LECTURE-13

Gel-based electrophoresis

Two-dimensional electrophoresis (continued)

TRANSCRIPT

Welcome to the proteomics course. In the previous lecture we started discussing about the gel-based proteomics. In that workflow, we talked about the two-dimensional gel electrophoresis and the different steps involved in performing the two-dimensional gel electrophoresis. As I mentioned, the gel-based proteomics includes various techniques such as SDS-PAGE, Blue Native PAGE, two-dimensional electrophoresis and advance forms of electrophoresis such as difference in gel electrophoresis or DIGE. In the last lecture I described how to perform a two-dimensional electrophoresis experiment. You need to follow a workflow and I give you different steps, which are involved to perform the 2-DE. The first step was the isoelectric focusing. Which means you want to focus the proteins based on its isoelectric point. I have demonstrated you some videos to give laboratory feel of the experiment how it can be performed. And now are continuing from the previous video.

In today's lecture, we will talk about the workflow of two-dimensional electrophoresis. The first step was already described in the last lecture and then we will discuss few more steps today.

In today's lecture we will focus on the equilibration step, how to equilibrate the IPG strip, how to perform SDS-PAGE which is protein separation in second dimension based on the molecular weight. Now, the fourth step is the staining procedure, how to visualize your gel. Often you have problems with low abundant proteins. Sometimes you are lucky to have good protein amount and you can try different types of staining methods depending upon your requirement and your protein yield. We will discuss different types of staining methods, which can be used to perform this step. So let's start with the first point, equilibration.

As briefly I described in the previous lecture, once you have performed the isoelectric focusing or IEF you need to prepare your IPG strip for the next step, which is SDS-PAGE. When you are performing the conventional SDS-PAGE, you pour the gel both stacking and resolving and then you want to make some wells using the combs and then you load your samples in different wells. When we talk about the two-dimensional electrophoresis it is different than doing the SDS-PAGE alone. The concept of the SDS-PAGE will involved, which will be the same, but here there will be a variation. You do not need to add the stacking gel. You need to prepare your protein sample, which is already focused inside the strip in the first dimension. Now, the IPG strip which already in the electrophoretic unit in the IEF instrument after that you want to prepare that protein can be separated further based on the molecular weight. Now, you need to add SDS to provide the negative charge and you need to do some treatment for reduction and alkylation. When you are taking the protein sample alone you have the flexibility of boiling the protein samples in the tubes and do all those treatment to denature the protein separately. But now your protein already focused in the IPG strip. So you do not have that flexibility here. You need to prepare your IPG strip in such a way that need to apply that on the top of SDS-PAGE gel.

So, I'll show you what steps are required in the equilibration. So, after the first dimension separation is done, you need to coat the protein with SDS-PAGE for making it compatible for SDS-PAGE. SDS is anionic detergent, which provides negative charge later on for separation in the electrophoretic field. The equilibration step performs the cleavage of both inter and intra-chains of disulphide bonds and it alkylates the sulfhydryl groups of cysteine residues. So there two equilibration steps, which are required for this preparative step; the first equilibration step and the second equilibration step.

Your recipe for equilibration buffer is ideally similar in both first and second equilibration which includes 6 M of urea, 2% of SDS, 0.375 M Tris-HCl, pH 8.8, 20% glycerol and then you need to make one variation in the composition of the buffer either you add Dithiothreitol (DTT) or Iodoacetamide (IAA). Please note that this recipe is given only for your reference. People try slight modification of this recipe for doing the equilibration

step. Now, as I mentioned in this buffer you can add DTT and that will used in the first equilibration step. The first equilibration step can be performed for 10 or 15 hours depending upon your strip length. Now once you have done the first equilibration. Now, once you have done the first equilibration you have to remove the solution and a new buffer for second equilibration. This buffer's recipe is similar to the last one except for one change; DTT is replaced by IAA.

You can add 135 mM of IAA during the second equilibration step. Now, this is very important beacause IAA can alkylate the residual DTT and it minimizes the vertical streaking. It also prevents the re-oxidation. So, you want to ensure that your protein is denatured again after doing the IEF process, it is coated with SDS molecules so that the protein can be be separated in the second dimension based on molecular weight. This step is performed for 10 to 15 min depending on the strip length and once both the equilibrations are done then you are ready to perform SDS-PAGE.

