HANDOUT

LECTURE-13

GEL-BASED ELECTROPHORESIS

TWO-DIMENSIONAL ELECTROPHORESIS (CONTINUED)

Slide 1:
The previous lecture initiated the discussion on gel-based proteomics, specifically 2-DE and the different steps involved in its performance. Gel-based proteomics also includes various techniques such as SDS-PAGE, Blue Native PAGE, 2-DE and advanced forms such as difference in gel electrophoresis or DIGE.

Slide 2:
This lecture will continue the discussion on the stepwise workflow of listed in this slide. The first step (Isoelectric focusing) has been covered in detail.

Slide 3:
This lecture will focus on:

- The 2nd step of equilibration of the IPG strip
- The 3rd step of performance of SDS-PAGE which is protein separation in second dimension based on molecular weight.
- The fourth step which is staining procedure to visualize the separated proteins on the gel, a step that needs to be optimized to suit the protein yield.

Slide 4:
During conventional SDS-PAGE, which involves protein separation solely in 1 dimension on the basis of mass alone, the protein samples are 'prepared' by boiling them in the presence of SDS and other reagents. This process denatures the proteins and involves reduction and alkylation of proteins. However, when SDS-PAGE is applied

as the second step of protein separation in the process of 2DE, boiling the protein samples is no longer possible. In this case, this step is replaced by a process called 'equilibration' explained in detail below.

Slide 5:

The equilibration process, taking place after protein isoelectric focusing in the 1st dimension, is designed to solubilize the proteins immobilized on the IPG strip . This step involves the cleavage of both inter and intra-chains disulphide bonds and alkylation of the sulfhydryl groups of cysteine residues, rendering the protein soluble and allowing coating with SDS, which is an ionic detergent that renders the protein negatively charged. The equilibration procedure occurs in 2 steps.

Slide 6:

- This first step involves breakage of disulphide bonds under reducing conditions and saturation of the strip with the detergent SDS.
- Equilibration Buffer: The base for this buffer is similar in both first and second step and includes 6 M urea, 2% SDS, 0.375 M Tris-HCl, pH 8.8 and 20% glycerol. The final ingredient, however differs between the first 2 steps. In the first step the base buffer is supplemented with Dithio tritol (DTT), while in the second step is supplemented with Iodoacetamide (IAA). Please note that the here recipe is only a reference, slight modifications can be added for optimization.
- The first equilibration step is performed for 10 or 15 hours depending upon strip length. Once this step is complete the solution is removed and the buffer for the next step is added.

Slide 7:

The second equilibration(which, as previously mentioned, requires the buffer to be supplemented with 135 mM iodoacetamide, an alkylating agent) is essential for:

- Protein alkylation to prevent reoxidation/reformation of the disuphide bonds
- Alkylation of the residual DTT, which minimizes vertical streaking

This step is performed for 10 to 15 min depending on the strip length. Once both the equilibrations are done, SDS-PAGE can be commenced.

Slide 8:
The following is the step-wise procedure for performing first and second equilibration steps.

- The strip is first equilibrated in the first equilibration buffer by placing it in a tray in a well containing the buffer
- Following completion of that step, the first equilibration buffer is removed and replaced by the second equilibration buffer
- The DTT reductant will be used for for the first step followed by IAA which functions as a scavenger of the excess reductant and alkylates proteins and prevents the re-oxidation.

Slide 9:

SDS PAGE: As an important note, when this process is applied as the sole method for protein seperation in 1DE, it requires both the addition of the stacking and resolving gels.

However, SDS-PAGE when performed in the 2nd dimension after isoelectric focussing of proteins does not require the stacking gel.

