

LECTURE-5

GEL-BASED PROTEOMICS

HANDOUT

PREAMBLE

Gel-based proteomics is one of the versatile fields of proteomics, which has provided us with tools that can be used for protein separation, characterization as well as quantification. The goal of proteomic studies is to detect altered protein expression and modifications associated with disease or to find molecular targets for biomarkers and therapy. Gel-based proteomics includes techniques like one-dimensional SDS-PAGE or Native PAGE, 2DE and DIGE. The proteins that are differentially expressed can be further identified using techniques like Mass Spectrometry. Gel-based proteomics mainly exploits the principle of electrophoresis; and provides information about protein properties such as molecular weight and isoelectric point. In this lecture we will provide an overview of the different gel-based techniques and their working principles.

OUTLINE OF LECTURE

1. Electrophoresis
2. Sodium Dodecyl Sulphate (SDS)-PAGE
3. Blue Native (BN)-PAGE
4. Two Dimensional Gel Electrophoresis (2-DE)
5. Fluorescence based Difference in Gel Electrophoresis (DIGE)
6. Comparison of gel-based techniques
7. Conclusions

BOX-1: TERMINOLOGIES

- **Gel-based Proteomics:** High-throughput techniques for separation of proteins from complex mixtures using polyacrylamide gels.
- **Electrophoresis:** The separation of charged molecules under the influence of an applied electric field.
- **IEF:** Iso-electric Focusing: Separation of proteins based on their isoelectric points (pI).
- **IPG:** Immobilized pH Gradient
- **SDS-PAGE:** Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis, which brings about further separation based on their relative molecular mass.
- **2-DE:** Two Dimensional Gel Electrophoresis is a protein separation technique that carries out separation using isoelectric focusing in first dimension, followed by SDS-PAGE in the second dimension.
- **2D DIGE:** 2D Difference in Gel Electrophoresis is an advanced form of 2DE that allows simultaneous analysis of test and control samples on a single gel by

TABLE FOR HISTORY AND EVOLUTION

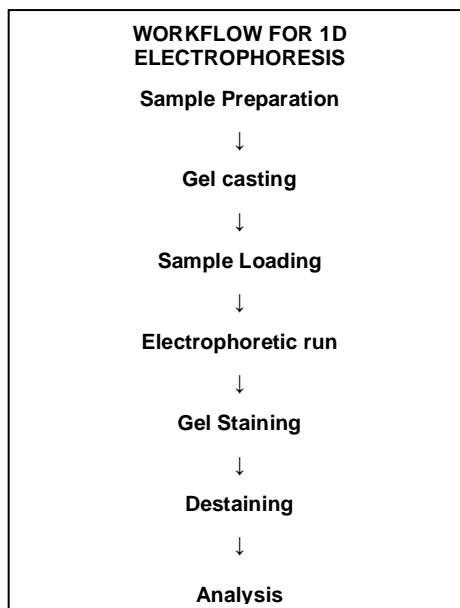
- **1930:** Arne Wilhelm & Kaurin Tiselius invented Electrophoresis.
- **1975:** 2-DE was reported independently by Klose and O'Farrell.
- **1990s:** Angelika Gorg contributed for development of IPG strips.

1. ELECTROPHORESIS

Electrophoresis is the widely used technique for protein separation and works on the principle of migration of charged molecules in a gel matrix towards the oppositely charged electrode, under the influence of an applied electric field. It is a powerful technique for finer protein separation and visualization of these separated proteins. Electrophoresis was invented by Prof. Tiselius in 1930 as the moving boundary method to study electrophoresis of proteins. It has been extensively used since then and numerous advancements have been brought about in this technique.

2. ONE-DIMENSIONAL GEL ELECTROPHORESIS

One Dimensional Gel Electrophoresis relies on the principle of separation of protein molecules on the basis of their charge to mass ratio and molecular weight. The low molecular weight proteins are able to migrate larger distances on the gel as compared to the higher molecular weight proteins. Polyacrylamide gels are formed from the polymerization of two compounds, acrylamide and N, N₁-methylene- bis-acrylamide (Bis, for short). Bis is a cross-linking agent for the gels. The polymerization is initiated by the addition of ammonium persulfate along with either β-dimethyl amino-propionitrile (DMAP) or N,N,N₁,N₁- tetramethylethylenediamine (TEMED). The gels are neutral, hydrophilic and three-dimensional networks of long hydrocarbons cross-linked by methylene groups. The commonly employed 1DE techniques include SDS-PAGE and NATIVE PAGE.



