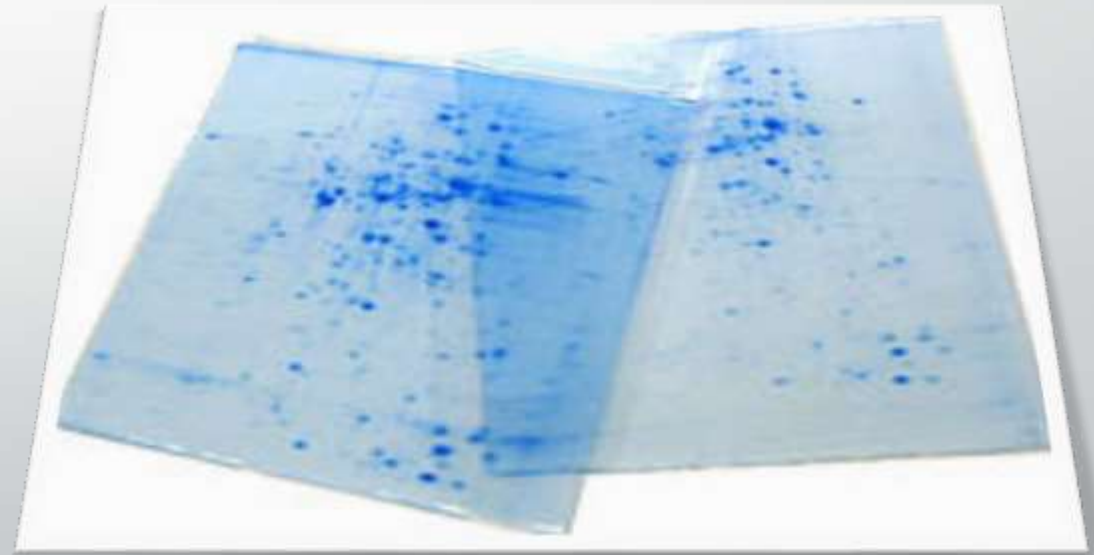


2-D gel Electrophoresis

Presented by saira sana



Introduction

- **Two-dimensional gel electrophoresis**, abbreviated as **2-DE** or **2-D electrophoresis**, is a form of gel electrophoresis commonly used to analyze or separate protein.
- 2-DE was first independently introduced by O'Farrell and Klose 1975



Prof. Dr. P. H. O'Farrell, Walter Sarstedt, Prof. Dr. Dr. J. Klose

Principle

- This technique separates proteins according to two independent properties in two discrete steps.
- **The first-dimension** : isoelectric focusing (IEF)
- **The second-dimension** : sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

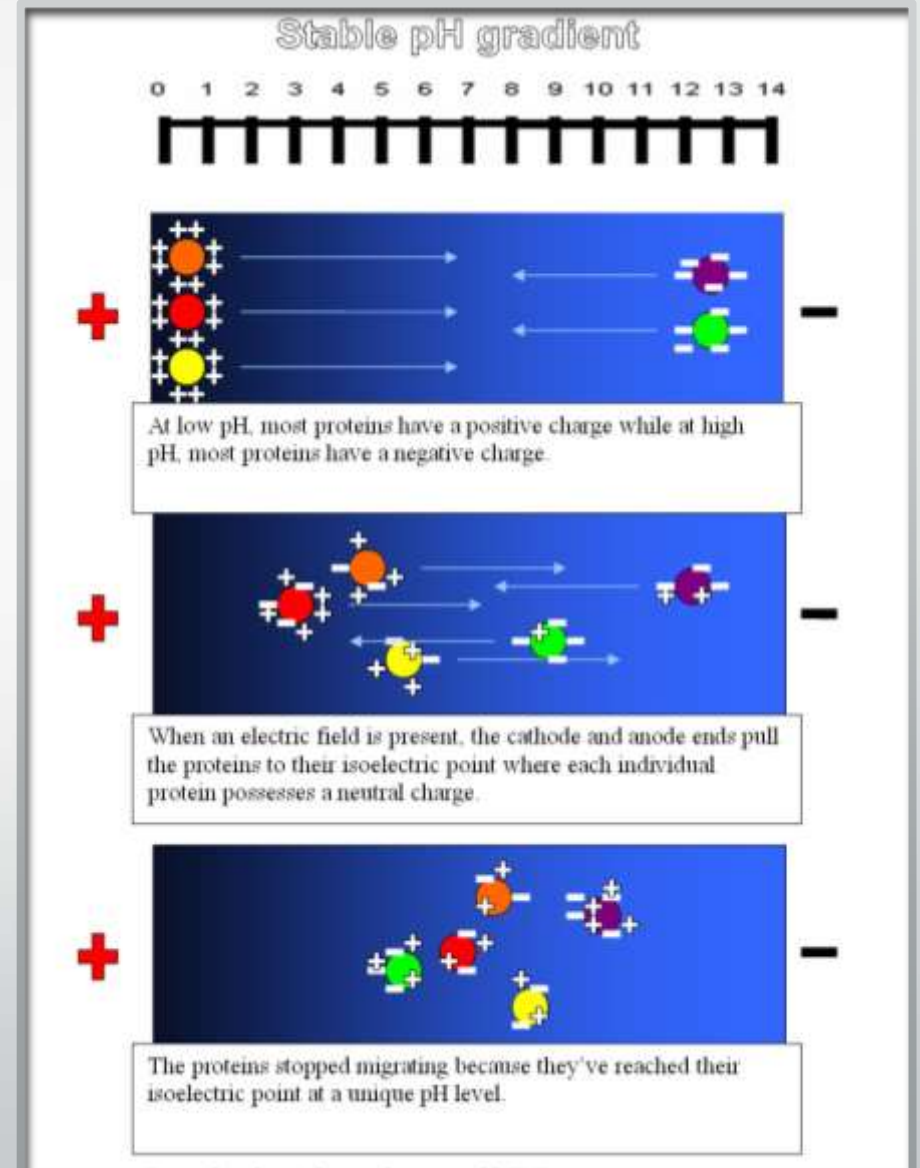
The first-dimension : isoelectric focusing (IEF)

