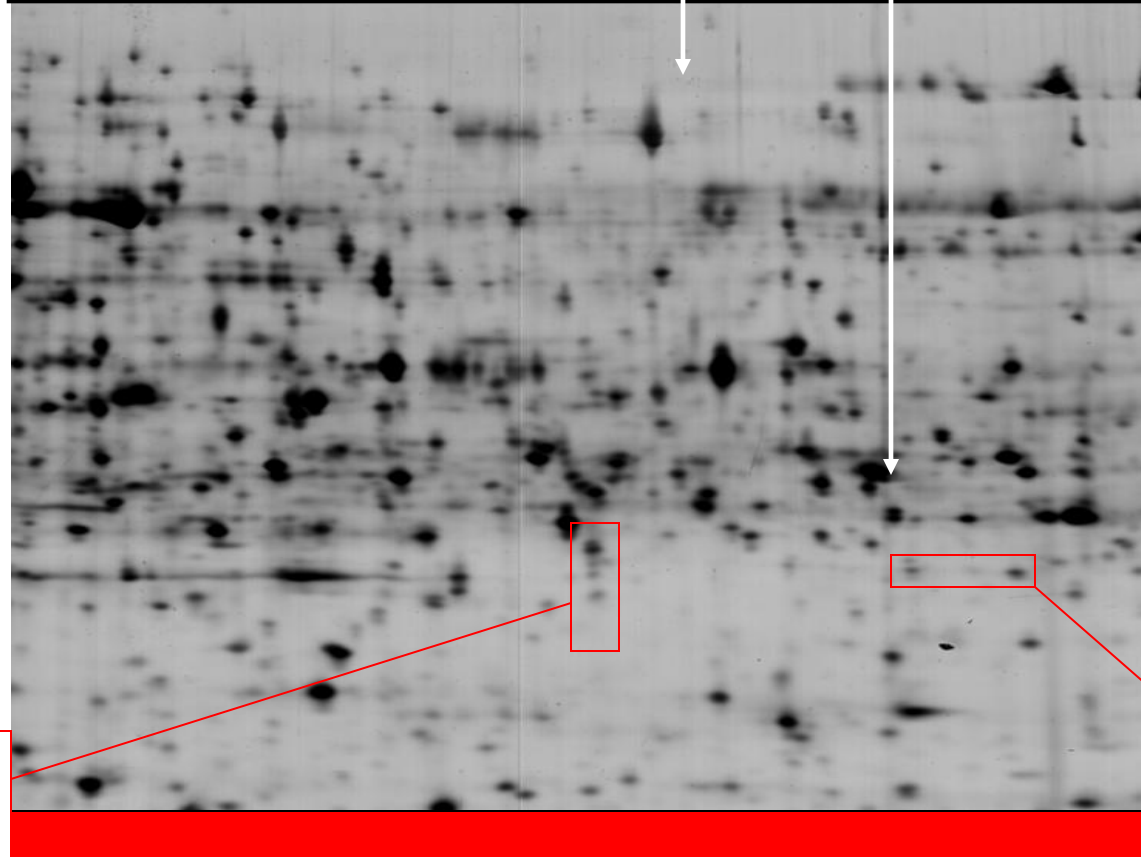
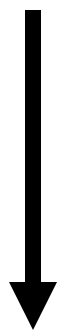
# SDS Gel

Negative electrode

pH 3 4 5 6 7 8 9 10



IPG strip-pressed down into the SDS-PAGE gel



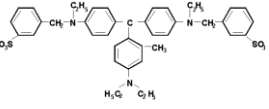
Positive electrode

Similar pI but different mw

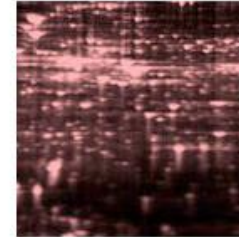
Similar mw but different pI

# Gel Stains - Summary

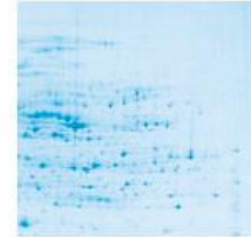


<u>Stain</u>	<u>Sensitivity (ng/spot)</u>	<u>Advantages</u>	
<b>Coomassie-type</b>	 <chem>Cc1ccc(cc1C2=CC=CC=C2N(C)C)c3ccc(cc3N(C)C)c4ccc(cc4)S(=O)(=O)c5ccc(cc5)N(C)C</chem>	5-10	Simple, fast
Silver stain	1-4	Very sensitive, laborious	
Copper stain	5-15	Reversible, 1 reagent negative stain	
Zinc stain	5-15	Reversible, simple, fast high contrast neg. stain	
<b>SYPRO ruby</b>	1-10	Very sensitive, <b>fluorescent</b>	