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- Acrylamide: matrix or gelling agent
- Bis-acrylamide: Cross linking agent
- SDS: Sodium Dodecyl Sulfate, Anionic detergent which imparts uniform negative charge on all the protein molecules which undergo separation

- APS: Ammonium Per Sulfate; Initiates polymerization
- TEMED: N,N,N',N'- tetramethylethylenediamine; Stabilizes the free radicals and promotes polymerization
- β -Mercaptoethanol: Acts as a reducing agent and breaks the Disulfide bonds.

Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis (SDS PAGE) is the technique which uses an anionic detergent namely Sodium Dodecyl Sulfate, which provides the uniform negative charge on protein molecules. This entails the electrophoretic separation to be brought about only on the basis of the molecular weight of the proteins.

Illustration: Electrophoresis techniques: SDS-PAGE

Electrophoresis is a powerful technique for protein separation and separated proteins can be visualized after subsequent staining steps. It is based on the principle of migration of charged proteins in an electric field. The polyacrylamide gel containing SDS is cast between glass plates as a vertical slab in the same buffer that is used for electrophoresis. The molecular dimensions of the pores can be controlled by varying the amount of N,N'-methylenebisacrylamide with free-radical cross-linking being facilitated by APS and TEMED.

Sample wells of uniform size, shape and separation are made using a comb, which is placed in the gel as soon as it has been poured. After the gel has polymerized, the comb is removed, providing a gel ready for the process. SDS is a negatively charged anionic detergent that binds to protein molecules and causes them to denature. The DTT used breaks any disulphide linkages that may be present. The binding of SDS causes the proteins to have a uniform charge-to-mass ratio, thereby allowing separation purely on the basis of molecular weight.

The protein samples are loaded into the wells with the help of a micropipette. Once the samples have been loaded, a direct current supply between 100-350 V is passed depending upon the size of the gel for a time sufficient to separate the protein mixture into discrete bands based on their molecular weight. Progress of electrophoresis can be observed with the help of tracking dye. The larger proteins are retarded in the gel and remain close to the point of application while the smaller proteins migrate further along the gel. The gel is then stained with either Coomassie or silver and viewed to observe the various discrete protein bands.

Illustration: SDS-PAGE data analysis

SDS-PAGE analysis is done to study the expression of a protein from the control and the sample, to detect the molecular weight of the protein using the molecular weight marker and to detect the quantity by the intensity of the protein. For this purpose software can be used.

- *Load the gel for analysis into the software. Specify the number of lanes and lane width, and drag to the image and the lanes will be created automatically.*
- *Once the parameters are set click "next".*
- *Specify the molecular weight of the standard. Now click on the marker to know the molecular weight of the sample in the gel.*

- *Specify the unit in which the quantity of the protein has to be displayed. Click on the band to know the quantification value of the protein band.*

3. Blue Native (BN) PAGE

Blue Native PAGE allows proteins to be studied in their native state and does not involve any denaturation step. The Coomassie dye used herein provides necessary charge to the protein complexes and further helps in their separation during migration on the gel. Thus the electrophoretic mobility mainly relies on the negative charge that is imparted by the dye, and the size and shape of the protein complexes that are being studied. It can be used for identification of multi-protein complexes and hence provides an integrative view of the protein, as they need to be separated in the native conditions.

1DE can be effectively used to determine the whole protein molecular weight, the molecular weight of individual components of a complex protein, detection of isoforms of a single protein and post-translational modifications. It can also be used to purify a particular protein from a complex mixture, which has to be further used for different applications and can provide an integrative view of the protein function.

Illustration: BN-PAGE

- *The polyacrylamide gel is cast between glass plates as a vertical slab in the same buffer that is used for electrophoresis.*
- *The gel is prepared by free radical-induced polymerization of acryl-amide and N,N'-methylenebisacrylamide in a suitable buffer. Ammonium persulfate (APS) and tetramethylethylenediamine (TEMED) are added to facilitate generation of free radicals and cross-linking. The molecular dimensions of the pores can be controlled by varying the amount of N,N'-methylenebisacrylamide.*
- *Sample wells of uniform size, shape and separation are made using a comb that is placed in the gel as soon as it has been poured. After the gel has polymerized, the comb is removed, which provides wells for loading the samples.*
- *The protein sample, present in a suitable buffer system, is mixed with the Coomassie blue dye, which provides the necessary charge to the protein complexes thereby facilitating their separation*

in the gel. Unlike SDS, the dye does not denature the proteins but binds to them in their native state.