- **Isoelectric focusing (IEF)**, also known as **electro focusing**, is a technique for separating different molecules by differences in their isoelectric point (pI).
- The **isoelectric point (pI, pH(I), IEP)**, is the pH at which a particular molecule carries no net electrical charge in the statistical mean.

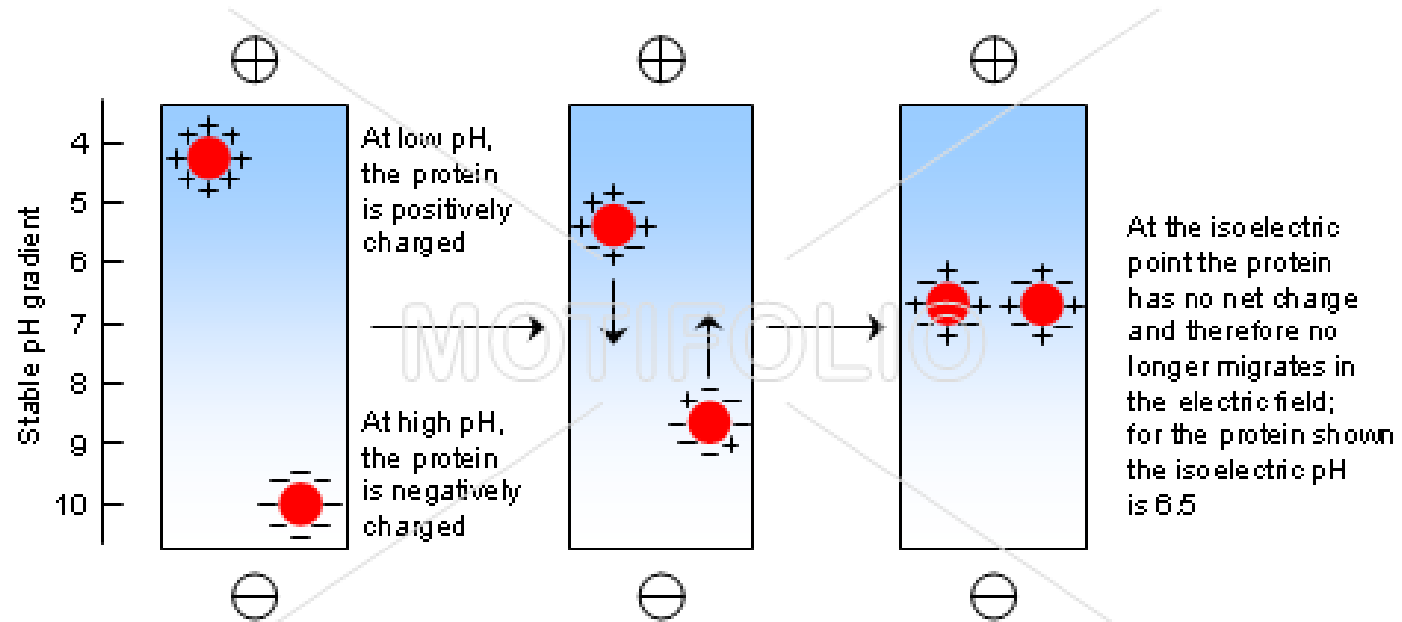
- IEF involves adding an ampholyte solution into immobilized pH gradient (IPG) gels
- **Amphoteric** is derived from the Greek word *amphoterioi* (ἀμφότεροι) meaning "both". Related words in acid-base
- IPGs are the acrylamide gel matrix copolymerized with the pH gradient