Flamingo



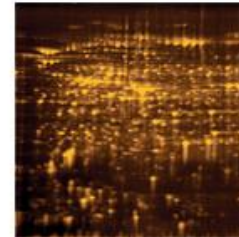
Coomassie Blue



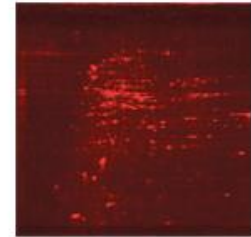
Silver



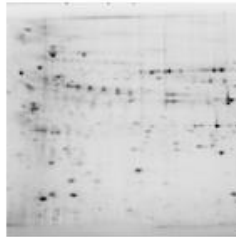
Oriole



SYPRO Ruby



Stain-free



## 2-D Video Tutorial

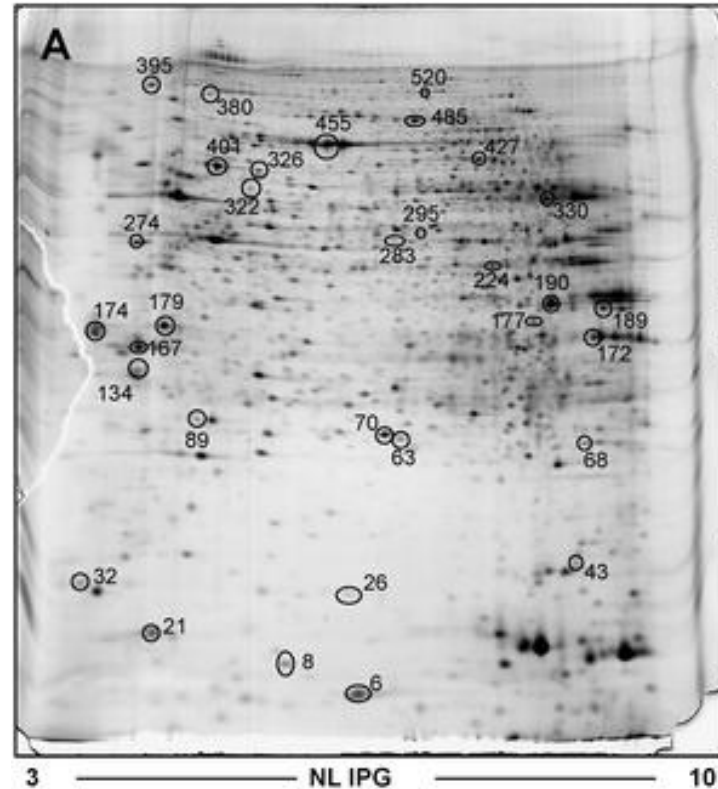
How to run a 2-D Gel from start to finish.