- The protein samples are then loaded into the wells with the help of a micropipette. Once the samples have been loaded, a direct current supply of around 100-350 V is passed depending upon the size of the gel for a time sufficient to separate the protein mixture into discrete bands based on their charge-to-mass ratio.
- Progress of electrophoresis can be observed with the help of tracking dye. The larger proteins are retarded in the gel and remain close to the point of application, while the smaller proteins migrate further along the gel. The gel is then stained with Coomassie brilliant blue and viewed to observe the various discrete protein bands.

Illustration: SDS-PAGE vs. BN-PAGE: Interactivity-1

Three tubes having protein samples containing insulin, myoglobin and hemoglobin are to be tested. Insulin is a 51 amino acid peptide hormone having a molecular weight of 5.8kD. It is composed of two chains linked by disulphide bonds and plays a very important role in maintenance of blood glucose levels. Myoglobin is an oxygen-binding, globular protein composed of a single polypeptide chain having 154 amino acid residues with a molecular weight of 16.7kD. It is made up of 8 alpha helices and is structurally related to hemoglobin. Hemoglobin is an iron containing, oxygen-transport protein. It is composed of four subunits (2a and 2b) linked together by salt bridges and other hydrophobic interactions with a mass of 68kD.

- How can one determine which tube contains which protein and then estimate the number of subunits present in each protein?
- Run all three samples by BN-PAGE.
- Run all three samples by SDS-PAGE.
- Run all three samples by both BN-PAGE and SDS-PAGE.

- *HINT:* A comparison of the electrophoretic separation profiles of hemoglobin, insulin and myoglobin using both BN-PAGE and SDS-PAGE will allow one to determine which tube contains which protein sample and how many subunits may be present in each protein.
- *BN-PAGE:* Since the molecular weight of each protein is known, the migration distance will indicate which band corresponds to which protein.
- *SDS-PAGE:* More than 1 band will appear for Hb and insulin indicating that they have multiple subunits. The thicker bands are indicative of more than one subunit of nearly identical molecular mass.

Illustration: SDS-PAGE vs. BN-PAGE: Interactivity-2

Which technique would be suitable for determination of molecular weight of the enzyme α -amylase? BN-PAGE or SDS-PAGE?

Determination of molecular weight of α -amylase:

SDS-PAGE is commonly used for determination of molecular weight of an unknown protein by running it along with other protein markers of known molecular weight. Mobility of the unknown protein can therefore be used to determine its molecular weight from a graph as shown above. Based on this information the molecular weight of α -amylase is 53kD.

4. TWO DIMENSIONAL GEL ELECTROPHORESIS (2-DE)

Two Dimensional electrophoresis separates proteins on the basis of isoelectric point and molecular weight. It can be effectively used for applications such as differential proteomic analysis, isoforms separation or analysis of post-translational modifications. In 2-DE the first separation occurs based on the isoelectric point of the proteins and the second separation, which is carried out orthogonally, occurs based on the molecular weight. Isoelectric focusing separates proteins based on their isoelectric point (pI) and results in immobilization of the protein at its isoelectric point at which the overall charge on the protein is zero. The IPG strips are equilibrated with DTT and IAA for reduction and alkylation, respectively. This makes the strip ready for the second dimensional run. In the second dimension, separation based on size occurs by employing SDS-PAGE. The gels are stained with Coomassie blue dye to stain the protein spots. Destaining follows this step and then the gels are scanned. The gel images thus obtained are analyzed and significant spots are excised and digested for further analysis by Mass Spectrometry.

Illustration: An overview of Two-dimensional electrophoresis (2-DE)

- *Electrophoresis is a powerful technique for finer protein separation and visualization of separated proteins. It is based on the principle of migration of charged proteins in an electric field. Electrophoretic techniques have developed significantly for improved protein separation and detection.*
- *Prior to isoelectric focusing in 2-DE, the commercially available IPG strips must be rehydrated. This is done by soaking them for 10-20 hours in the protein sample, which is contained in a suitable buffer solution. Once the strips are rehydrated, they are covered with mineral oil to prevent evaporation of solution and drying of the gel.*
- *Once the IPG strips have been rehydrated, the strip is placed in the tray and the sample is added through the sample cup followed by the cover fluid to prevent drying of gel. In passive loading, the gel strip is placed face down in the cover fluid containing the sample for 10-12 hours after which it is run in an electric field.*
- *IEF - These loaded strips are then focused on an isoelectric focusing unit by passing current. The various proteins of the sample mixture migrate in the electric field and come to rest when the pH is equal to their pI i.e. they become neutral and are no longer affected by the electric field.*

Progress of electrophoresis is monitored by means of a tracking dye like bromophenol blue (BPB), which is a small molecule and therefore migrates ahead of all other proteins.