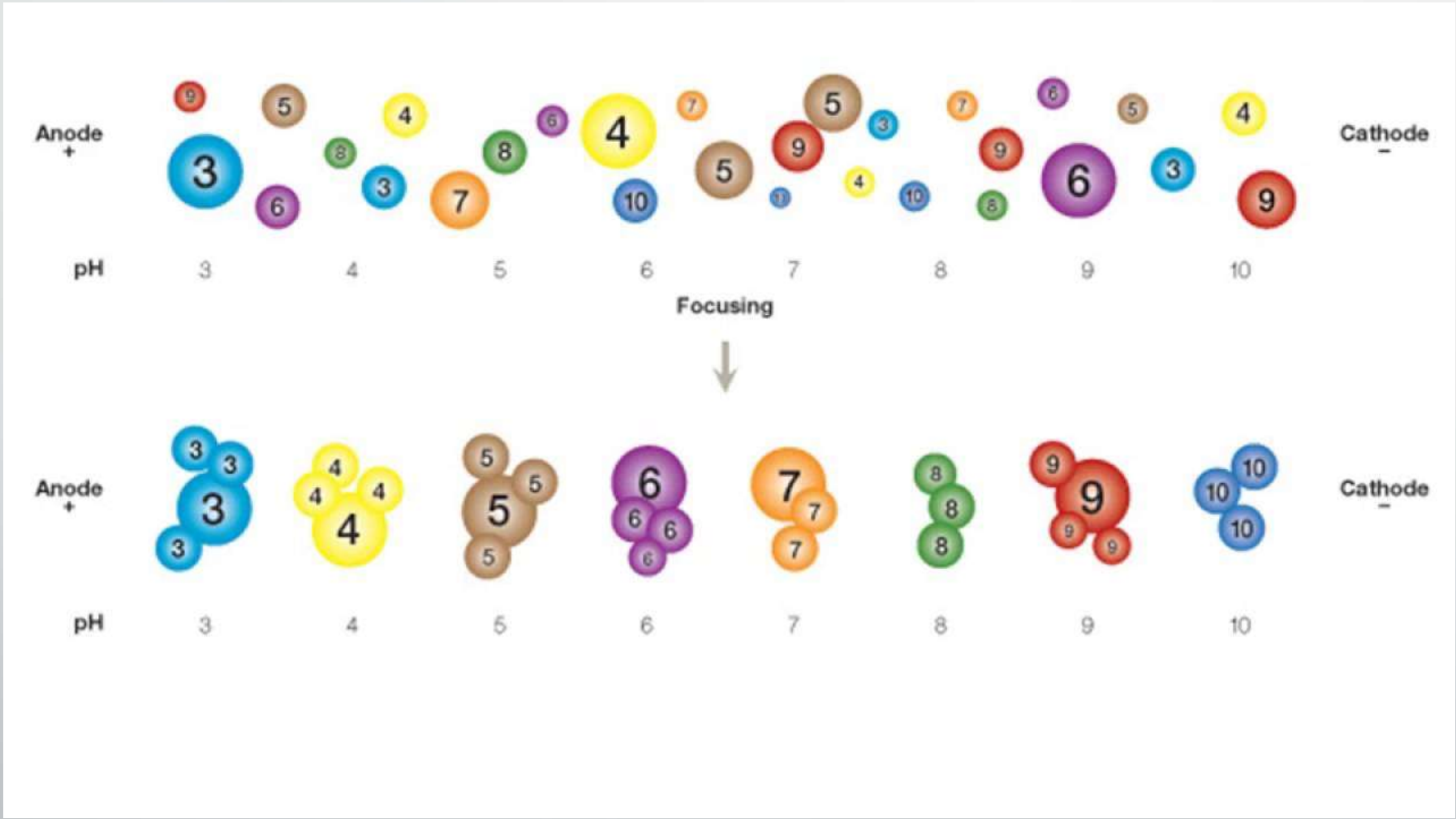


- A protein that is in a pH region below its isoelectric point (pI) will be positively charged and so will migrate towards the cathode (negatively charged electrode)
- As it migrates through a gradient of increasing pH, however, the protein's overall charge will decrease until the protein reaches the pH region that corresponds to its pI.
- At this point it has no net charge and so migration ceases (as there is no electrical attraction towards either electrode).
- As a result, the proteins become focused into sharp stationary bands with each protein positioned at a point in the pH gradient corresponding to its pI



Separation of protein molecules by isoelectric focusing





Isoelectric Focusing (IEF) Protocol

- **First step : rehydration** : The 2D electrophoresis sample rehydration buffer, also as known as the sample buffer, is used to denature and solubilize protein samples, and rehydrate the IPG strips.
- The first step in sample preparation is selecting and/or preparing a suitable sample rehydration buffer. Due to the large variety of proteins, there is no universal sample rehydration buffer.
- The sample rehydration buffer must:
 - Maintain proteins in solution during rehydration of the IPG strips and IEF
 - Not have any effect on the pI of the protein

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- The buffer typically contains a denaturing agent (urea or urea/thiourea), solubilizing agent (non-ionic or zwitterionic detergent and ampholytes), and reducing agent (DTT)

