Swayam Course - Analytical Techniques

Week: 9, Module 23 - 2D Electrophoresis & DIGE

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Learning Objectives:

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1. Introduction to 2D electrophoresis

Definition: 2D Electrophoresis is a method for separating and identifying the proteins in a sample by displacement in 2 dimensions oriented at right angles to one another.

2-D electrophoresis is experiment used for the analysis of complex protein mixtures extracted from cells, serum, tissues, or other biological samples. These technique seperates proteins in two discrete steps: the first-dimension separation by isoelectric focusing which separates proteins according to their isoelectric points and the second-dimension step, SDS-polyacrylamide gel electrophoresis which separates proteins according to their molecular weights. Each spot on the two-dimensional gel corresponds to a single protein species in the sample. Thousands of different proteins can thus be separated, and information such as the protein pI, the apparent molecular weight, and the quantitative expression of each protein is obtained.

2. Methodology

2.1. Sample preparation

Sample preparation includes solubilization of all proteins and elimination of all other biological compounds that might interfere with this first separation. As the first separation is always almost isoelectric focusing, emphasis is on use of uncharged chemicals for extracting the proteins, denaturing them and keeping them in solution. The standard combination includes a combination of chaotropes and detergents such as urea thiourea and CHAPS.

2.2. Isoelectric focusing

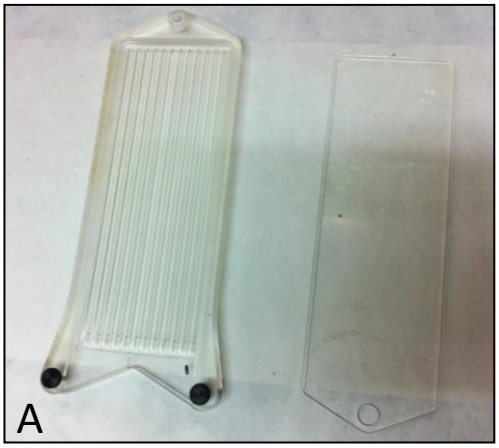
Isoelectric focusing is the first stage of separation of proteins based on their intrinsic charge or the isoelectric point. Isoelectric point of a protein is the pH at which the protein has no net charge. Above its isoelectric point, a protein has a net negative charge and migrates toward the anode in an electrical field. Below its isoelectric point, the protein is positive and migrates toward the cathode. 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. The technique is capable of extremely high resolution with proteins differing by a single charge being fractionated into separate bands. Molecules to be focused are distributed over a medium that has a pH gradient created by aliphatic ampholytes. An electric current is passed through the medium, creating a "positive" anode and "negative" cathode end. Negatively charged molecules migrate through the pH gradient in the medium toward the "positive" end while positively charged molecules move toward the "negative" end. As a particle moves towards the pole opposite of its charge it moves through the changing pH gradient until it reaches a point in which the pH of that molecules isoelectric point is reached. At this point the molecule no longer has a net electric charge due to the protonation or deprotonation of the associated functional groups and as such will not proceed any further within the gel. The gradient is established before adding the particles of interest by first subjecting a solution of small molecules such as polyampholytes with varying pI values to electrophoresis. IEF can be performed using IPG strips. These are rehydrated with 250 µl of rehydration buffer (8 M urea, 2 M thiourea, 2% CHAPS, DTT 0.003%, and IPG buffer, pH 3-10, 0.5%). Samples are applied by cup loading and IEF is performed as per program. IEF is stopped when a total volt-hours (VhT) is achieved. Temperature is set at 20˚C. Strips are covered with cover fluid throughout the run period.

2.3. Equilibration

The purpose of the inter-dimension equilibration process is to coat the proteins separated in the isoelectric focusing gel with SDS, so that they become mobile in the second dimension. This is always achieved by

equilibration of the isoelectric focusing gel in a SDS-containing buffer**.** The equilibration fulfils two functions: One, the disulfide bridges are reduced and the cysteine residues alkylated with iodoacetamide and two, the proteins are equilibrated with SDS so that they become negatively charged for the electrophoresis. Take a plastic tube that is filled with 10ml fresh SDS solution containing 65mM DTT and one tube with 10 ml of fresh SDS solution containing 135mM iodoacetamide. Equilibrate the IPG strips with gentle motion 15 minutes each in both tubes and finally rinse the strips with a few ml SDS –running buffer.