Slide 10:

SDS-PAGE is one of the most widely used electrophoretic techniques that separates the proteins based on their size or molecular weight. The molecule SDS or sodium dodecyl sulphate is an anionic detergents providing negative charge. It binds at a ratio of 1.4 g of SDS per gram of protein to provide a similar charge to mass ratio to all the proteins undergoing seperation. Therefore, while protein migration in the electric field will be based on the negative charge, the uniform negative charge bestowed on the proteins will ensure that their seperation occurs purely on the basis of their molecular

weight. in the electricIDE SDS-PAGErequires the preparation the protein for denaturation, which is achieved by boiling them in SDS, ß-mercaptoethanol and DTT. This performs the functions of denaturation and the breaking of disulphide bonds. Since the workflow of 2DE would not permit boiling, this step is replaced by the equilibration steps which acheives the same objectives, as previously discussed.

Slide 11:
Components used in preparation of the SDS-PAGE gel:

- Acrylamide is the gelling agent and provides the material for the formation of the gel matrix while Bis-acrylamide is a cross linking agent. Addition of acrylamide and bis-acrylamide in a fixed ratio acheives the crosslinking of acrylamide by the cross linker.

Slide 12:
However, additional reagents are required to polymerize the gel, including APS or ammonium per sulfate which initiates the polymerization process by generating free radicals, TEMED, a free radical stabilizer that also promotes polymerization and ß-mercaptoethanol, an agent not essential to gel formation but one that promotes gel polymerization and also assists in protein denaturing by breakage of disuphide bonds.

Slide 13:
Once gel formation is complete, boiling of proteins needs to be carried out if performing regular, 1D SDS-PAGE. The negatively charged SDS molecules in the boling mixture will bind to the folded proteins, helping denaturing the proteins and providing them a net negative charge.

Slide 14:
Once the protein preparation is completed, proteins can be loaded on the SDS-PAGE gel (which in 1DE, consists of 2 different gels, the stacking gel wherein protein is added and the resolving gel wherein the proteins are eventually seperated) and the current will be applied. Then based on the molecular weight the lower molecular weight proteins will migrate the farthest and the higher molecular weight proteins remain near the top of the resolving gel. Depending upon the protein size you can separate the proteins of

lower and higher molecular weights in the SDS-PAGE gel. A well charectarized molecular weight ladder is added to determine the mass of unknown proteins. Note: A difference between the 1D and the 2D electrphoresis workflow remains that the need for the comb, placed in the stacking gel, to form wells in 1D SDS-PAGE, which is not so for SDS-PAGE in 2DE wherein the focussed IPG strip is placed in the slot of a flat well formed at the top of the SDS-PAGE gel. To emphasize the point, the 2 DE SDS-PAGE gel only serves as the resolving gel to achieve protein seperation, with there being no requirement for the stacking gel required in 1D SDS-PAGE, as the process of protein 'stacking' or 'loading' has already been achieved in the first dimension or IEF.

Slide 15:
SDS-PAGE is one of the commonly used methods for determining the molecular weight of non-charectarized proteins. If you run the standar protein molecular weight markers along with your unkown protein, you can extrapolate the molecular weight of the sample protein.

Slide 16:
Shown here is an image of a Coomasie blue-stained SDS-PAGE gel wherein, going from left to right, the first lane contains the standard protein molecular weight markers, and the remainder of the lanes contain sample proteins whose molecular weight is to be determined. Commercial molecular weight ladders can have various ranges: for example in this case, from top to bottom, 150 kDa to 10 kDa. One can determine the molecular weight of the unknown protein by comparing the position of its band to the corresponding bands in the leftmost lane containing the molecular weight markers.

Slide 17:

The following animation is designed to walk through the steps involved in SDS-PAGE,

wherein the various steps involved in separating proteins based on their molecular weight are detailed.

Casting the gel

First the polyacrylamide gel is cast in between the glass plates as a vertical slab in the same buffer which is used for electrophoresis. The molecular dimensions of the pore can be controlled by the varying the amount of NM-methylene bis acrylamide with free radical cross linking facilitated by APS and TEMED.