- SDS-PAGE - The IPG strip is equilibrated in a reducing agent like DTT followed by an alkylating agent, iodoacetamide, which prevents reformation of the reduced bonds. This strip containing the separated proteins is then placed on the SDS-polyacrylamide gel slab for further protein separation in the second dimension based on their molecular weight.
- The proteins on the IPG strip are then subjected to SDS-PAGE by applying a direct current between 100-350V depending upon the size of the gel. Any proteins that may have been present as a single band on the IPG strip due to similar isoelectric points can now be separated on the basis of their molecular weight with smaller proteins migrating farthest.
- View of a sample gel, which has been run by 2-DE and stained with Coomassie blue, is shown. Each spot provides information about the MW and pI of the proteins.

Illustration: Passive and Active Rehydration

A representative protocol of passive and active rehydration is demonstrated in following animation.

- Passive rehydration- Prepare the sample for loading on the strip equivalent to 600 μ g protein concentration for 18cm IPG strip. In case user is loading for 7cms and 24cms, user can calculate protein accordingly.
- Load the sample in the reswelling tray/manifold and avoid air bubbles.
- Remove the strip from -20°C and allow it to thaw.
- Place the strip on the reswelling tray with the gel side facing down and keep it for half an hour.
- Add cover fluid to the strip to prevent evaporation. While adding avoid air bubbles. The strip must be covered with oil.
- Overnight process helps the IPG strip to get rehydrated with protein sample in the absence of electric field. Once the passive rehydration is over, the strip is ready for IEF run.
- Active rehydration - In active rehydration, sample is loaded in a single electrode manifold unit in case of single strip or strip loaded into manifold directly if many strips are there. Unlike use of rehydration tray in passive rehydration, for active rehydration manifold is used.
- The electrode assembly are held together and placed at the correct position by the plastic support. The system goes in for automatic calibration for display, buzzer and light to check for working condition.
- Connect the instrument properly. ON the system and Select active rehydration protocol for 5hrs at 20V/50mA for each strip. Now after the active rehydration step user proceeds for IEF step.

Illustration: Isoelectric Focusing

Proteins exhibit unique isoelectric property and these properties are exploited for the separation of individual proteins from a pool of proteome. A representative protocol is demonstrated in following animation.

- Place the manifold on the table. Take a clean tissue paper and clean all the lanes of manifold to make it free from dust and dry it completely.
- Place the instrument on leveled surface., Take the manifold and position it inside the groove of the instrument. Ensure that the surface is level by placing the round spirit level and adjust if necessary at the back edge of the instrument.
- Pick the strip end with forceps from reswelling tray (which is kept for passive rehydration). Drain out excess oil by tapping it on tissue paper without folding the strip and keep it straight. Place the positive side of strip on the positive end of instrument with gel side up.

- *Cut the paper wicks into 2 pieces. Add distilled water to the wicks until it gets wet. Place it on the strip end such one end of the wick overlaps the end of the gel on the strip. The paper wicks absorbs impurities /salts and aid in current conductance.*
- *Add cover fluid to the well containing the strips and to the other wells. Maintain the level of cover fluid must not over flow.*
- *Place the electrodes on either side of the tray such that the tip of the electrode touches the paper wick and is immersed in the cover fluid if not add some more oil and close the lid.*
- *Connect the instrument properly. ON the system and Start the software by clicking on the icon. Select the specific protocol for the sample to run the IEF. User can change the protocol according to their requirements. User can change the select the strip length and define the number of strips running in the experiment.*
- *The set protocol is reached, when the sample preparation is perfect and the IEF run without any interference. The set protocol will not reach if the sample preparation is imperfect having impurities and may interfere during the run*
- *In case of improper run replace the wicks with the new one. Restart the run. The run continues once the wicks are changed.*
- *Protein stops moving at a point when the net charge is zero which is known as Isoelectric point. Stop the run once the set voltage is reached and the run is completed*
- *Remove the IPG strip carefully without bending. Store the strip at -80°C until further processing. Once the equilibration buffers are prepared the strip can be taken out to carry out 2D run.*

Illustration: Equilibration of IPG strips

Separated proteins in the strip undergoes equilibration step so that the multi-subunit proteins can be separated. This step is helpful for prevention of reunion of the separated proteins and stabilization of the separated protein in the gel. A representative protocol is demonstrated in following animation.