<https://www.youtube.com/watch?v=3wFh0z7so8w>

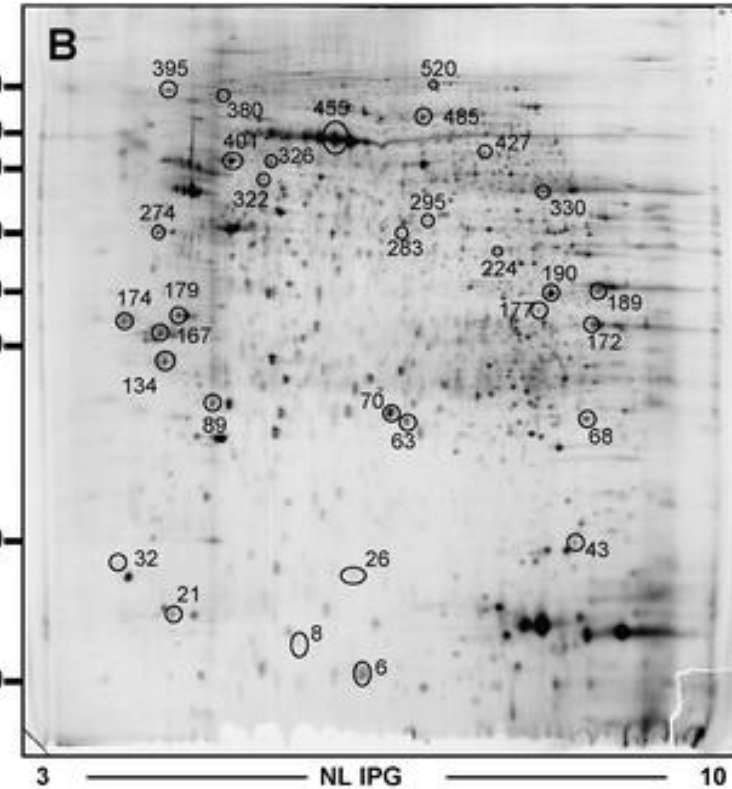
# 2D Gel Results

- On a typical 2D gel there are 2000 spots
- if a single gene is changed between two cell lines about 20 spots will be different

Healty condition



Disease condition



Marked spots refer to proteins with varied level between healty and disease



## FROM THE SPOT TO THE PROTEIN IDENTIFICATION:

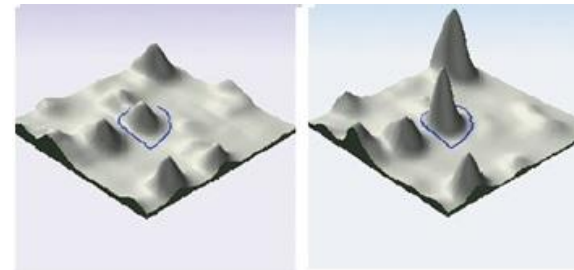
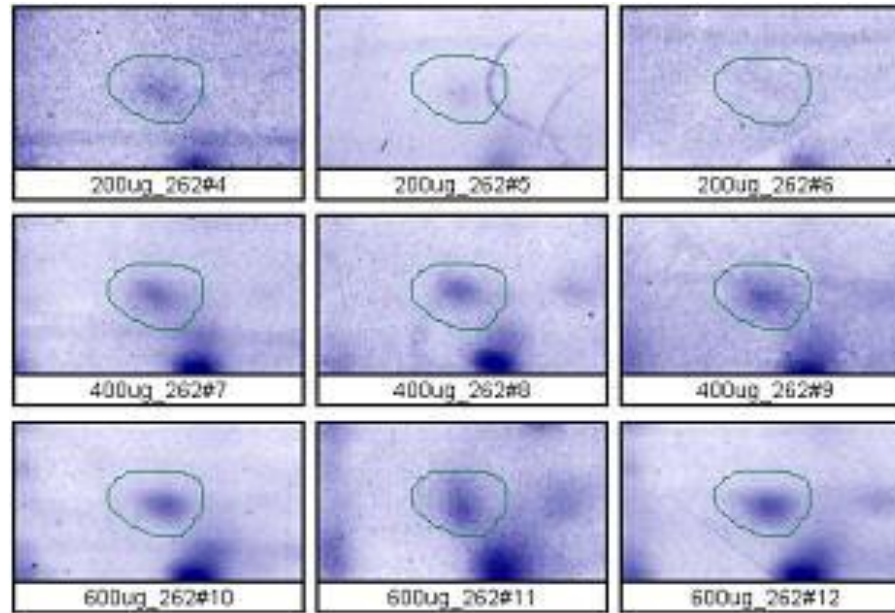
- 1) Differentially expressed proteins, identified by image analysis software, can be marked on a printed image
- 2) Spots can then be manually cut from the gel.
- 3) Identification of proteins is typically accomplished by in-gel digestion of protein spots
- 4) followed by mass spectrometric analysis of extracted peptides.