I'll show you in this slide the step-wise procedure for performing first and second equilibration steps. So, first strip can be equilibrated in the first equilibration buffer by adding inside a tray in a well and then you change that to 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 and prevents the re-oxidation. So here, you can see how the different bonds are broken from the disulfide and IAA is preventing the re-oxidation, alkylating the residues.

After having discussed the equilibration step, now we can talk about how to perform SDS-PAGE. As I mentioned when you perform SDS-PAGE alone for separating your regular protein extract then you need to add both stacking and resolving gels. Now, in this case when you are using SDS-PAGE only in the second dimension of two-dimensional epectrophoresis then you do not need to add the stacking gel. You have already focused the protein in the gel in the first dimension based on the IEF. Now, for the second dimension sepearation you have already prepared the strip to be loaded on

the SDS-PAGE gel. So, the next step can be similar to what one can expect in regular SDS-PAGE.

SDS-PAGE is one of the widely used electrophoretic techniques which separates the proteins based on their size or molecular weight. So, this molecule SDS or sodium dodecyl sulphate which is an anionic detergents provides negative charge. It binds at a ratio of 1.4 g of SDS per gram of protein to provide almost similar to mass ratio. Therefor, you protein separation will be mainly based on the charge in the electric field. As I mentioned there is a step required to prepare the protein for denaturation, so you need to boil your proteins in SDS, ß-mercaptoethanol and DTT to denature the proteins and to remove or break any disulphide bonds. Since you can not do this boiling step in two-dimensional electrophoresis where you have already focused your IPG strip in the first dimension and hence you need to do the equilibration step. Rest of the steps will be very uniform.

So, let me describe you the role of the components which are being used in SDS-PAGE. Acrylamide provides the matrix or gelling for making the gels and bis-acrylamide is a cross linking agent and by adding acrylamide and bis-acrylamide in a fixed ratio one can make the gel by adding the gelling agent and the cross linking agent.

But that is not sufficient to polymerize the gel, you need to add few more reagents which include APS or ammonium per sulfate which initiates the polymerization process, TEMED which is a free radical stabilizer which also promotes polymerization. Although, ß-mercaptoethanol is not used in making the gel but it breaks the disulfide bonds. I hope you have been able to understand role of each components which are being used in SDS-PAGE either during the protein preparation of SDS-PAGE or making the gel to separate the protein.

Now, once this step is done you need to boil your protein sample if you are performing a regular SDS-PAGE. The negatively charged SDS molecules will bind to the folded proteins. SDS molecules denature the proteins and provide the negative charge.

Once the protein preparation is done you can load the protein on the SDS-PAGE gel and based on the molecular weight the lower size proteins will migrate the farthest and the higher molecular weight proteins remain near the top surface of the gel. Depending upon the protein size you can separate the proteins of lower and highed molecular weights in the SDS-PAGE gel. You can add ladder or known molecular proteins to determine your unknown proteins. Since we are talking about two-dimensional electrphoresis workflow, I think its important to clarify that in 2-DE when you use SDS-PAGE you do not need to make these wells by adding a comb. Because you already have a flat IPG strip which has done the first dimension protein separation. So in this case you need make a flat well so that your IPG strip can be placed on top of the SDS-PAGE gel. Again to remind you, here you are only making the resolving gel for better resolution and separation of the protein. The stacking part is not done in the 2-DE which has already been done in the first process of IEF.

The SDS-PAGE is one of the very commonly used method for determining the molecular weight of unknown protein. If you run the standard protein markers along with your unkown protein you can determine the molecular weight of the unknown proteins.

I am showing you one SDS-PAGE image in which from the left to the right the first lane is the standard or protein with known molecular weight. In this well you can see that there are different bands visible which are the known molecular weights. Some times starting from 150kDa to 10 kDa of molecular weight range. Then we have different wells and in each well we have unknown protein for which we want to determine the molecular weight and after staining with coomassie blue you can see the pure band, as in this case. So, one can determine the molecular weight of the unknown protein by performing the SDS-PAGE.

I'll now describe you the animation and show you the steps involved in SDS-PAGE. In this animation you will see various steps involved in separating proteins based on their molecular weights. First of all the polyacrylamide gel containing SDS is cast 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 amount of NM-methylene bis acrylamide with free radical cross linking facilitated by ammonium per sulfate or APS and TEMED. Next step is to create the wells on these gels. The sample wells of uniform size and 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 to provide the gel ready to process. Now you can prepare the sample to load on the SDS-PAGE gel. SDS is 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 you boil or heat your protein samples to denature the proteins. Now the negatively charged SDS molecules will bind to the proteins and denature them. The binding of SDS causes the proteins to have uniform charge to mass ratio. Thereby allowing the separation purely based on molecular weight. The protein samples can be loaded in the wells by using a micropipette. As you can see in the animation you use different wells to load different types of samples.

