Comprehensive Molecular Diagnostics and Advanced Gene Expression Analysis

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Lecture 29 : Proteomic Techniques – Differential in-gel electrophoresis

Namaskar. Welcome back. So, we are continuing with module 6 that is basic techniques of proteomics and today's topic is differential in gel electrophoresis right. So, this is very familiar the workflow these are the basic steps by which we approach any proteomic workflow all right. So, till our further lectures in the last class we discussed till MS that is mass spectrometry. Now regarding the next step that is database searching we will be discussing when we are discussing the bioinformatic tools and the processes all right.

So, we will be continuing our discussion with DIGE and protein microarray. So, definitely our today's topic is differential in gel electrophoresis as I mentioned this acronym as mentioned in short form as DIGE. So, we will be discussing DIGE under the following concept now before we learn what is DIGE and what is the need of DIGE we should be acquainted with the conventional 2 dimensional gel electrophoresis that is 2DE all right. And the limitations what are the limitation of 2D page ok polyacrylamide gel electrophoresis ok this should be capital polyacrylamide gel electrophoresis then it will be very easier for us to understand the need.

So, we will be discussing how DIGE comes the principle of DIGE the workflow of DIGE what is the internal standard in differential gel electrophoresis we will also be discussing the advantages as well as the pitfalls of DIGE all right. So, we have lot to discuss. So, let us start with and before going forward you know thus when you here or when you learn when you read the term differential gel electrophoresis or difference gel electrophoresis it is the same thing all right many text book many article might not mention differential in gel electrophoresis, but it is the same procedure ok. So, let us first discuss conventional 2 dimensional gel electrophoresis we already discussed it when we are discussing the overview right. So, you already know that is the powerful technique which is widely used all right in mainly in separating complex mixture of protein from various sources maybe it may be cell or tissue or any other biological sample.

So, what actually I mean ultimately in 2 dimensional gel electrophoresis what we get is a this type of so called dirty looking sheet of final output diagram all right where if there are so many spots, but each spots correspond to a protein all right it may be protein of interest and then we compare the spots with another gel who may have a different set of proteins then we can compare the expression or what the amount of protein many things we can do. But the and what happens how did we do 2D electrophoresis just as a revision 2D electrophoresis definitely happens in 2 steps. So, first step happens in first dimensional one side that is known as isoelectric focusing where the proteins are resolved that means, separated according to their isoelectric points very important P i small p capital I isoelectric point on immobilized pH gradient strips or IGP strips ok that is what is happening in the first dimensions there is a immobilized pH gradient strip like this where proteins will get separated. So, this is first dimension and then sodium dodecyl sulfate poly acrylamide gel electrophoresis called SDS page is happening per in a perpendicular field to that the electrical field is applied to this direction. So, in one dimension and second dimension the all the proteins or mixture of proteins from a complex mixture individual proteins are separated or resolved all right.

Now, there are limitations definitely it is much better much much better than one dimensional gel electrophoresis, but in spite of that in spite being so good in an helpful proteomic procedure the main there are few limitation number the first limitation is when it comes to handling multiple sample at a time that is the main shortcoming where 2D page becomes non user friendly what I can say it requires so many more gels to it requires it becomes very labor intensive all right we need more gels we need more number of gels we need more manpower we need I mean may much more power supply or we need to do the experiment one after another. So, it will take more time. So, either it will need more resources or if in case of limited resources we need more time all right. So, that is not good if we have so many samples where we need to do them together. So, that leads to a very low throughput that is the number of results per unit time by 2 dimensional gel electrophoresis is much lower when there are many samples to be analyzed all right.

So, what do we do I mean till now if we had many samples we have to run more than one gel the number of samples we need to run as many gel as the number of sample mainly because of the fact there are and and and what if we need to know proteins that are similar in property all right both their p I and molecular weight, but are inherently different in some way there is no way to know because we can use only one dye in conventional 2 dimensional gel electrophoresis and both will be stained suppose blue dot and in if we do finally, a autoradiography ultimately the final picture it will be shown as a black dot and it might look very similar 2 black dots might overlap and appear as a blob all right. So, moreover the thing is when we are doing multiple gel runs all right and when we are loading sample user to user variation is unavoidable not

only that even if it is possible that we give the load the sample. So, accurately there might be difference in the gel itself or there might be some difference in the electrical field because this is a biological material whenever we apply an electrical field there will be heat production. So, all these things whenever we are running 2 exact samples in unison or in parallel across all the samples it we have seen that results will vary all right. For example, we are using a dye cypro ruby here 6 different gels were run from the same sample and at the end we are seeing that there are differences.