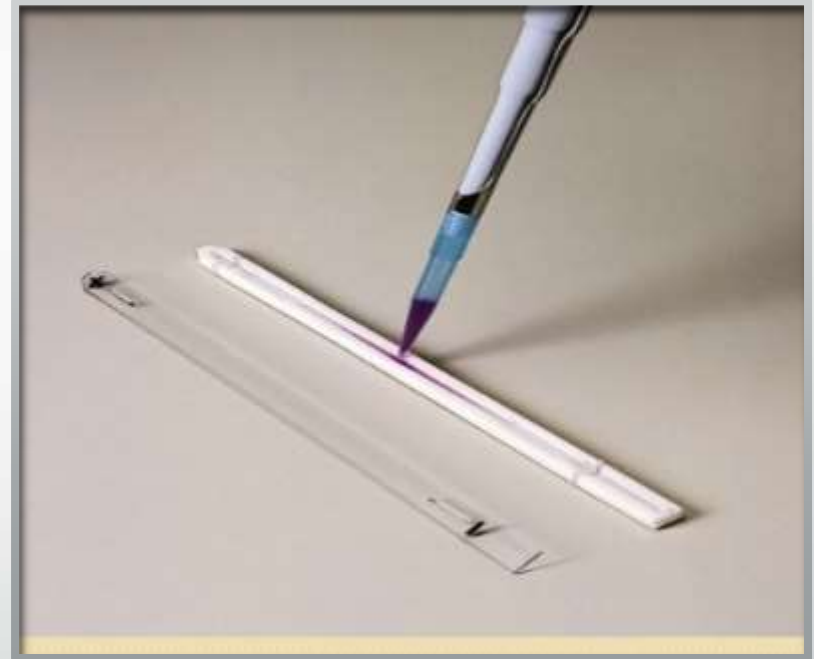
Component	Function	Final concentration	Comments
Urea	Denatures and solubilizes proteins	<ul style="list-style-type: none"> • 8 M urea or 9 M urea in some cases • For urea/thiourea solution, use 5–8 M urea with 2 M thiourea 	<ul style="list-style-type: none"> • Urea solutions are prepared fresh or stored frozen at -200°C. • Deionize urea solutions on a mixed-bed ion exchange resin using manufacturer's recommendations. • Thiourea is used to increase solubility of some proteins (Rabilloud, 1998)
Detergent	Solubilizes the proteins and helps to maintain the proteins in solution during rehydration and IEF	Total detergent concentration range is 0.5–4%	Use non-ionic or zwitterionic detergents such as CHAPS, NP-40, not sds
Reducing agent	Cleaves the disulfide bonds in the proteins	DTT or DTE at 20 mM to 100 mM	<ul style="list-style-type: none"> • Use DTT or DTE (dithioerythritol). • β-mercaptoethanol is not recommended for reduction
Ampholytes	Helps in solubilizing proteins and aids in maintaining the pH gradient 0.2–2%	Note: Higher ampholyte concentration requires longer focusing times.	<ul style="list-style-type: none"> • Use the appropriate carrier ampholytes based on the pH range of the IPG strip. • May use ampholytes pH 3–10 for all pH ranges of IPG strips.

Prepare the reswelling tray:
Level the re-swelling tray and slide the protective lid completely off. Ensure that the tray is clean and dry.

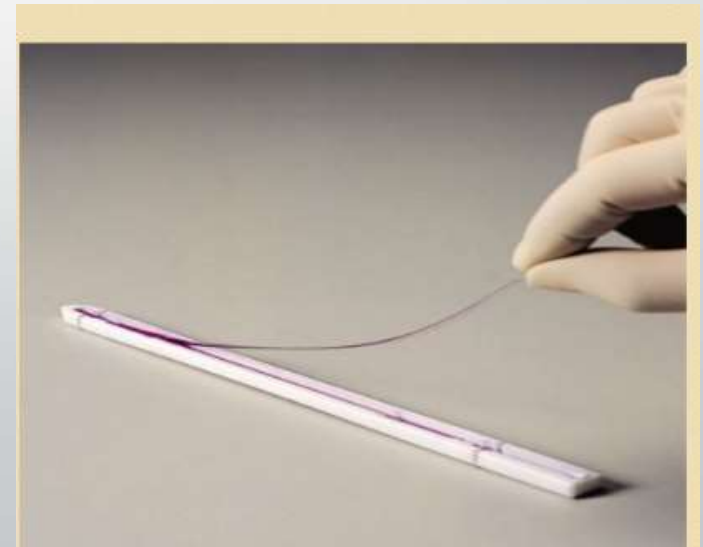
IPG Strip Reswelling Tray



Apply the rehydration (with sample) solution: Mix suitable protein sample with appropriate volume of rehydration and IPG buffer and pipette the mixture to the reswelling slot. Note: expel the solution slowly to the center of the slot, and remove any large bubbles.