Once all the samples are loaded then you can take this unit and move into an electrophoretic apparatus. A direct supply between 100 to 350 volt is passed depending upon the size of the gel for a time sufficient to separate the protein mixture into the discreet bands based on their molecular weight. The 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 then can be stained either with coomassie or silver stain and viewed to observe the various discreet protein bands present in each of the sample. For example, the left in the figure below is silver stained and the right image 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 unknown protein samples whose molecular weight you want to determine can be separated in different wells.

So, I hope after looking at the images you are clear how to make gels to separate the proteins based on their molecular weight. I'll show later on a laboratory demonstration how to use SDS-PAGE gel for separating proteins in two-dimensional electrophoresis.

Let us now move to a variation of SDS-PAGE known as Blue-Native PAGE. In SDS-PAGE we are providing a denaturing environment for denaturing the protein. Whereas in the Blue-Native PAGE we want to separate the proteins in the native conditions and by applying these 2 techniques together one can obtain a very unique and different information often related to the 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 two techniques together to get the integrated information. I will briefly describe you here Blue-Native PAGE (BN-PAGE). Please keep in mind this not going to be used for 2-DE but often people use the native form for 2-DE separation.

In the Blue-Native PAGE the protein analysis is performed under the native conditions. Here you are not going to boil your protein sample with SDS and providing different type of denaturing condition. You need to add a sample with coomassie dye, which provides the necessary charge required for protein complexes to separate in the gel. But as I mentioned unlike SDS, this dye will not denature the proteins but it will bind in their native state itself.

People apply the 2-D Blue-Native for various applications such as studying about the multi-protein complexes or MPCs. Identification of MPCs is not possible by using the SDS-PAGE or denaturing environment but can be identified by using the Blue-Native PAGE. In BN-PAGE the electrophoretic mobility of MPCs is established by the negative charge of bound coomassie dye, its size and shape of the complexs. So this technique can provide the integrated view of the protein function.

I will now show you an animation, which describes how to perform Native PAGE.

The polyacrylamide gel is cast between the glass plates as a vertical slabs in the same buffer that is 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.

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 process.

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 it is shown in the animation you have different wells to load different protein samples. You can use one of the wells to add one of the known or standard markers and the other wells can used to separate the unknown proteins. Once all the samples are loaded then this unit can be placed into an electrophoretic unit and direct current supply of aroung 100 to 350 volt can be passed depending upon the size of the gel for a time sufficient to separate the protein mixture into discreet bands based on their mass, charge to mass ratio. The progress of electrophoresis can be observed with the help of tracking dye.

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After watching this animation I hope you are able to understand how to perform native PAGE and these concepts can be applied for using the 2-DE.

Now you are familiar that how you can apply both SDS-PAGE and BN-PAGE and you can obtain unique information about subunit composition and different types of isoforms from different proteins and you need to apply both the techniques together. Now you are also familiar how to determine the molecular weight of an unknown protein. So as I mentioned you have seen the similarities in the concepts of classical SDS-PAGE and BN-PAGE and this concept can be applied in 2-DE. There's a little variation here because you do not need to add a comb, you have to have a flat comb for making a well to add your IPG strip and you do not need to add separation for a stacking gel. So in this laboratory demonstration video I'll show how to make SDS-PAGE for 2-DE. Equilibration process will be demonstrated as well as how to run SDS-PAGE gel for 2-DE.

Protein separation by SDS-PAGE: SDS-PAGE which constitutes the second dimension of 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 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 intersperced separator sheets depending on the number of the gels to be run. The assembly should be tightly packed such 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 chamber of the casting assembly. APS acts as a free radical generator while TEMED acts a catalyst for 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 solution of SDS across the top to ensure that the gel does not become dry.

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, bromo-phenol blue along with DTT. Place the assembly on a mechanical shaker for 10 min. The DTT enables the cleavage of all disulfide bonds present in the proteins on 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 the DTT is replace by Iodoacetamide. Transfer the IPG strip into this solution after draining out any excess liquid from the first. 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.