- *Equilibration buffer-I: it consists of urea as denaturing agent; SDS, provide uniform negative charge to the protein; DTT, as the reducing agent of disulphide bond; glycerol, as the stabilizing agent of polyacrylamide gel.*
- *Equilibration buffer-II: it consists of urea as denaturing agent; SDS, provide uniform negative charge to the protein; IAA, as the acetylating agent of reduced disulphide bond; glycerol, as the stabilizing agent of polyacrylamide gel.*
- *Place the IPG strip in the equilibration tray with gel side up. Add the equilibration buffer-I to IPG strip and keep it on shaker for 15min. The strip must be covered with the buffer. Dithiothreitol in equilibration buffer reduces the disulphide bond and helps to maintain all proteins in their fully reduced state. Remove the strips from the well and place it in the new well.*
- *Add equilibration buffer-II to the strip and keep it on shaker for 15min. The strip must be covered with the buffer. IAA acetylates the reduced disulphide bond and prevents its oxidation, back folding and aggregating of protein subunits. Remove the strips from the well and place in the new well. Wash the strips with tank buffer after second equilibration step. Now the strip is ready for SDS-PAGE run.*

Illustration: Second Dimension Separation

Proteins exhibit different molecular weight depending on the amino acid composition. This property is exploited to separate proteins in second dimensions on SDS-PAGE gels. A representative protocol is demonstrated in following animation.

- *Prepare the gel casting template unit. Space within the glass plates helps to hold the gel or gel is formed within this space. For easy separation of glass plates, plastic sheets are used.*
- *Prepare 12.5% resolving gel cocktail consisting of 49 ml acrylamide and bisacrylamide 30ml 1.5M Tris -HCl(pH 8.8), 10% 1.2 ml SDS and 10% 1.2 ml APS, 165ul TEMED and 120ml water. Add APS at the last minute. Pour the solution at once and avoid air bubbles.*

- *Spray 0.1% SDS to prevent drying off the gel, and helps to produce even surface on top of the gel. When the gel is getting polymerized, start the process of equilibration for IPG strips.*
- *Add equilibration buffer-I to the strip and keep in shaker for 15 min. The strip must be covered with buffer. Dithiothreitol in equilibration buffer reduces the disulphide bond and helps to maintain all proteins in their fully reduced state. Remove the strips from the well and place in the new well. Add equilibration buffer-II to the strip and keep in shaker for 15 min. The strip must be covered with the buffer. IAA acetylates the reduced disulphide bond and prevents its reformation of disulfide bonds.*
- *The tank buffer consists of tris base, glycine provides proper conductance and SDS adds negative charge to protein for separation.*
- *Wash the strips with tank buffer after second equilibration step. Now the strip is ready for 2D run. Transfer the glass plate with gel slab into glass plate holder to keep IPG strips.*
- *Place the strip in the gel and ensure that there shouldn't be any gap or air bubbles between the gel and the strip.*
- *Agarose sealing: Agarose need to be liquid hot before use. Seal the strip and the gel using agarose sealing solution and wait till it solidifies.*
- *Place the glass plate containing strip and dummy plates in the gel holding unit. All the grooves in the unit need to be filled. In case if user is running only two gels, place each gel unit on either side of the unit. It helps for the even flow of voltage across the unit.*
- *Prepare 1x SDS buffer from 10x. Pour the buffer into the chamber till the maximum level(without the gel cassette) in the lower tank is achieved. The lid acts as upper reservoir for buffer chamber. Once the buffer is added till the max level the unit is ready for 2D run.*
- *Connect the setup, if not properly connected the power back will show error. Set the voltage 100V for 1hr and 300V for 4hours. The water bath temperature is set at 20°C, the 2D separation is exothermic reaction. To remove the excess heat water is circulated inside the unit.*
- *SDS denatures the proteins and give negative charge to the proteins which allows the proteins to move from negative to positive terminal based on the pore size of the gel the proteins are separated on the basis of their molecular weight*
- *Place the gel in the distilled water to remove excess SDS that may interfere in staining. Now gel is ready for staining process.*

Staining methods

Different staining methods are commonly used to stain the 1D or 2D gels.

1. Coomassie Brilliant Blue (CBB): Coomassie blue dyes (R-250 & G-250) are low cost, organic dyes that are easy to use for staining of proteins that have been separated by electrophoretic techniques. Gels are soaked in the dye solution dissolved in methanol and acetic acid, after which excess stain is then washed off with a destaining solution. The higher affinity of proteins towards the dye molecules, allows the protein bands to be selectively stained with sensitivity of 8-100 ng without significant staining of the background. These dyes are also compatible for further MS-based applications.