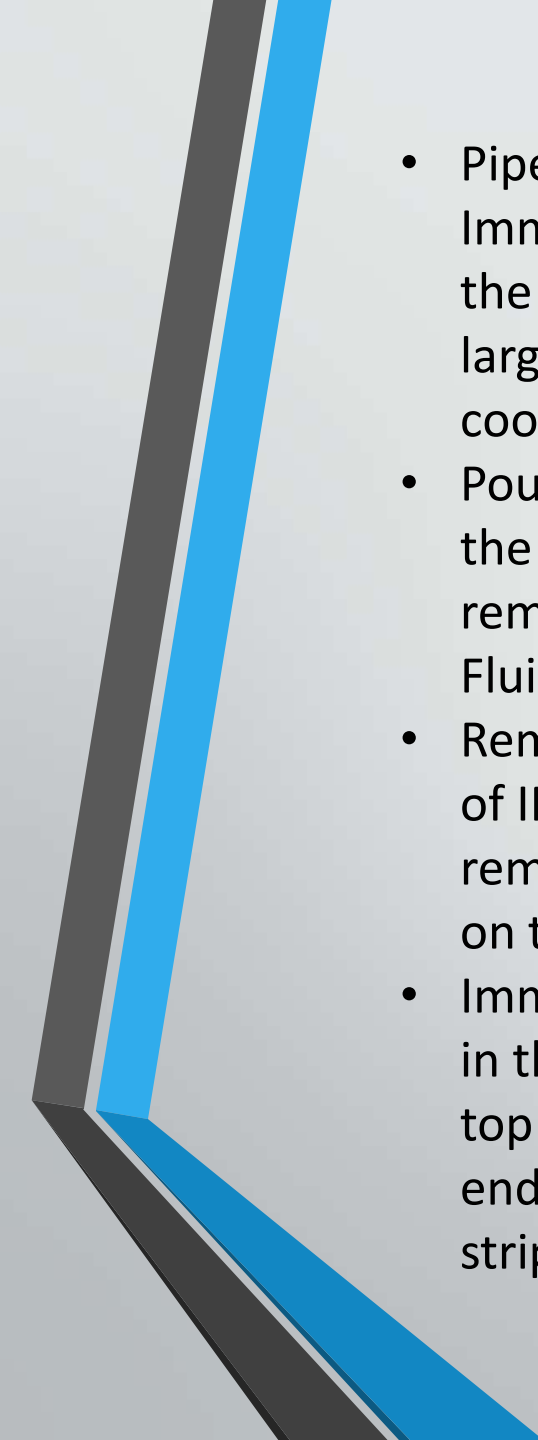


Placing the IPG strip: Remove the protective cover from the IPG strip. Position the IPG strip with the gel side down. Gently lift than place the strip down while sliding it back and forth along the surface of the solution. Be careful not to trap bubbles under the IPG strip.



- Overlay each IPG strip with 1.5 to 3 ml of IPG Cover Fluid (mineral oil) to prevent evaporation and urea crystallization.
- Slide the lid onto the reswelling tray and allow the IPG strips to rehydrate at room temperature overnight. (A minimum of 10-16 hours is required for rehydration)
- Clean all components of the IEF apparatus .
- Confirm electrical connections
- Position the cooling plate on the unit and ensure that the surface is level. Set the temperature on Thermostatic Circulator to 20 o C.



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- Pipette about 10 ml of IPG Cover Fluid onto the cooling plate and position the Immobiline DryStrip tray on the cooling plate so the red electrode connection of the tray is positioned at the top of the plate near the cooling tubes. Remove any large bubbles between the tray and the cooling plate to increase the contact cooling surface.
 - Pour about 5-10 ml of IPG Cover Fluid into the immobiline DryStrip tray. Place the DryStrip aligner into the tray on top of the IPG Cover Fluid slowly and remove the large bubbles between the two surfaces. Avoid getting IPG Cover Fluid on top of the aligner at this point.
 - Remove the IPG strip from its slot in the Re-swelling Tray carefully. Hold one end of IPG strip with forceps and rinse the IPG strip with Milli-Q water completely to remove excess rehydration solution and thus prevent formation of urea crystals on the gel surface during IEF.
 - Immediately transfer the rehydrated IPG strip to adjacent grooves of the aligner in the immobiline DryStrip tray. Place the strips with the point (acidic) end at the top of the tray near the red electrode (anode) and gel side face up. The blunt end should be at the bottom of the tray near the black electrode (cathode). All strips must have the same length.

- **Prepare electrode strips:** Cut two IEF electrode strips to a length of 110mm. Moisten each strip with 0.5 ml Milli-Q water and dump the electrode strips on filter paper. The strips should be damp – too much moisture within the strips will cause uneven focusing of the sample. Set aside.
- Place electrode strips across the cathode and anodic ends of the aligned IPG strips. The electrode strip must at least partially contact the gel surface of each IPG strip.
- Align each electrode over an electrode strip, ensuring that the marked side corresponds to the side of the tray giving electrical contact. Press electrode down to contact the electrode strips



- Cover the IPG strips with mineral oil to prevent evaporation and urea crystallization.