A pictorial description of the 2D electrophoresis methodology is presented here below. Steps in rehydration is shown in **pictures A to H.** Steps in Iso-electric focusing is shown in **pictures I to P**. Steps in SDS PAGE is shown in **pictures Q-R.**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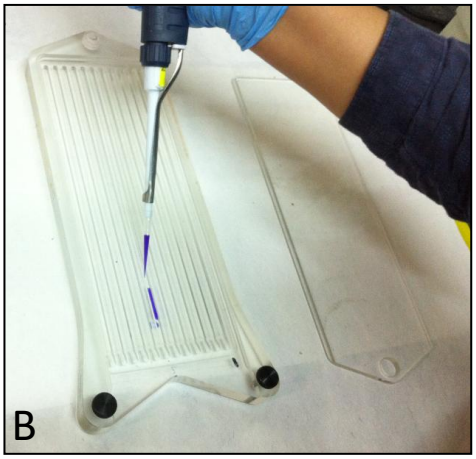
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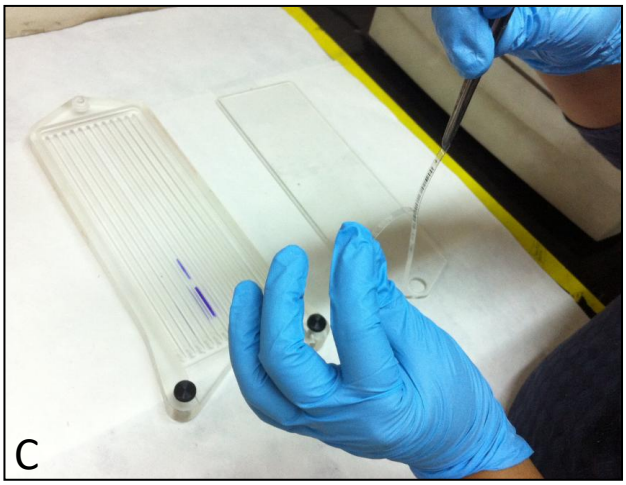
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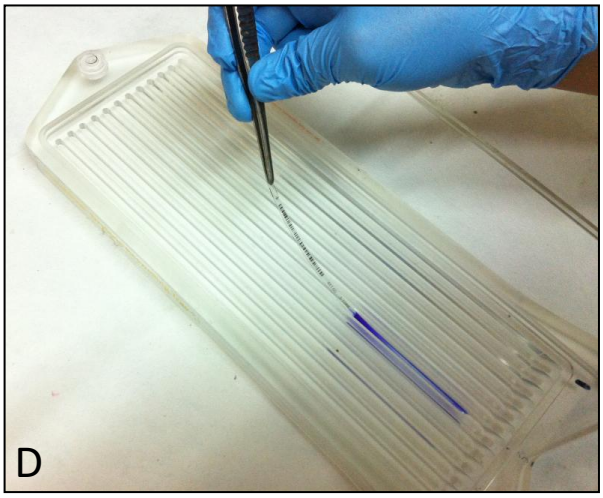