2. Silver staining: Electrophoresis gels are saturated with silver ions in the form of either silver nitrate or as an ammonia-silver complex after fixing the proteins in the gel. The less tightly bound metal ions are subsequently washed off and the protein-bound silver ions are reduced to metallic silver using formaldehyde under alkaline conditions in presence of sodium carbonate or citrate buffer solution. Although as little as 1 ng of protein can be detected by silver staining, the gel-to-gel reproducibility remains an issue. Compatibility of silver stains with MS is another issue, which has however, been overcome in recently introduced silver stains.

3.SYPRO Ruby Red: This is a ruthenium-based metal chelate fluorescent stain that provides a single step protein staining procedure with low background staining in polyacrylamide gels. They have been observed to be as sensitive as the silver stains (0.25-1 ng) with the linear dynamic range extending over three orders of magnitude,

thereby showing better performance than CBB and silver stains. This stain can also be combined with other dyes thereby allowing multiple detections in a single gel.

4. SYPRO orange: This dye is less sensitive than SYPRO Ruby Red but is also capable of detecting proteins in SDS-PAGE gels in a rapid single step process without the requirement for any destaining procedure. As little as 4-30 ng of protein can be detected by this fluorescent dye and it is compatible for further MS-based applications. Two other similar dyes having comparable sensitivities and similar excitation and emission wavelengths are SYPRO Red and Tangerine.

5. Cyanine dyes: They are water-soluble derivatives of N-hydroxy succinimide that covalently bind the ϵ -amino groups of a protein's lysine residues and are spectrally resolvable as they fluoresce at distinct wavelengths. The labeled protein samples can therefore be mixed and run on a single gel, thereby eliminating the problem of gel-to-gel variations, the principle employed in difference gel electrophoresis (DIGE). Cy3, Cy5 and Cy2 having sensitivities of 0.1-2 ng are most commonly used for proteomic and MS-based applications.

6. Lightning fast/Deep purple: A fluorescence-based stain obtained from the fungus *Epicoccum nigrum* that can be used for detecting proteins in 1-D and 2-D gel electrophoresis with sensitivity down to 100 pg protein. Stained proteins are excited by near-UV or visible light with maximum fluorescence emission occurring at around 610nm. These dyes are suited for further use with Edman or MS applications.

7.Pro-Q-Diamond: This fluorescent dye is capable of detecting modified proteins that have been phosphorylated at their serine, threonine or tyrosine residues. They are suitable for use with electrophoretic techniques or with protein microarrays and offer sensitivity down to few ng levels, depending upon the format in which they are used. This dye can also be combined with other staining procedures thereby allowing more than one detection protocol on a single gel.

Illustration: Staining methods

Detection techniques using organic and fluorescent dyes for gel-based proteomics have developed significantly over the last few years allowing researchers to carry out high-throughput studies with increased sensitivity.

Coomassie Brilliant Blue (CBB)

- *The completed electrophoresis gel is placed in a tray containing the Coomassie blue staining solution, typically R-250, that has been dissolved in an aqueous solution of ethanol and acetic acid. Negatively charged Coomassie dye interacts with proteins through ionic and non-covalent interactions. This tray is then placed on a mechanical rocker that allows for uniform contact of the gel with the solution by means of gentle rocking.*
- *The stained gel is then placed in a destaining solution consisting of 50% methanol & 10% acetic acid to remove any excess dye that may be bound to the gel. The gel is finally removed and viewed by means of a scanner where the blue protein bands can be easily detected.*

Silver staining

- *The completed gel is first placed in a fixing solution of methanol and acetic acid that fixes the protein bands in the gel and minimizes any diffusion. This must be subjected to gentle shaking for around 30 minutes after which the silver stain solution is added. The gels are rocked gently in order to allow for proper and uniform staining.*
- *The silver stained gels are first washed to remove any excess stain and then placed in a developing solution where the silver ions get reduced to metallic silver. Formaldehyde in an alkaline solution in presence of sodium carbonate or other alkaline buffers is commonly used for this process. This gel can then be viewed as dark bands against a light background as shown in the figure on the right.*

SYPRO Ruby Red

- *The completed gel is first placed in a fixing solution of methanol and acetic acid that fixes the protein bands in the gel and minimizes any diffusion. This must be subjected to gentle shaking for around 30 minutes after which the gel is soaked in fluorescent SYPRO Ruby Red stain solution. The gel is rocked gently for uniform staining with the ruthenium-based chelate dye.*
- *The gel can be left overnight in the dye solution and then washed with a methanol-acetic acid solution. The MS-compatible gel is then viewed using UV or Laser-scanning instruments with an excitation wavelength near 280 or 450 nm. The stain has two excitation maxima and emission maxima of 610 nm.*