SDS-PAGE: Drain out the excess liquid of the second equilibration solution from the IPG strip using a tissue paper. Soak the IPG strip for few minutes in the same buffer which is to be used in gel tank. Then carefully place strip in between the glass plates and gently push it in until it rests on the gel surface without any gaps. Place the filter paper containing the molecular weight marker beyond the positive end of the stip. Then pour the luke warm overlay 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 level does not rise above the mark indicated. Place the seapartion 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 present on the IPG strip carry a large negative charge due to activity of SDS which denatures the proteins and binds to the polypeptide backbone at a constant weight ratio. This ensures that the proteins present get separated exclusively on the basis of their molecular weights 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.

Now, we'll talk about how to stain the gel and visualize the protein spot because so far you were able to separate the protein based on their pI, molecular weight and now everything is on a transparent gel. You do not know how well your separation has occurred. So, now you need to visualize your spots. Different types of staining methods have been developed and those can be applied to visualize protein bands or your protein spots depending upon what type of gel you are using.

I am giving you an overview of staining techniques. Let me describe you few staining methods in more detail. Let us start with coomassie blue staining.

This is one of the most commonly used stains for protein detection in polyacrylamide gels. The sensitivity is good but not very sensitive as compared to silver or SYPRO Ruby but its easier for performing staining, higher stability and is cheaper and has good compatabilty with mass spectrometry. All of these make coomassie blue as a stain of choice for most of the laboratories worldwide. Now there are some advanced forms of coomassie stains being developed, including biosafe coomassie stain which is non-hazardous made keeping in mind the environmental scenario. So it can be disposed as non-hazardous waste. The biosafe coomassie is also a ready to use stain and a single reagent protein stain.

I'll show an animation on how to perform the coomassie blue staining. In this animation I will show you how the staining can be performed by using coomassie brilliant blue stain. The completed electrophoresis gel is placed in a tray containing the coomassie blue staining solution, typicall R 250. That has been disolved in an aqueous solution of methanol or acetic acid. The negatively charged coomassie dye interacts with protein through ionic and no-covalent interactions. After adding the stain, the tray is placed on a mechanical rocker which allows uniform contact of the gel with solution by means of gentle rocking. After overnight staining you have to drain out the solution. 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 finally be viewed as shown in this image here, showing a 2-DE gel with coomassie brillaint blue and it can be scanned by using a scanner and these protein spots can be easily viewed.

So in this animation you were able to see after performing the 2-DE electrophoresis how to add coomassie stain on your gel, do staining and destaining procedure so that you can visualize your blue spots on the transparent background. Each of the spots can be now used further for image analysis purpose. Most of the time, if you are lucky and if your protein extraction protocol is good you have good protein yield. But often your samples are very challenging. Despite your good extraction protocol and your good laboratory skill, still you are unable to obtain a very good protein quantity. So, how to move forward? You still want to do the protein separation but coomassie blue may not be sufficient to visualize the spot on the gel. Now, you need some more sensitive stains such as silver stain.

So, I will describe you how to do silver staining. Silver staining is more sensitive than coomassie blue dye. First of all when you have run your 2-DE gel you need to do the protein fixation in the gel by adding methanol and acetic acid. The staining procedure involves silveramine complex which is bound to the tungstosilicic acid. The silver ions are transferred from tungstosilicic acid to the proteins. Now providing alkaline solution with formaldehyde it can reduce the silver ions to the silver metallic form and a brownish tinge can be seen on the spot, which can be further analysed. But if you allow this reaction to occur continuously you will see a very dark background on the silver stained gel. So, you need to stop the reaction by adding acetic acid.

I will not show you the animation on how to perform silver staining. In this animation on silver staining I will describe you the process how to stain the gel using silver stain. The competed gel is first placed in the fixing solution of methonol and acetic acid that fixes the protein band in the gel and minimises any 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 place 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 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.

So let us see what we've learnt today. In summary we started with two-dimensional electrophoresis workflow, the first step equilibration was discussed today. IEF was discussed in the previous lecture. Then we talked about how to perform protein separation in second dimension. During that course we talked about SDS-PAGE and BN-PAGE and then we talked about different types of staining method. We discussed in detail about coomassie staining and silver staining. In the next lecture we will continue with this workflow of performing 2-DE, we will talk about few more sensitive stains available, how to use those? Then how to analyse images using different softwares and then to perform the statistical analysis of those so that we can identify the interesting protein spots for a biological question. I hope now you are able to follow the workflow of 2-DE and we will continue in next lecture. Thank you!