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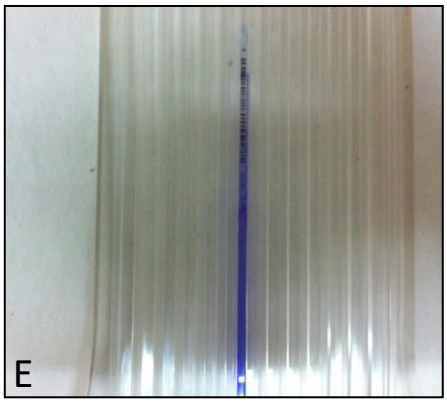
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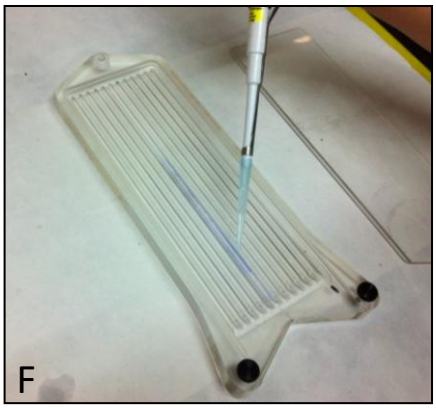
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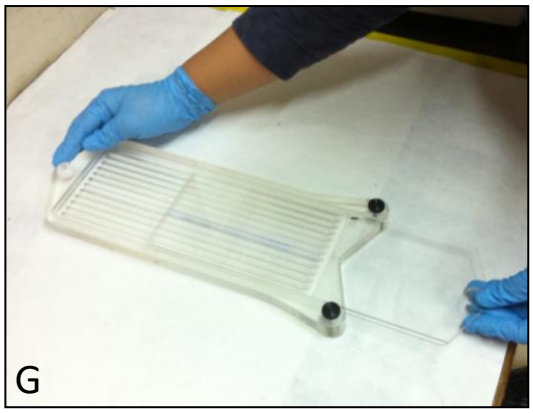
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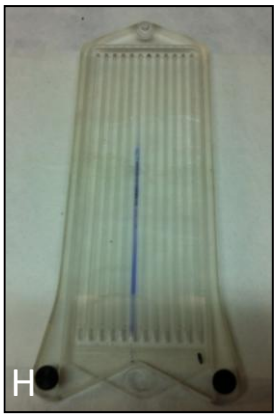
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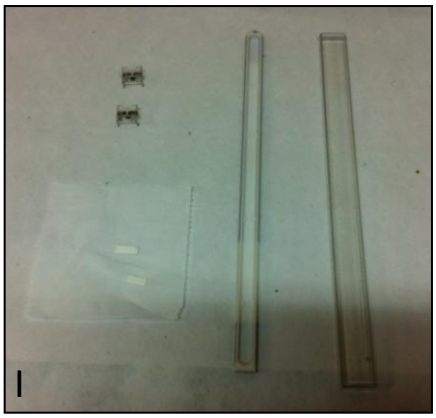
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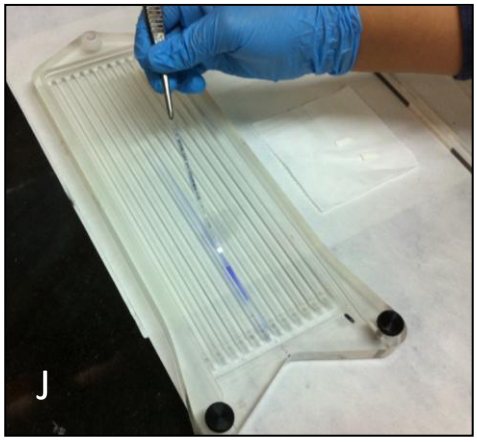
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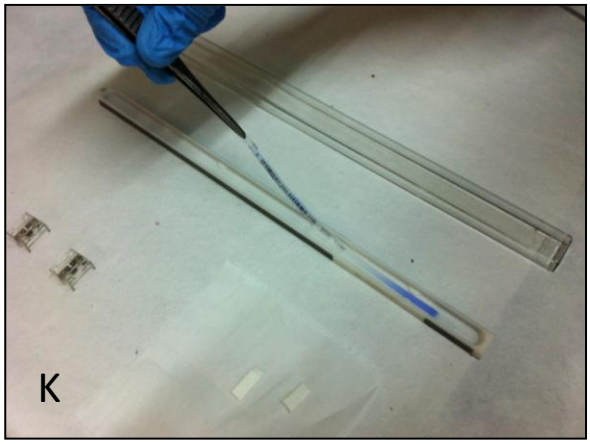