Cyanine dyes

- *In this detection technique, the dyes are mixed with the samples prior to electrophoresis. Each protein sample as well as the internal standard is labeled with a differently fluorescing cyanine dye, which allows all protein samples to be simultaneously run on a single 2-DE gel.*
- *This gel can be viewed by illuminating it alternately with excitation wavelengths corresponding to the various Cy dyes. This technique minimizes gel-to-gel variations and allows a large number of samples to be run using fewer gels, thereby proving to be extremely useful for large-scale proteomic applications.*

Illustration: Methodology for gel scanning and 2D data analysis

The proteome obtained by the 2-DE process is analyzed to identify the significant spots by comparing the control and the treated samples. It offers a flexible solution for the comprehensive visualization, exploration and analysis of 2D gel data. A representative protocol is demonstrated in following animation.

- *Gel Scanning – Place the gel in the middle of the scanner platform, avoid air bubbles. Click on shortcut icon or you can start the Scanner Control software using the Start menu. The Scanner Control window appears. Preview window appears. User needs to set the parameters before going for scanning. During scanning, check the image in the Image Quant Preview window for saturation. Saturated pixels appear in red. If the image appears saturated, you might need to expose a clean screen to the sample for a shorter amount of time. If the image appears faint, you might need to expose a clean screen for a longer amount of time. If the image appears usable, continue with the next scan and save the image. Naming of gel must be proper with project name, type of sample etc.*
- *Create project, Load the control and treated gel images in folder for analysis. Once the gels are loaded cropping need to be carried out. Images rotating need to be carried out to align all the gels in same orientation; image processing does not affect original image files. The bold horizontal grid line plays the role of landmark to help you visualize the rotation. Flip icon is used when gel images are scanned in the wrong direction; you can Flip Horizontally or Vertically to produce their correct mirror image.*
- *You can crop your gel images with the crop tool. This creates new gels that only contains the selected area and removes the outer area. Once the gels are cropped the images are ready for the analysis.*
- *Once gels have been added to a project and you have taken a good look at them, you are ready to detect spots. A spot delineates a small region in the gel where protein is present. This shape is automatically differentiated by a spot detection algorithm and quantified; its intensity, area and volume are computed. To detect the spots you need to define the optimum spot detection parameters. The parameter smooth helps to detect all real spots and split the overlapping ones. Subsequently, Saliency and Min Area values helps out to filter the noise.*
- *After spot detection, zoom each and every spot to check for a real spot. The software detects dusts and artifacts, which need to be removed from analysis.*
- *User must detect dusts, artifacts from the detected spots and remove it from analysis. If user finds unwanted spot, he can delete it and if he finds spots exactly at the same position across the gels, such spots can be landmarked. Landmark of spots helps in matching. Once land marking is over, save the gels and import the gels into matching folder.*
- *Matching of gels must be carried out after land marking. Matching algorithm first matches the landmarked spots, then matches the nearby spots. In case if less number of matches are produced, create few more landmarks and try again. Land marking must be performed in such a way that it should cover entire gel area.*

- *Vector lines helps to check the matching process, how correctly the gels have matched with each other. If the vector lines are of same length and in same direction, we can say that matching is OK. The overlay option helps user to check the profile of each gel, and to detect matching pattern.*
- *Once overlay is done, and user is satisfied with matching user can proceed with the data analysis. Now save the workspace and drag the images into classes folder.*
- *The overlay option helps user to check the profile of each gel, to detect matching pattern.*
- *Once overlay is done, and user is satisfied with matching one can proceed with the data analysis if not again restart the matching.*
- *Spots across the gel can be selected and compared to know their pI, volume, intensity, MW etc.*
- *The protein spots can be represented in the 3-D form with peak height denotes its intensity and can be rotated to view in different angles. This helps user to make a rough calculation for the fold difference expression of protein between the samples. To make accurate calculation for fold difference, user must do the statistical analysis of the data.*
- *Click on the statistical analysis table to get statistical information of the spots. The data can be used to analyze fold difference between the spots, to determine increased and decreased spot intensity, to generate a histogram for distribution of spots and calculate t-test, ANOVA, and other statistical analysis.*