Voltage and time

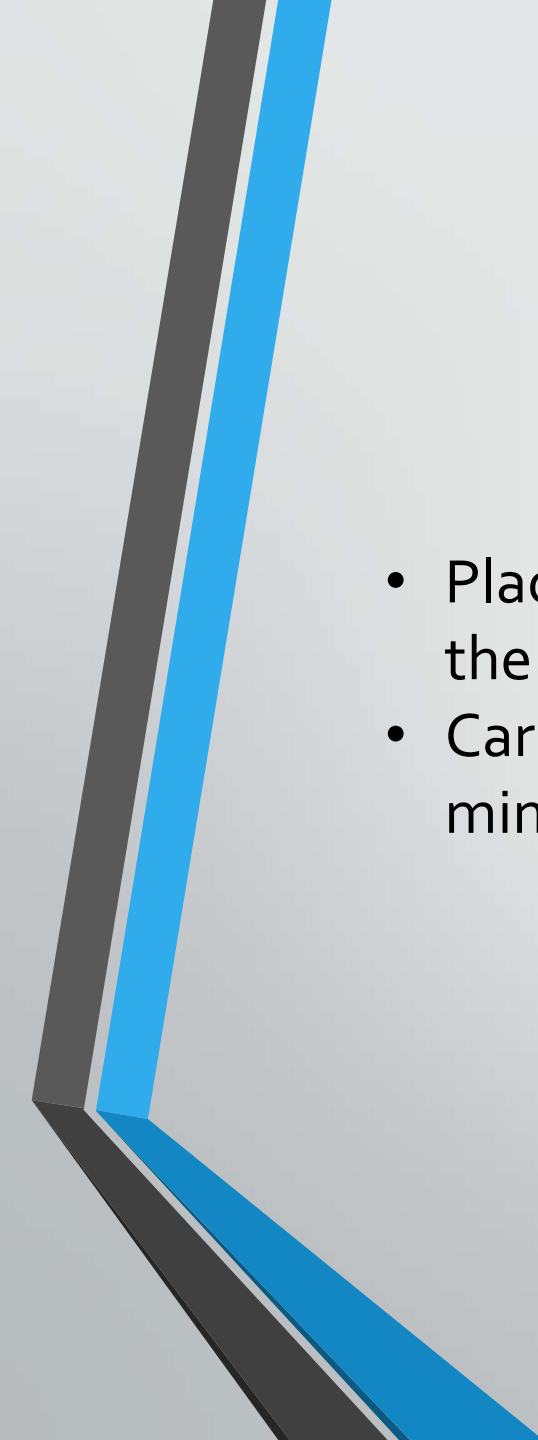
	Voltage	Time	ramp
Step 1	200	1 min	Gradient
Step 2	3500	1.5 hr	Gradient
Step 3	3500	3 hr	step



IEF APPARATUS

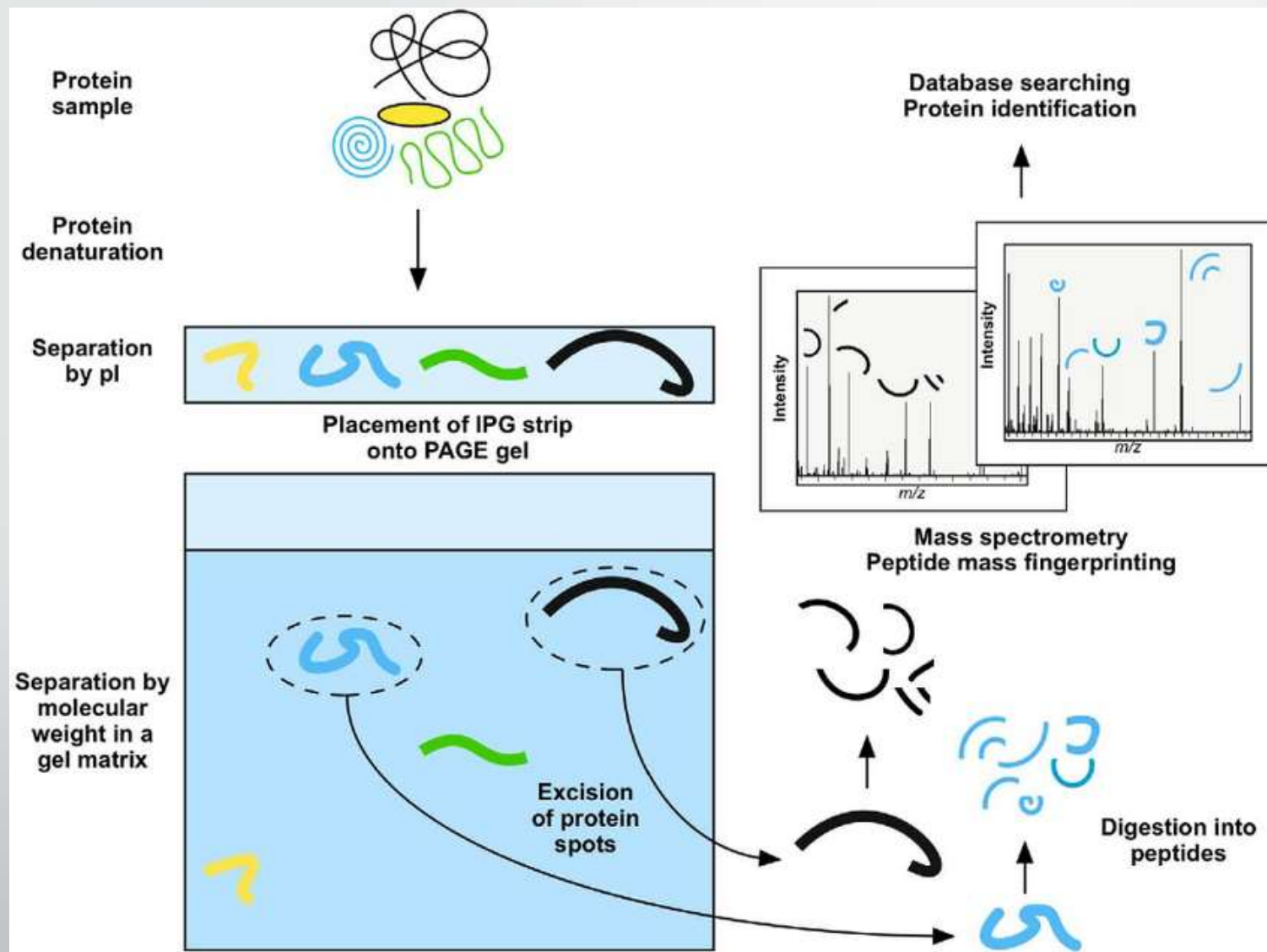
- After the run remove the IPG strips from the tray. At this point you can store it at -20 C or go for 2nd dimension.
- **IPG STRIPS EQUILIBRIUM**
- Prepare equilibration buffer I(DTT) and II(Iodoacetamide)