# 2D Gel Post Analysis

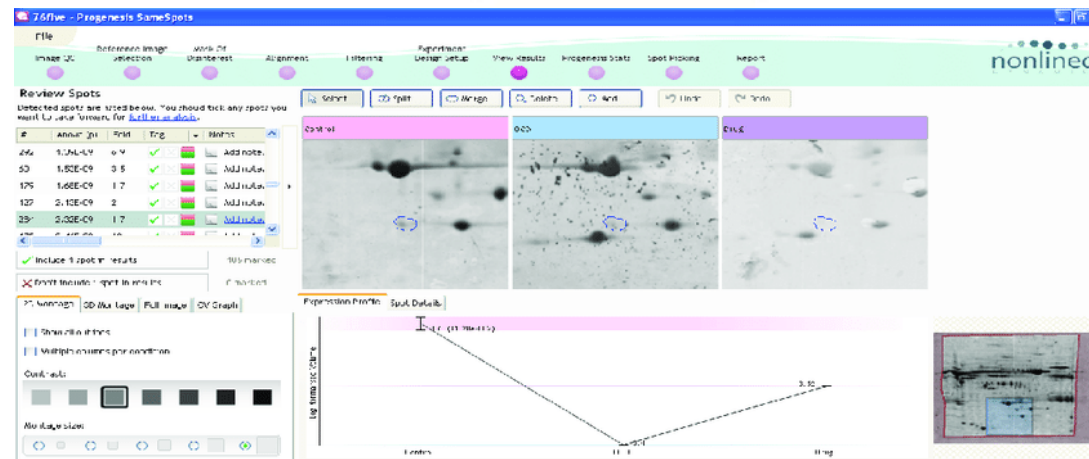
Which spots show a difference between gels??

- Gel images need to be digitized for further analysis
- Compare gel images and determine what bands/spots are different
- Requires software to compare gels: they compare pixel by pixel

Apparent difference-  
need to extract spot for  
MS...



scanner



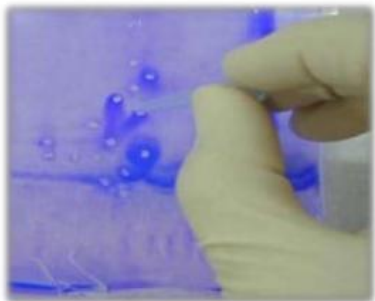
SameSpots™ v5 | 2D Gel  
Image Analysis Software

# In-gel digestion: Extracting a Gel Spot (1)

Identification of proteins is typically accomplished by in-gel digestion of protein spots

## Cut out spots

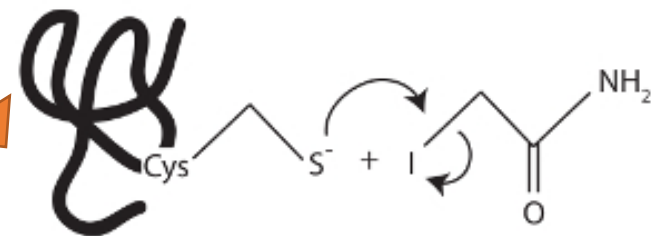
- 1** • Spot picking (gel excision) – manual or mechanical?