Creating wells in the gel and sample preparation

The next step is to create wells in the gel. Sample wells of uniform size, shape and separation are created 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, leaving the gel ready to be run. Next, the sample to loaded on the SDS-PAGE gel is prepared. SDS is a negatively charged anionic detergent that binds to protein molecules and causes them to denature. The DTT breaks any disulfide linkages that may be present. During the process, heat (from boiling) begins process of protein denaturation. The negatively charged SDS molecules also bind to the proteins and denature them. The binding of SDS causes the proteins to have uniform charge to mass ratio, thereby allowing separation purely based on molecular weight. The protein samples can be loaded in the wells by using a micropipette. As seen in the animation, one protein sample is added per well.

Loading of sample on the gel

Once all the samples are loaded then you can take the SDS-PAGE unit shown here and move it into an electrophoretic apparatus. A direct supply between 100 to 350 volts is passed depending upon the size of the gel for a time period sufficient to separate the protein mixture into discrete bands based on molecular weight. The progress of electrophoresis can be observed with the help of a tracking dye.

Electrophoretic apparatus

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.

Processed gels

The gel then can be stained either with coomassie or silver stain and viewed to observe the various discrete protein bands present in each of the sample lanes. For example, the gel on the left in the in the figure here is silver stained while the one on the right is of the gel stained with coomassie. You can load the molecular weight marker or standards as shown in the first and the last lanes and then the unknown protein samples whose molecular weight you want to determine can be separated in different lanes.

Slide 18:
A variation of SDS-PAGE known as Blue-Native PAGE is now discussed. In SDS-PAGE, a denaturing environment is provided to disrupt the protein 3D structure. In contrast, Blue-Native PAGE separates proteins in the native conditions. A combination of these 2 methodologies provides unique information about protein isoforms, subunit compositions, molecular weights and different types of post-translational modifications. This information can be obtained either alone from the SDS-PAGE or from Blue-Native PAGE or by combining these 2 techniques together to get integrated information. The following slides will describe Blue-Native PAGE (BN-PAGE). In this scenario, it is not a part of 2-DE but often the native form is used for 2-DE separation.

Slide 19:
In the Blue-Native PAGE the protein analysis is performed under the native conditions hence does not involve the boiling/denaturing step. The sample is added with coomassie dye which provides the necessary charge required for the protein separation in the gel. But as previously mentioned, unlike the SDS procedure, this dye will not denature the proteins but it will bind to the proteins in their native states.

Slide 20:
The 2D BN-PAGE procedure is used for various applications including study of multi-protein complexes or MPCs. Identification of MPCs is not possible by using the denaturing environment of SDS-PAGE but can be identified by using Blue-Native

PAGE. In BN-PAGE the electrophoretic mobility of MPCs are determined by the negative charge of bound coomassie dye, as well as size and shape of the complex. This technique can hence provide an integrated view of protein function.

Slide 21:
The following is an animation which describes how to perform Blue-Native PAGE.

ANIMATION 2

The polyacrylamide gel is cast in between glass plates as a vertical slab in the same buffer that used for electrophoresis. The gel is prepared by free radical- induced polymerization of acrylamide and bis-acrylamide in a suitable buffer. APS and TEMED are added to facilitate the generation of free radicals and cross linking. The molecular dimensions of the pores can be controlled by varying the amount of NN-methylene bis-acrylamide.

Casting the gel

Once the solution is poured the, sample wells can be created. The sample wells of uniform size and shape and spearation 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 can be removed and the gel is ready for the procedure.

Preparing wells in the gel

The protein sample present in the suitable buffer is mixed with coomassie blue dye which provides the necessary charge to protein complexes thereby facilitating their separation in the gel. Unlike SDS, the dye does not denature the protein but binds to them in the native state. The protein samples are then loaded into the well with the help of a micropipette. As shown in the animation below, each well or lane receives a single protein sample, while the standard molecular weight markers are added in other lanes

Loading the samples into the gel

Once all the samples are loaded then the gel unit can be placed into an electrophoretic unit and direct current supply of aroung 100 to 350 volts can be passed depending upon the size of the gel for a time sufficient to separate the protein mixture into discrete

bands based on their mass and charge to mass ratio. The progress of electrophoresis can be observed with the help of tracking dye. The larger proteins are retarded and remain close to the point of application while smaller proteins migrate further along the gel. The gel can be then stained with coomassie blue and viewed to observe various discreet protein bands.