So, just imagine when we are comparing 2 different samples if there is a difference between one sample there is a variation in so much variation in single sample then it is difficult to standardize and compare 2 different proteins of interest all right. So, that is very important the differential analysis is difficult and therefore, we cannot confidently claim statistically that our result is valid. What we see and we claim has got no validity unless it is statistically valid for any research all right. So, all boils down to the fact that when we have 2 gel one is control and one is treated the question arises are the spot differences real can you really say that this is due to experimental not due to experimental variation not by chance and actually due to the differences in biological or experimental results. So, the fact that 2 dimensional gel electrophoresis has got low sensitivity and reduce dynamic range I discussed in the lecture class previously when we discussed 2DE that proteins with very small molecular weight and beyond the p i limit of this immobilized strips will not be able to be separated right and moreover there are gel to gel variation.

So, there are reasons why these variations can occur as I discussed. So, let us discuss in brief number one is system related variation. So, as I told electrophoretic condition all right whether it is in the first dimensional gel that is IPG isoelectric focusing or in the second dimensional 2D page sample application variation user to user variation not only that these software's these 2D gel diagrams we do not note spots manually all these are analyzed using densitometric softwares all right. So, user to user variation in picking up spots all these can lead to variations in the exact same sample even if we run in duplicate all right. So, there are also other inherent biological variation for example, if we are trying to mimic a condition in animal model and then we are trying to run the similar condition in a human model for some sample for example, and if we expect a similar distribution that might also not happen because there are inherent biological variation from different animal from different subject to subject and that cannot be I mean parallely that cannot be accounted in conventional to the 2D page all right.

And lastly there actually there can be actual difference due to drug state drug treatment life life cycle change life cycle change for example, in aged or in young sample. So, whatever be the situation we cannot confidently say if the definitely a still date 2 dimensional gel electrophoresis was the most advanced proteomic method of choice

because we did not have anything better right, but with advancement of technologies we identified and we expect more and more we identified the shortcomings and we expected more. So, what is our expectation what is the researchers expectation from 2D gel electrophoresis? Number one there should be least gel to gel variation when we run two different gel in duplicate there should not be any spot difference whatsoever all right. Even if there is a normal biological variation there must be a way where we can normalize. If there are multiple samples we do not need multiple gels to run we need least number of gels all right.

There is protein loss as I told you one sample whenever it is done in 6 different gels somehow one spot was there this spot is not present here. So, protein has been lost. So, this should be accounted all right. Next we can differentially analyze different protein expression I mean different protein expression should be analyzed would be able to be analyzed. So, this is known as differential analysis and lastly whatever be our result we should be confident enough that our change is statistically significant our result our observation is statistically significant.

So, based on all these things the advancement or the technological innovation in 2 dimensional gel electrophoresis was differential in gel electrophoresis. Please mind it this is not much different from how 2D gel electrophoresis happens there is just a minor tweak in the process majority of the process remains the same and then the analysis process is much advanced that is why it gives much advantages compared to conventional 2D gel electrophoresis all right. So, we should always know that DIGE is not a replacement of 2 dimensional gel electrophoresis DIGE is an aid it is a much more improved version of conventional 2D electrophoresis traditional 2 dimensional gel electrophoresis all right. So, basically since it is a more evolution of a conventional gel electrophoresis what it does is it is actually based on the principle of again the same thing densitometric analysis. What is densitometry? In case of blots for example, in western blot or any proteomic experiment we get or in in even we discuss DNA gel electrophoresis right.

So, the blot can be the band can be due to DNA can be due to protein can be to RNA. So, we pick up those bands and there are softwares which quantifies the intensity of these bands all right, but in case of 2D gel this is the picture of densitometry this is the concept of densitometry. So, based on the amount of the band intensity we can say how they are comparable high and low by calculating the area under the curve this is the basic principle of densitometry. In case of 2 dimensional gel electrophoresis again we get spots and these spots will be analyzed from gel to gel and again they can be analyzed using densitometric software to give us the result about the quantitative value. What is new in DIGE is the use of fluorophores all right.

So, fluorophore tags or fluorescent dyes are first mixed with the sample all right. These dyes they coherently bind with epsilon amino group of lysine what are the dyes I will be mentioning very soon. So, mind this is a very important question where the dye fluorescent dye covalently binds in DIGE to the epsilon amino group of lysine important question for as an assignment exam everything and for your knowledge also. So, when covalently binding of the dye has happened prior to sample application then we can actually load multiple sample in the same 2D platform. So, isoelectric focusing and SDS page is happening just like conventional 2D, but all those multiple samples since they are tagged with different dye can be analyzed in one single gel.