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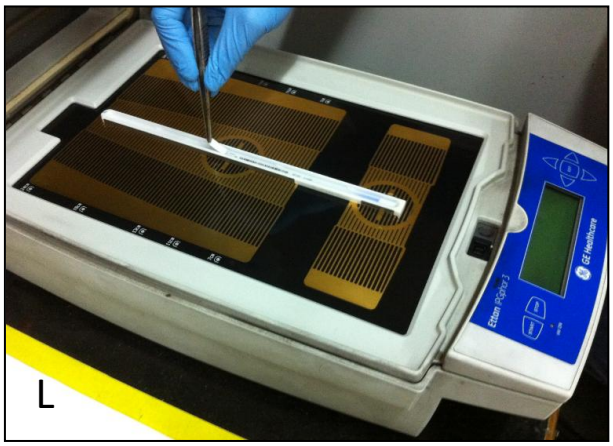
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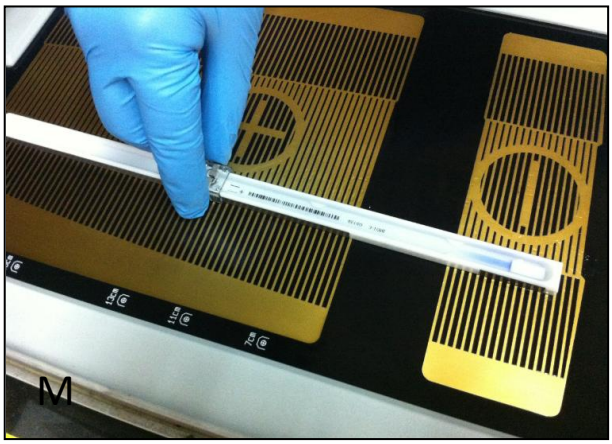
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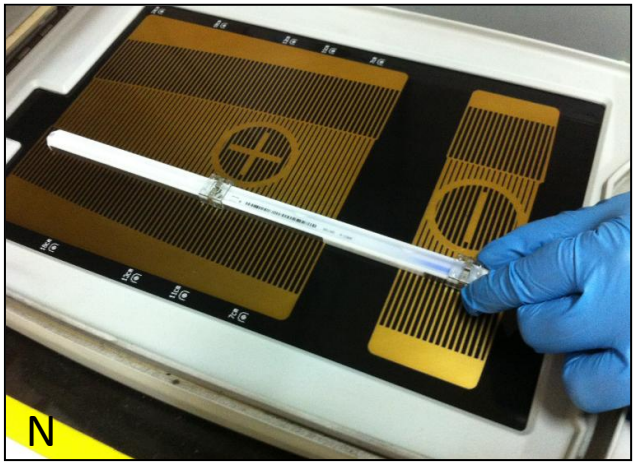
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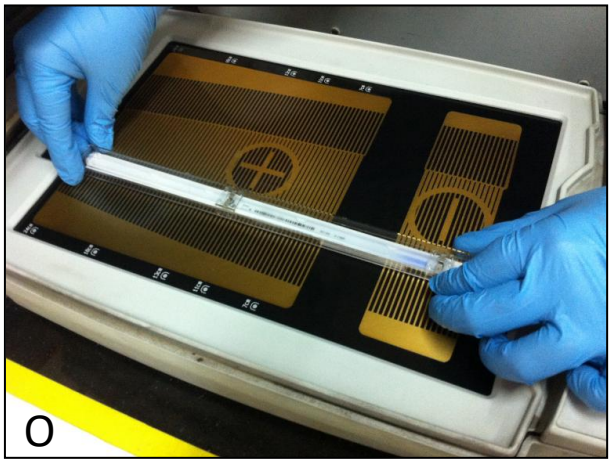
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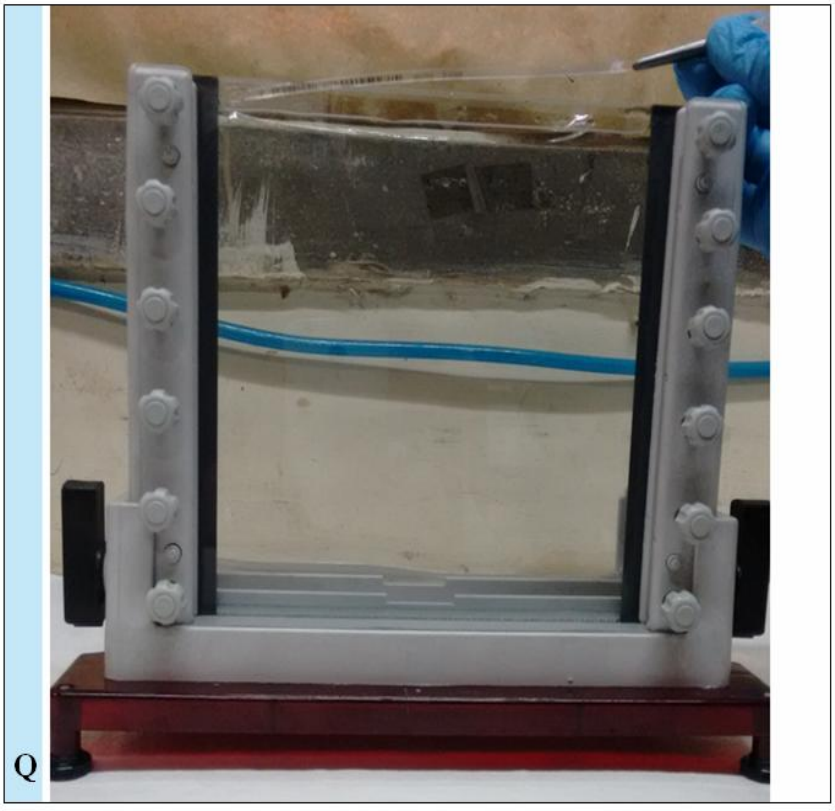
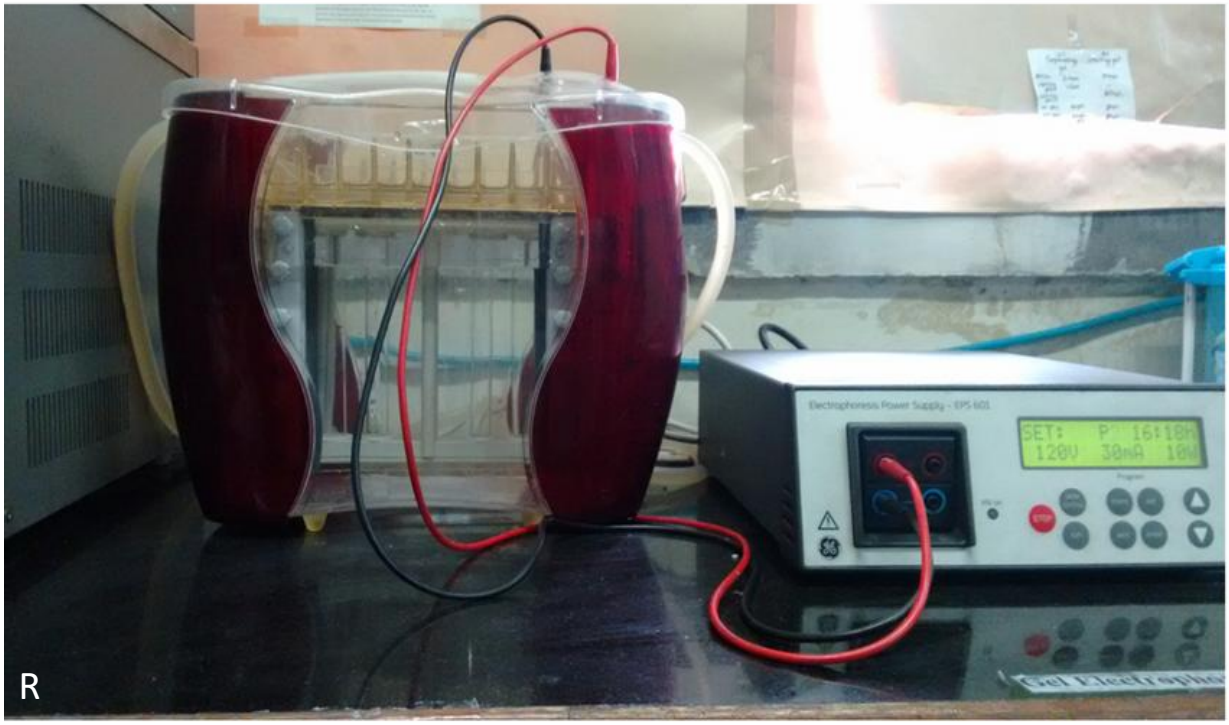
2.4. SDS PAGE