Running the gel

This animation should help clarify the performance of native PAGE. The same concepts can be applied for seperation in the second dimension if performing in 2-DE.

Slide 22:
After covering both SDS-PAGE and Native-PAGE, the following section will test the readers ability to obtain unique and different information by applying both SDS-PAGE and Native-PAGE together.

Slide 23:
The following laboratory demonstration video demonstrates how to carry out SDS-PAGE for 2-DE. The equilibration process as well as the running of the gel for 2-DE will be demonstrated.

LABORATORY DEMONSTRATION

Protein separation by SDS-PAGE: The initial steps of SDS-PAGE in 2-DE involves the assembly of gel apparatus, gel casting, equilibration of IPG strip followed by the placement of the IPG strip on the gel and subsequent protein separation.

Assembly of gel apparatus and gel casting: Clean all the components of the gel assembly thoroughly with water and dry them with paper towels. Carefully arrange the glass plates in the gel-casting assembly, interspersed with separator sheets depending on the number of the gels to be run. The assembly should be tightly packed so that there are no leaks. Close the casting assembly and tighten the screws provided. Prepare the gel-casting solution containg acrylamide and bis-acrylamide, Tris-HCl, SDS, APS and TEMED and pour it with the help of a funnel into the central camber of the casting assembly. APS acts as a free radical generator while TEMED acts a

catalyst for the polymerization reaction. Ensure that the solution spreads evenly such that the top edge of the gel is uniform. Allow the solution to stand for the polymerization to occur and spray the SDS solution across the top to ensure that the gel does not become dry.

Assembly of the gel casting unit

Equilibration of IPG strips: Place the thawed IPG strip in the tray. Over it pour the first equilibration buffer consisting of urea, Tris-HCl pH 8.8, glycerol, SDS and bromo-phenol blue along with DTT. Place the assembly on a mechanical shaker for 10 min. DTT enables the cleavage of all disulfide bonds present in the proteins in the IPG strip. Next, add the second equilibration solution to another well of the tray. This solution has the same composition as the first one except that DTT is replaced by IAA. Transfer the IPG strip into this solution after draining it of any excess liquid from the previous step. Again place the assembly on the shaker for 10 min. IAA prevents the reformation of disulfide bonds by binding to the free sulphydl groups that were obtained after DTT treatment.

Equilibration of IPG strip

SDS-PAGE: Drain excess liquid from the second equilibration step from the IPG strip by blotting on paper towels.Soak the IPG strip for few minutes in the same buffer which is to be used in the gel tank. Then carefully place the strip in between the glass plates and gently push it in until it rests against the gel surface without any gaps. Place the filter paper containing the molecular weight marker beyond the positive end of the stip. Then pour a luke warm overlay of agarose solution over the strip to prevent the gel from drying out. Assemble the remaining plates in the gel assembly and carefully lower the entire apparatus into the gel tank containing the buffer solution. Ensure that the buffer does not rise above the mark indicated. Place the seperating chamber above this and pour the buffer into the upper chamber. Close the gel assembly and connect the apparatus to the electricity supply. Set the appropriate voltage and begin the run. All the proteins in the IPG strip carry a large negative charge due to SDS which denatures the proteins and binds to the polypeptide backbone at a constant charge to weight ratio.

This ensures that the proteins present get separated exclusively on the basis of their molecular weight rather than their mass to charge ratios as in Native-PAGE. The proteins with lower molecular weight have high mobility and migrate further through the gel pores, while the higher molecular weight proteins remain closer to the point of application. This allows efficient separation of the proteins in the second dimension.