This is very similar to the to what we read during evolution of Sanger sequencing. So, at first each and every base was being done as soon as capillary electrophoresis came with the use of fluorescent tags chain fluorescent chain terminators everything could be loaded in one gel only we did not need 4 separate lanes. I hope you remember if you do not remember that I suggest you also review that video after this class all right, but I am confident that you already remember that. So, since we are using different fluorophores it is very easy to run the electrophores with multiple sample in one single gel. So, before going to the details this is basically the overview all right we will be explaining each and every workflow in detail.

So, what happens suppose there are 2 samples or mixture of 2 proteins. So, proteins from 2 different cells suppose control and treated all right. Number 1 we are treating the control sample or sample 1 with 1 fluorophore or 1 type of fluorescent dye and the second type with another fluorescent dye very simple we run both the dye both the sample in a same gel all right. So, now both the fluorophores will have different fluorescent property. So, suppose excitation and emission of 1 fluorophore will be different than the excitation emission of wavelength of another fluorophore.

So, we can actually scan different proteins by simply exposing them to different fluorescent excitation wavelengths all right. So, these 2 are then scanned on the different fluoro fluorescent wavelength excitation wavelengths all right suppose this is wavelength 1 and this is wavelength 2. Then all the spots are detected something was co spot detection with the internal standard we will be discussing that very soon and ultimately the image analysis software can analyze and overlay the images and then they can give us a completely differential reading of what protein has expressed and what is the status of protein in first mixture compared to the second mixture and everything is normalized in 1 single gel in 1 run. So, there is no need of running even duplicate gels for the single sample all right. Now this was a very compact explanation.

So, let us see what happens ok the this diagrams have been cited from the article which is in proteomics this is the article detail you can go and read the article is very

well explained article, but I have made it very simple for you guys. So, first of all sample lysis sample should be lysed based on the source we should treat sample with any type of lysis buffer. So, that can be 8 molar urea, 2 molar thiourea, 4 percent chaps, 10 10 micro molar 10 milli molar tris. So, depends on the amount of protein. Now in this article how they what they did was they use 4 to 8 picomole per dye per microgram of protein for 30 minute or nice very important all those things should be cooled all right and thereafter these are quenched the excess dyes are removed with free lysine and reduced to DTT that is full form is dithiothreitol ok dithiothreitol very important whenever you see DTT mind it it is used in relation to disulphide bond in relation to protein lysis buffer all those things ok.

Very important there is a this is also important that DTT is a content of lysis buffer how we are lysing the sample as well as dithiothreitol has got direct action in the workflow of DIG. Now regarding the dyes what dyes we are using these are mainly cyanine dyes these are named C these abbreviated as CY. So, CY 2, CY 3, CY 5. So, all these are used. Now you see sample A has been tagged with or mixed with C a CY 3 and sample B has been mixed with CY 5 ok.

Now internal standard again we will be discussing is a pool or a mixture of both from both sample A and B and it is tagged with a different dye CY 2 all right then they are run and then the whole mixture is analyzed. Now in reality we are actually scanning at three different wavelengths ok this is the first step basic sample lysis and labeling with cyanine dye. Thereafter the samples are I mean gels are bonded between low fluorescence glass plates why do we have to low fluorescence glass plate because this is not simply staining with a dye. So, for example, standard two dimensional gel electrophoresis we can use stains like Coomassie blue COO MASIE or Amido black those are the common stains that we use to stain protein those are also used in western lots ok. However here we are actually using fluorescent dye that are not visible to the naked eye right and we need to see whether the these are emitting any fluorescence.

So, definitely if we use a plate who itself has got fluorescence of its own then it will interfere with the results. So, where a special type of glass plates that is low fluorescence glass plates are used for 2D gel electrophoresis all right. So, gels are bonded after the gel is done the gels are bonded between low fluorescence glass plates all right and the standard separation is already happening separation first dimension by selective focusing and second dimension by SDS page. So, and in the next step it is very easy to understand. So, this gives to the gives way to the next step that is imaging image analysis all right.

So, fluorescent detection by multi wavelength scanner. So, there is a fluorescent the equipment that can actually scan the gel across multiple wavelength what is the picture

and for each wavelength that is being scanned each picture is obtained each film each picture is obtained that can be overlaid using the software the computer ingenuity and it can be analyzed. Next is image analysis with software all right. So, image analysis with software what happens after we get an image. So, thus there is a software that is built in the equipment depending on the equipment those software can have multiple names.