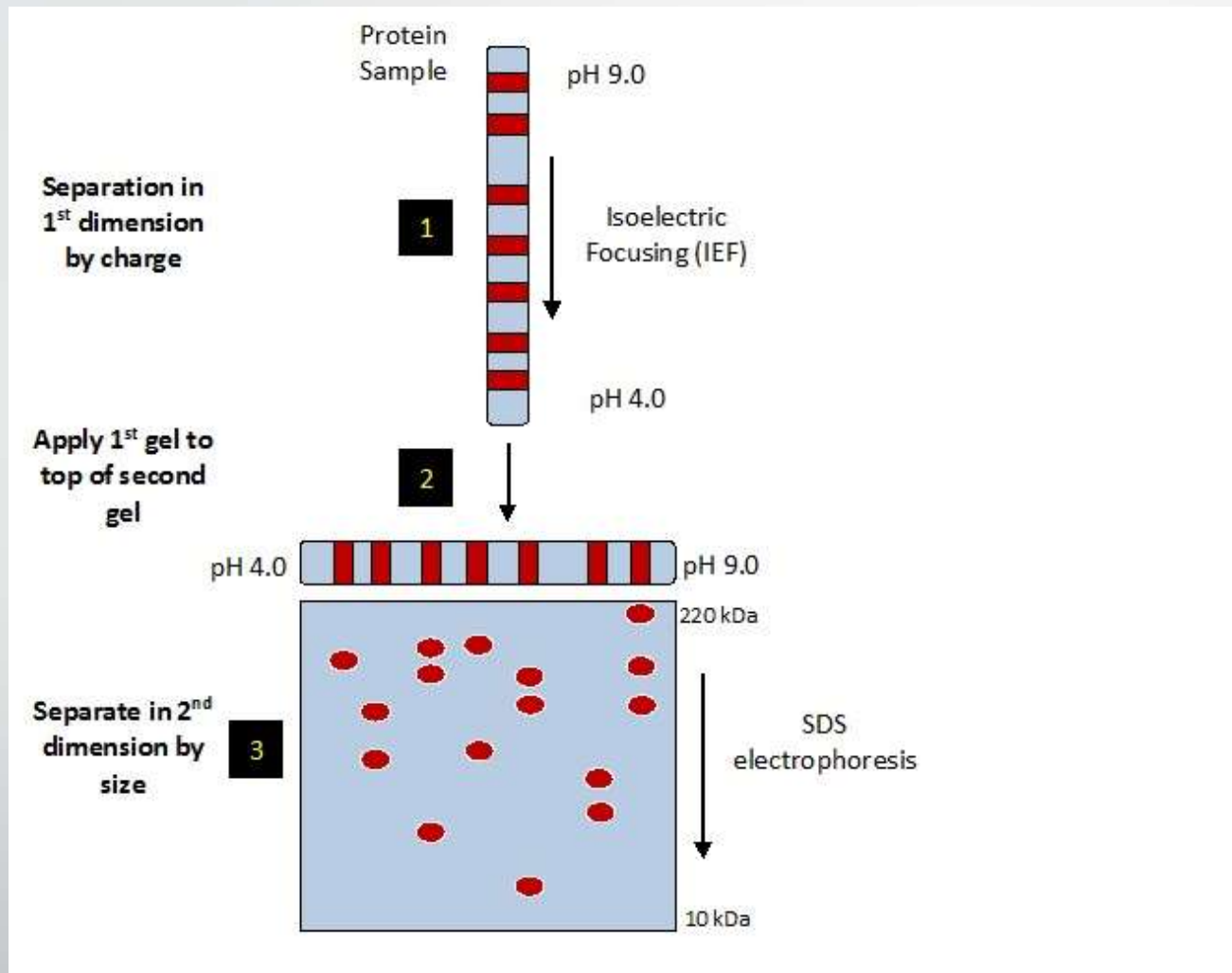
2-D equilibration buffer I components	2-D equilibration buffer II components
6 M Urea	6 M Urea
2% SDS	2% SDS
0.375 M Tris-Hcl (PH -6.8)	0.375 M Tris-Hcl (PH -6.8)
20% Glycerol	20% Glycerol
2% (w/v)DTT	2.5% Iodoacetamide