Prior to SDS-PAGE, each strip is equilibrated with SDS equilibration buffer, first with 10 ml of SDS equilibration buffer containing 10mg/ml DTT for 15 minutes, followed by SDS equilibration buffer containing 25 mg/ml iodoacetamide for another 15 minutes. The strips are then loaded and run on 10% homogenous acrylamide gel cast on *SE 600 Ruby gel apparatus* (GE Healthcare, USA). The strips are then sealed with agarose sealing solution to maintain continuity between the acrylamide gels and the immobiline dry strips. Separation in second dimension is carried out with running parameters was set as constant voltage of 20 V at 20 ºC for 30 minutes, followed by 120 V until the bromophenol blue dye front had run off from the bottom of the gels.





2.5. Gel staining

2.5.1. Coomassie staining

The gel is immersed in a 0.1 % Coomassie brillant blue G-250 (50 % methanol, 10% acetic acid) solution for 1 hour. When preparing the Coomassie Blue reagent is advisable to filter the solution before use. This is followed with destaining steps in which the gel is immersed in a 40 % methanol solution. The solution is replaced several times to wash out the unbounded dye. The time required for developing will depend upon gel size, thickness, and of course protein loading. Gel destaining can be carried out overnight by reducing methanol concentration. Once bands or spots start to show up, it is recommended to discard the staining solution and leave the gels in Milli-Q water for 24 hours.

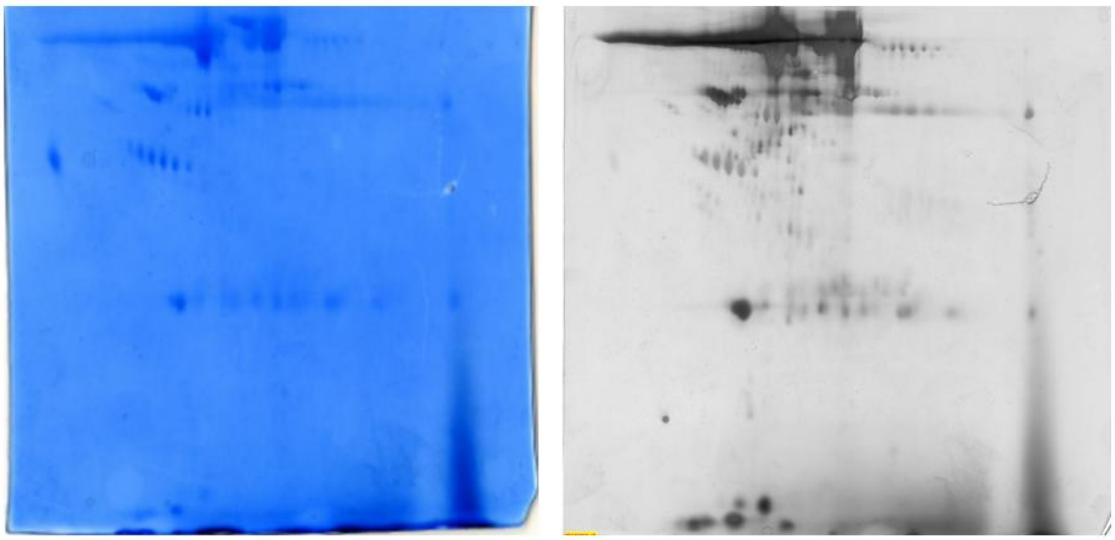
2.5.2. Silver staining

After SDS-PAGE, the gel is immersed in 250 ml fixing solution (45% methanol, 45% water, 10% acetic acid) for overnight. Once fixation is over, fixing solution is removed; repeated washing with water is done to remove traces of acetic acid. Then, 250 ml sensitizing solution (50mg sodium thiosulfate, 250ml water) is added. Sensitization is done for 1 minute with gentle shaking. Gel is washed with distilled water for three times for 1 minute each. 250 ml silver solution (0.5mg silver nitrate, 50µl formaldehyde, 250ml water) is added to the gel and kept shaking for 30 minutes. Gel is then washed with distilled water three times for 1 minute each. After washing, the gel is developed by adding 250ml developing solution

(15g sodium carbonate, 250µl formaldehyde, 500ml water) for 4 to 6 minutes and transferred to stopping solution (1.25g glycine, 250ml water) once the protein spots had reached desired intensity.

2.5.3. Colloidal coomassie staining

The gel is allowed to fix in this fixing solution (50% Ethanol + 3%Phosphoric acid + 47%H_2O) overnight. Excess fixative is removed and gels are washed three times for 30 minutes per wash. The gels are equilibrated in *Neuhoffs solution* (16% Ammonium Sulfate + 25%Methanol + 5% Phosphoric acid + 54%H_2O) for one hour. Coomassie Brilliant Blue G250 powder (1g/L) is added to each staining tray and stained for 3 days. Normally spots can be seen by 24-48 hours but for optimal staining the step can be extended for 3-4 days. The gel is stored in 5% acetic acid solution at 4°C.