Placing IPG strip on the gel

Slide 24:
The next part will discuss how to stain the gel and visualize the protein spots , following protein seperation based on their pI & molecular weight. At this point, all the seperated proteins are present within a transperant gel matrix. Spot visualization is now required in order to interpret the results. Different types of staining methods have been developed that can be applied to visualize protein bands or spots depending upon what type of gel being used.

Slide 25:

The following is an overview of various staining techniques, starting with the coomasie blue staining.

Slide 26:
Coomasie blue staining is is one of the most commonly used stains for protein detection in polyacrylamide gels. While it not as sensitive as compared to silver or SYPRO Ruby, it is widely used as it is easy to use, and is less expensive and more stable than these other alternatives and is also compatible with mass spectrometry. Certain improved forms of this dye have been generated, such as the biosafe form, which offers an attractive alternative given its non-hazardous, environmentally friendly nature. It can be disposed as non-hazardous waste. Furthermore, the biosafe coomassie is a ready to use, single reagent protein stain.

Slide 27:
The following animation demonstrates how to perform coomassie blue staining.

ANIMATION 4

This demonstrates how staining can be performed by using the coomassie brilliant blue stain. The completed electrophoresis gel is placed in a tray containing the coomassie blue staining solution. The negatively-charged coomassie dye interacts with protein through ionic and non-covalent interactions. After adding the stain, the tray is placed on a mechanical rocker which allows uniform contact of the gel with the solution by means of gentle rocking. After overnight staining, the solution is drained and the stained gel is then placed in a destaining solution which contains 50% ethanol and 10% acetic acid to remove any excess dye that may be bound to the gel. Again this process involves overnight steps or atleast 10-12 hours gentle shaking on the rocker. The stained gel can be scanned by using a scanner and these protein spots can be easily visualized.

Each of the spots can be now used further for image analysis purposes. Most of the time, protein extraction and seperation methods work well enough to yeild a good amont of protein that can be easily visualized with Coomasie. However, certain extractions, for various reasons and despite good laboratory procedure, could be tricky, resulting in low yeilds, necessitating use of more sensitive stains such as the Silver stain.

Slide 28:
The silver staining technique:

- Protein fixation in the gel is achieved by adding methanol and acetic acid.
- Staining chemistry:
    - The stain contains a Silveramine complex which is bound to the tungstosilicic acid. The silver ions are transferred from tungstosilicic acid to the proteins. These silver ions are then reduced by an alkaline solution containing formaldehyde to yeild the metallic silver form, which results in a brownish tinge indicating the protein spot. Allowing this reaction to proceed for longer periods however results in a dark background on the silver-stained gel. Hence it is stopped by the addition of acetic acid at a suitable point.

Slide 29:
Following is the animation demonstrating the silver staining technique

ANIMATION 5

This animation on silver staining will describe the process of how to stain the gel using silver stain. The completed gel is first placed in the fixing solution of methonol and acetic acid that fixes the protein band in the gel and minimises diffusion. This step must be subjected to gentle shaking for around 30 min after which the silver stain solution is added. After adding the silver solution, the gels are rocked gently in order to allow proper and uniform staining. The silver stained gels are first washed to remove any excessive stain and then placed in a developing solution where the silver ions get reduced to metallic silver. Formaldehyde in an alkaline solution in the presence of sodium carbonate or other alkaline buffers are commonly used for this process. Again, a gentle rocking will allow for the development of a uniform staining pattern. Finally the gel can be viewed as dark bands or dark spots against a light background. As you can see in this 2-DE gel image the spots having the dark spots are stained with silver stain.

Slide 30:
In summary, this lecture covered the following; the 2-DE workflow, the steps of equilibration, followed by principles of protein seperation in the 2^{nd} dimension, including SDS-PAGE and BN-PAGE amd different types of staining methods, including coomassie staining and silver staining. The next lecture will continue with the workflow of performing 2-DE, covering a few more sensitive stains available, and image analysis using different softwares, followed by statistical analysis of obtained images to identify the proteins of interest.