Illustration: Methodology for the Spot picking

- *Once the significant spots of interest are identified from the 2D-analysis software, spots can be picked either manually or by robotic arm for protein identification by MALDI-MS. Spot excision can be carried out on normal DIGE-gels or stained gels soon after the electrophoretic run.*
- *Clean the laminar airflow thoroughly with ethanol. Blower is kept ON to avoid any contamination like keratin from user actions. For manual picking the gel need to be placed on the supportive material, so that the stained spots are easily visible for naked eyes. User can start manual spot picking with help of scalpel and transfer the gel pieces to tubes. If spot is small perform manual picking once, if large spot perform picking twice or thrice to cover the spot completely. Transfer the gel pieces into fresh eppendorf tube.*
- *Need to be stored at 4°C in freezer, before MS analysis.*
- *Transfer the gel pieces into fresh eppendorf tube. The gel pieces need to be placed in distilled water to avoid shrinking due to evaporation. Need to be stored at 4°C in freezer, before MS analysis.*
- *Before automatic spot picking, the preparative gel from the 2D run electrophoresis need to be scanned for protein spots to be visible. The preparative gel soon after 2D run need to be taken for scanning. Depending on landmarks, the robotic arm fixes the co-ordinates for other spots to be picked.*
- *The DIGE scanning for preparative gel needs to be carried out depending on the settings based on dye used.*
- *Soon after the instrument is ON, it goes for calibration. User must wait and after calibration the display pops up for scanning.*
- *The image from DIGE scanner and image from spot picker need to be overlaid. The DIGE image helps user to annotate the spots and spot picker image helps user to fix the landmarks of spots for the robotic arm to perform picking.*
- *The user must select the significant spots for global or differential protein expression profiling, and annotate them accordingly. Depending on the spots selected the robotic arm starts picking individual spot in one go. Try to annotate at the middle of the spot, to get maximum concentration of protein from a gel piece.*
- *Gel pieces need to be stored at 4°C in freezer prior to the MS analysis.*

5. FLUORESCENCE BASED DIFFERENCE IN GEL ELECTROPHORESIS (DIGE)

There are various limitations associated with 2-DE due to the gel-to-gel variations and manual artifacts, which emerge mainly from inconsistency in sample preparation, and then from the subsequent gel running itself, during the 1st and 2nd dimensions. These limitations eventually lead to the lack of reproducibility. This has necessitated the need for the development of a technique, which would overcome these limitations and help in solving the purpose of protein studies in a better way. DIGE technique will be discussed in detail in next lecture.

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6. COMPARISON OF TECHNIQUES

TECHNIQUE	ADVANTAGES	DISADVANTAGES	APPLICATIONS
1DE	<ul style="list-style-type: none"> • Easy technique to check for protein purity in a given protein extraction. 	<ul style="list-style-type: none"> • Large number of proteins cannot be separated from a complex mixture with good resolution. • It cannot be used to study whole proteome or to analyze complex fluids like serum or cell lysates. 	<ul style="list-style-type: none"> • Test protein purity • Study protein expression
2DE	<ul style="list-style-type: none"> • Powerful technique for simultaneous separation of thousands of proteins. • Highly sensitive visualization of proteins as small differences in protein expression levels can be detected with statistical confidence. • Relatively easy to handle and affordable 	<ul style="list-style-type: none"> • Laborious and time consuming. • Requires 800-1000 ug of protein as the starting material. • In one gel, only one sample can be analyzed. • Requires procedures like staining or fixation to be done after the second dimension gel electrophoresis 	<ul style="list-style-type: none"> • Study global and differential protein expression and resolution of complex proteins (Chen et al. 2004) • For Biomarker Discovery (Lescuyer et al. 2007)
DIGE	<ul style="list-style-type: none"> • Requires less amount of starting protein material. • Highly reproducible and sensitive as two different samples can be analyzed on the same gel and so the differences in protein expression levels are purely attributed to biological variations. • Better quantitative comparison is attributed to the use of internal control. • No need for fixation or destaining as fluorescent dyes are used for sample labeling. 	<ul style="list-style-type: none"> • Only two different types of samples can be analyzed on a single gel. • Spot excision is a problem since they are not visible to the naked eye and hence require aids such as robotic Spot picking. • Fluorescence detection gives high background at times when signals from different labels may get mixed. 	<ul style="list-style-type: none"> • In Cancer studies (Bai et al. 2010) • For Biomarker discovery (Uemura et al. 2009) • For studying post translational modifications (DeKroon et al. 2012)

7. CONCLUSIONS

This lecture provides an overview of different gel-based techniques and a brief idea about different applications. The scope of these tools can be further explored depending upon the purpose for which it has to be utilized. Gel-based techniques have been widely used since their inception because of its user-friendliness and because of the vast amount of data that can be procured from the analysis of these results.

8.REFERENCES

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