3. Applications of 2D electrophoresis

1. Protein profiling: snap shot representation of 'most' proteins in particular organism or tissue

2. Comparative proteomics for biomarker discovery: helps to know differentially expressed proteins in different phenotypic states

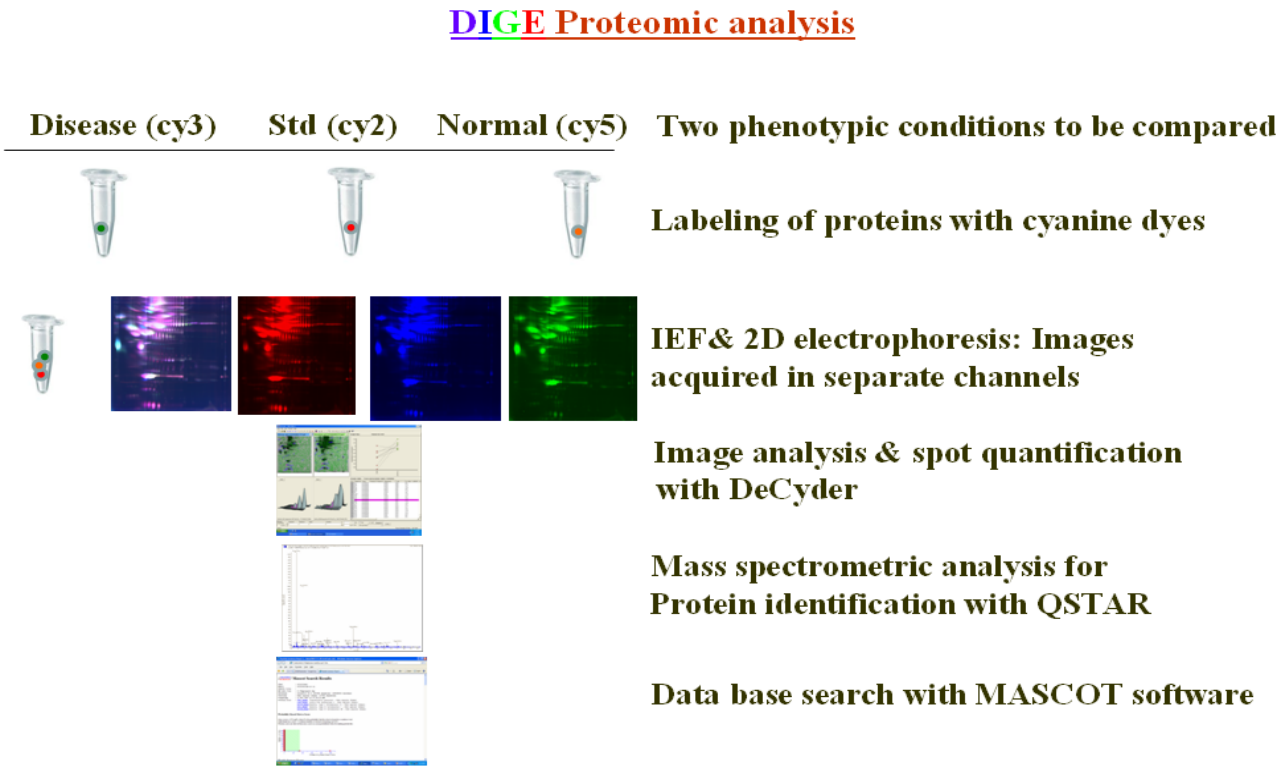
4. Modifications of 2D electrophoresis: DIGE

2-D Fluorescence Difference Gel Electrophoresis (2-D DIGE) is a variant of two-dimensional gel electrophoresis that offers the possibility to include an internal standard, so that two samples can be subjected to the same experimental conditions and can be easily compared and accurately quantitated for differential protein expression. This technique incorporates an internal standard, made by pooling all samples in an experiment, and is run on every 2-D DIGE gel. This means that there is a standard for

every spot on the gel, and that all gels within the same experiment are quantitatively linked. When we have several samples, 2-D electrophoresis can be difficult due to the need for large numbers of replicates. Because the internal standard virtually eliminates gel-to-gel variation, technical replicates are not necessary when one performs 2-D DIGE.

4.1. **Methodology used in DIGE**

Protein samples and the internal standard are each labeled with one CyDye DIGE Fluor minimal dye. These labeled samples are combined, run on an isoelectric focusing gel in the first dimension, and separated by SDS-PAGE in the second dimension. The ability to multiplex different CyDye DIGE Fluor minimal dye-labeled samples on the same gel means that the different samples will be subjected to exactly the same first- and second-dimension running conditions. Consequently, the same protein labeled with any of the CyDye DIGE Fluor minimal dyes and separated on the same gel will migrate to the same position on the 2-D gel. This limits experimental variation and ensures accurate within-gel matching. A diagrammatic representation of the methodology is provided below.



Differentially expressed spots across biological replicates are marked as shown below.

CyDye: DIGE fluors are available as (A) minimal and (B) saturation labeling dyes.