dehydrated

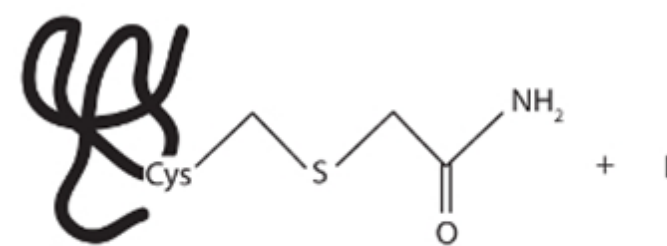
**1**  
Destain  
Gel Slices

200µL Destain Soln.  
2 x 30 minutes at 37°C



Protein with sulfhydryl group

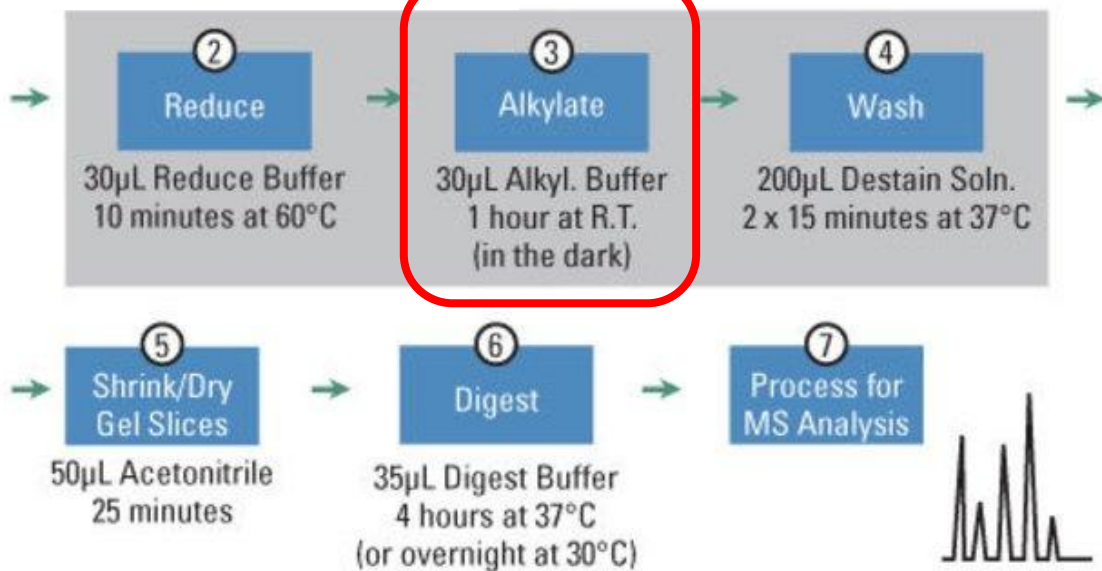
Iodoacetamide



Protein with protected sulfhydryl group

Iod

## 2 Trypsin Digestion of Gel spots

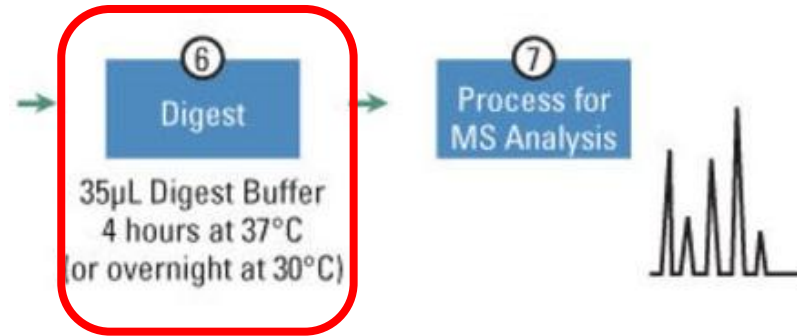


# In-gel digestion: Extracting a Gel Spot (2)

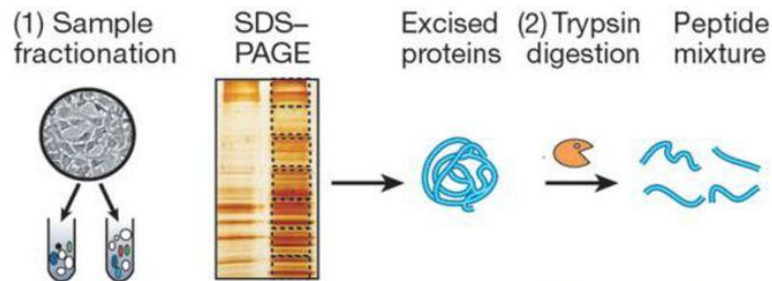
Identification of proteins is typically accomplished by in-gel digestion of protein spots

## Why trypsin?

- It is cheap and easy to isolate
- It has a high specificity which is easy to predict
- It is a robust enzyme
- It works over a range of concentrations and conditions
- It works very well on denatured proteins
- It generates peptides of a good length for mass spec analyses
- All tryptic peptides contain at least an Arg or Lys with their positive charge



## Proteomics workflow: (2) trypsin digestion



Therefore, in stage 6 proteins are **degraded enzymatically** to peptides, usually by trypsin.

This yields peptides with C-terminally protonated amino acids (K/R) which is beneficial in subsequent peptide sequencing.

Table 1. Distribution of peptide fragment length from 20,639 proteins

Enzyme/reagent	Residues cleaved	Total fragments	Avg. fragment length
Trypsin	K/R	662,981	8
Lys-C	K	359,140	16
Asp-N	D	321,655	18
CNBr	M	150,605	38
Hydroxylamine	N-G	36,643	152
Dilute acid	D-P	35,574	166