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- Place IPG strips gel side up and add 4ml of equilibration buffer –I ,place the centrifugal tube on shaker for 20min.
 - Carefully take out the strip and place in the equilibration buffer –II for 20 min .take out IPG – strips and rinse with water

The second-dimension : sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

- This separates proteins by their sizes (molecular).
- Normal SDS PAGE is done in this stage
- Stacking is replaced by IPG strips and normal running gel separates molecules





- Gel runs at 70 volts
- After finishing run place the gel into CBB dye and leave on shaker for night.

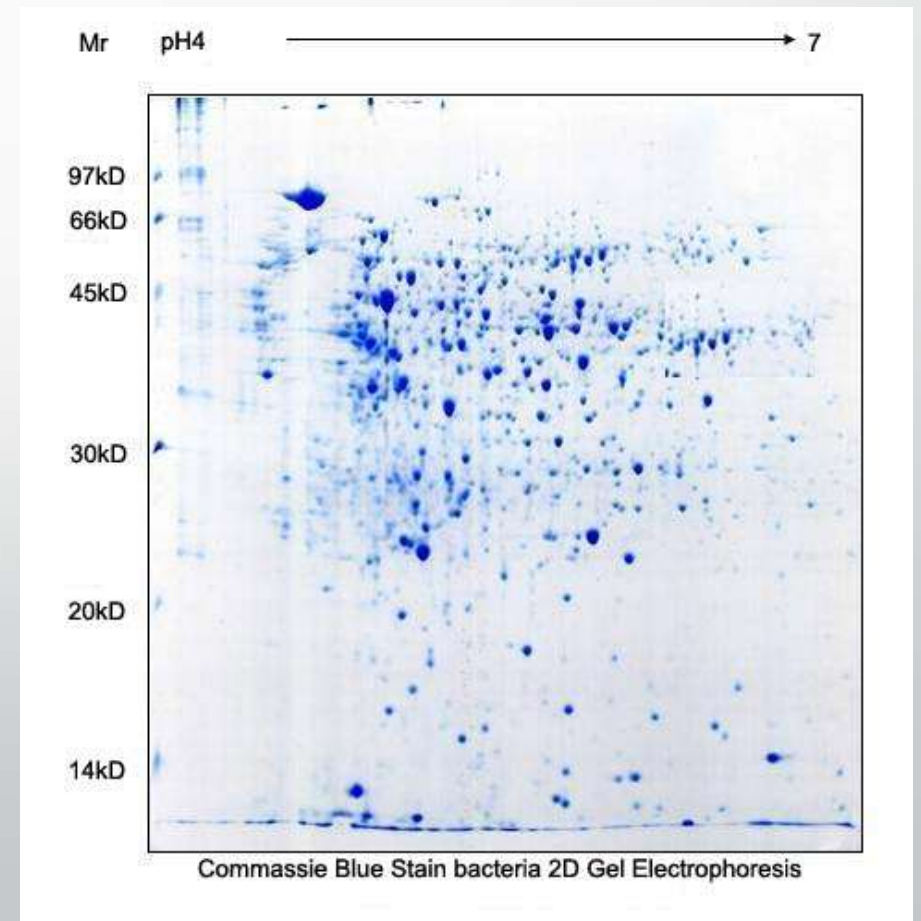


Image analysis

- 2-D gel image will be analyzed using PD Quest software
- In quantitative proteomics, these tools primarily analyze bio-markers by quantifying individual proteins, and showing the separation between one or more protein "spots" on a scanned image of a 2-DE gel .

Advantages

- Very sensitive
- High resolution up to 10,000 different proteins
- Unbiased search
- Spatio temporal expression