Minimal dyes are intended for general 2-D application use where sufficient amounts of sample are available. The cyanine dyes (Cy2, Cy3 and Cy5) with different absorption and emission spectra are N-hydroxy succinimidyl ester derivatives that are covalently tagged to the ε-amino group of lysine residue of proteins, and replace the ε-amino group positive charge with the positive charge of the dye. The binding of the dyes introduce small but matched increases in molecular weight to the protein. Since these dyes are hydrophobic, to prevent precipitation of proteins, only one lysine residue per protein is labeled (minimal labeling). Minimal labeling limits the fluorescence intensity and thus the sensitivity of the stain. The dyes are all charge-matched and molecular mass-matched to prevent alterations of the isoelectric point, and to minimize dye-induced shifting of labeled proteins during electrophoresis.

Saturation dyes are designed to be used for applications where only small amounts of sample are available, for example in Laser Capture Microdissection. which saturate cysteine residues. The dyes are mass and charge-matched; but there is a smaller chance of protein modification and isoelectric point shift. Saturation cysteine dyes have superior sensitivity to minimal lysine labeling. But the limitation is that if a cysteine amino acid is not present in a protein, then the saturation dyes will not be able to label it, making the protein undetectable.

Advantages of DIGE

1. High sensitivity. The CyDye fluorescent dyes have a sensitivity of 0.2 ng/spot, as compared to the sensitivity of Coomassie at 100 ng/spot. This allows us to run smaller amounts of protein on the 2D DIGE gels and results in much better spot resolution than traditional 2D gels stained by Coomassie.

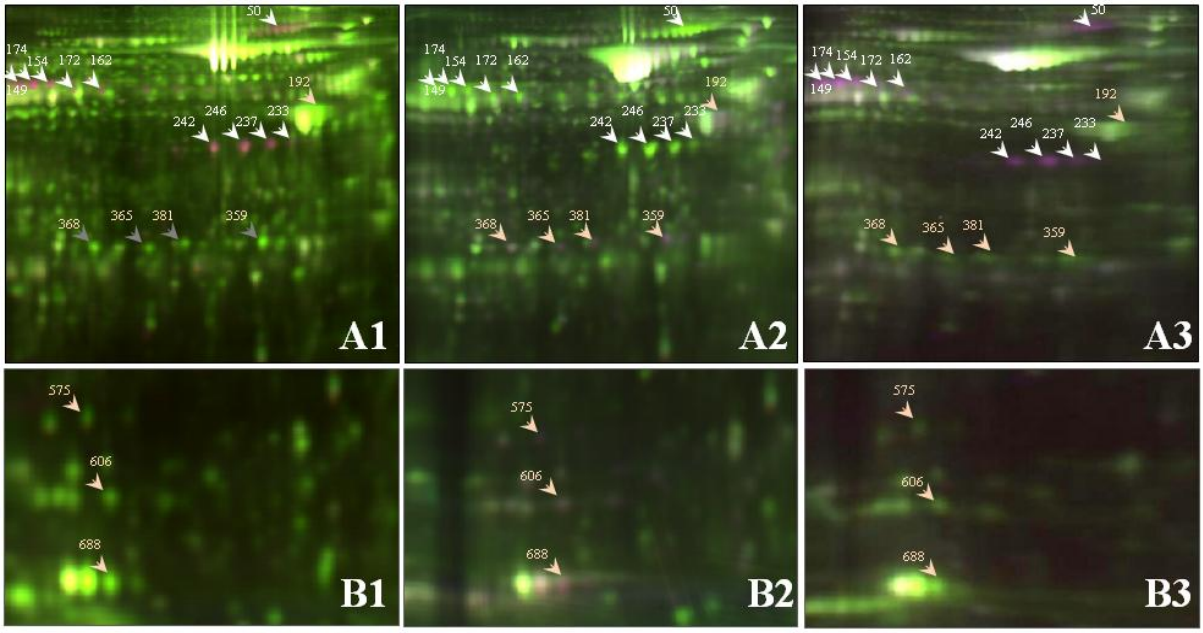
2. High accuracy. Discrete signal from each dye with minimal cross-talk contributes to high accuracy. High spot resolution enables accurate software-aided spot quantitation and protein expression comparison between different phenotypic protein samples. Differences of protein expression with as small as 10%, protein isoforms and post-translational modifications are easily visualized.

3. Fewer number of gels. Since the protein expression from 3 different samples are compared in the same gel, fewer gels are required. Also, there is no need to run technical replicates as in standard 2D as incorporates internal standard for normalization.

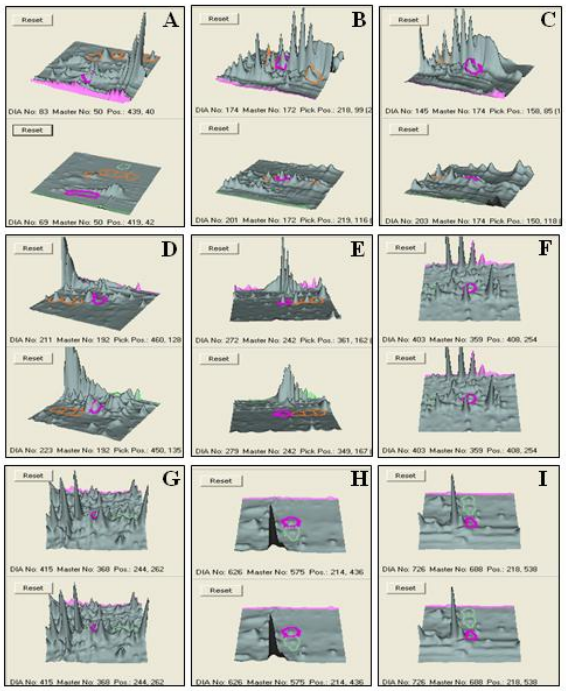
4. Fast turn-around. Fluorescent scanner, software aided in-gel analysis and spot picker enable fast turn-around time.

5. Cost effective. Though the pricing of cyanine dyes are on the higher side, the quality of the gels, quality of differential expression analysis and gel picture quality more than compensates the cost.

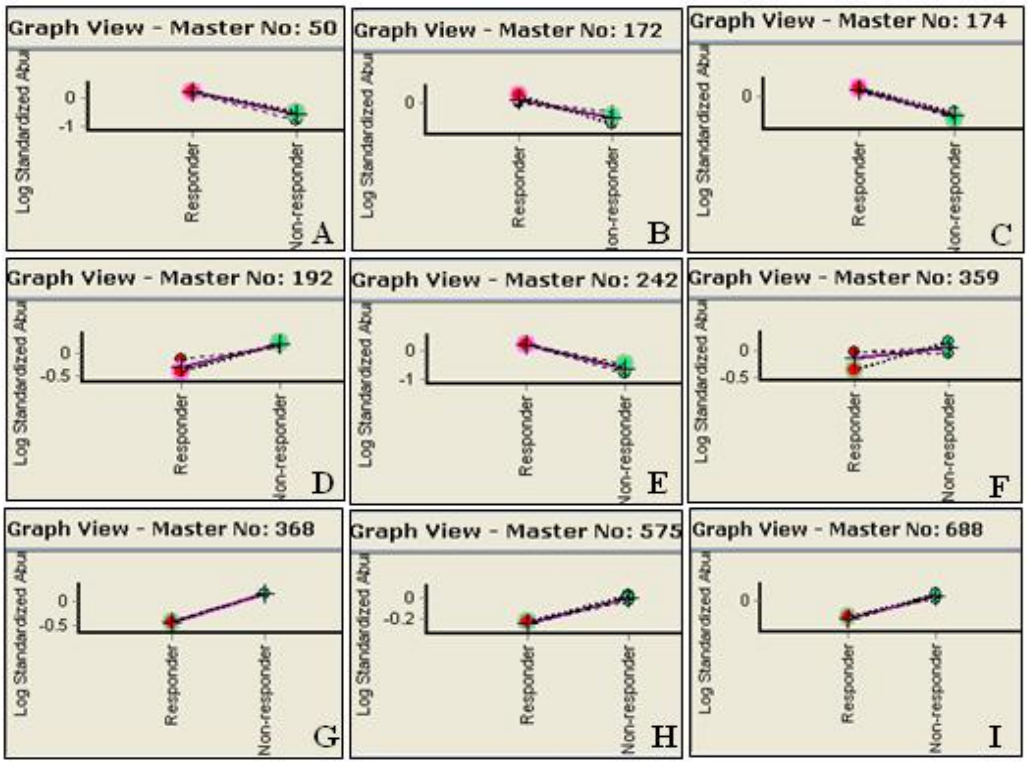
6. **Uniform experimental conditions.** Allows detection of up to three pre-labeled protein samples and standards on the same 2-D electrophoresis gel.



The marked spots are relatively quantified using software as shown below.



The relative quantification is analyzed for consistency across many sets of gels as shown below.



These spots are picked, trypsin digested and identified by mass spectrometric analysis which will is explained in a different module.

5. Advantages and Dis-advantages of 2D electrophoresis

5.1. Advantages od 2D Electrophoreis

1. Cost effective
2. Not very difficult
3. Qualitative and Quantitative analysis is possible
4. Unbiased search

5.2. Dis-advantages of 2D Electrophoresis

1. Single protein can make multiple spots therefore number of proteins is less than the number of spots
2. Abundant proteins constitute the major fraction of the spots
3. Separation is dependent on the gel concentration and size
4. Basic, hydrophobic and membrane proteins are not easily separated
5. Sensitivity is as good as the staining technique

SUMMARY

- 2-D electrophoresis is experiment used for the analysis of complex protein mixtures extracted from cells, serum, tissues, or other biological samples.
- Methodology used in 2D electrophoresis involves solubilization of the proteins, reducing the non-protein contamination, iso-electric focusing on IPG strips, SDS PAGE electrophoresis & staining for visualization of spots
- Applications of 2D electrophoresis include protein profiling and differential expression analysis
- DIGE is a improved modification of 2D electrophoresis which allows qualitative and relative quantification of proteins between different phenotypic isolates
- There are certain advantages and dis-advantages of 2D electrophoresis and have to be factored in depending upon the availability of protein, sensitivity of mass spectrometric machine, cost and tissue availability