So, mainly what do they do they do spot detection there are multiple hm modes for example, cross gel spot matching. So, whether the spots are matched in between the gels they can automatically determine the fold increase the amount of protein that has been expressed or the spot how much it is bigger or how dense it is or what is the amount of fluorescence it is exciting emitting all right all these can be calculated and it can also be normalized simultaneously by this ratio psi 5 to psi 2 means the protein by the internal standard of both the I mean the ratio of the protein to the internal standard is actually done for both the gels and thus they help in base normalization which is actually helping in statistical analysis of the two gels or the two conditions that we are studying over here all right. Now, this is all about DIG where we can study the differences ok we can study the expression if we just need to study the expression we can stop here. However, if something unknown is happening all right if we already know that this is the amount of protein that is happening or this is the amount of protein that should vary or we just need to show there is some variation we can definitely show all right. However, we also not only we should separate we should also be able to analyze for analysis next steps are important.

So, you see even after the analysis the gels that are tagged by fluorescent dyes are basically colorless. So, what do we need to do? We need to now that all the spots we already know that the spot differences are not by chance thus these are the spot difference these are the areas what are which might have spots etcetera. Then we again stain the gel this time we use the traditional colloidal stales for example, Kumasi blue, Cypro ruby for protein the reasons are for protein visibility validation of the gel quality aids spot matching means suppose our fluorescent equipment has given spots in the area suppose this is the this is our gel fluorescence mind it is colorless the fluorescent read study has given 4 spots arbitrarily I am just do drawing the spot this does not happen in reality. If the same spots are also visible using the traditional dye we will be 100 percent sure that these are not artifacts ok mind it we do not need to do post stain unless we want to characterize these proteins and now our goal is to characterize the protein we want to know the nature of the protein. So, how to do that? So, first we need to see what the proteins are ok there are important utilities I mean these are already mentioned alright accurate gel to gel comparison addressing challenges ensuring a reliable results yes I agree, but the main reason why we need to visualize ok again very important because minimal labeling may not ensure co migration of the labeled and unlabeled proteins because we are actually using very very very minimal amount of dye to start with

alright.

So, whether the proteins are actually separate using 2 dimensional gel electrophoresis we will not be able to actually get unless there is a proper physical stain. So, you see I mean this might appear confusing to you in one side we are seeing that we are saying that DIG is aging 2 dimensional gel electrophoresis and again now we are saying we have to use the conventional 2 dimensional gel electrophoresis stains to aid DIG analysis because these 2 are going hand in hand I already told you this is not an this is not a replacement of 2D gel electrophoresis we need some components of 2D gel electrophoresis to I mean make the process much better. So, number one was the procedure of running 2D gel electrophoresis and next is using the stains that we use in conventional gel electrophoresis to stain in post staining phase ok. Next so, after that after we have already stained the gels which are already analyzed using fluorescent analyzer using the software. So, then the gels are scanned they are matched with dye labeled images ok.

So, we are 100 now 100 percent sure the ok there is a spot there is also a spot in dye labeled images. Now, the our protein of interest lies in this spot why we are so, determined to locate physically where the spot is in in spite of the fact the computer has already given us the good picture because now we want to get hold of that protein. So, we need to cut that gel excise that gel ok we need to subject it to trypsin digestion some standard procedure and then we need to detect them using mass spectrometry mind it these things are also were also done in traditional 2 dimensional gel electrophoresis is nothing new what happened in 2D gel electrophoresis after we got the sample using the standard dye we are excising the gels and we areagain lysing and detecting by MS, but often there was found the thing is unless we can confidently say unless there we rule out the variation the unless we normalize the thing MS will simply tell you what is the protein that is present MS is will not tell you about the quantity very very very important alright it will tell you about the character of the protein, but by DIG by fluorescent labeling density matrix we already now know about the quantity and now our next goal is to characterize with the help of mass spectrometry. So, this completes the entire process of DIG. So, we should discuss about internal standards.

So, what is internal standard why do we actually need to use internal standard mainly to match and normalize the protein patterns across different gels right. Main why because we want to negate the problem of inter gel variation if we run the processes in the same way there has to be some variation due to the reasons I have already mentioned, but if we somehow can locate a common spot alright this common spot will have to be rendered in the same way. So, it may be so that during our loading the common spot may be appearing similar or may be appearing big or small what software will do it will get hold of that common spot which is for example, stain by psi 2 that is a mixture of all the

proteins and they will normalize because they are dividing the ratio as I told you psi 3 by psi 2 and psi 5 by psi 2. So, after all these things we can surely say that using internal standard the results are much more comparable or alright. So, internal I mean internal standard in 2D electrophoresis why it is used mainly it is a reference sample generally it is a pooled sample from pooled protein from both the samples and individual included in each gel alongside individual sample for normalization and quantification alright as I told you.

In case for example, the in our experiment it is labeled with psi 2 generally the standard procedure is labeling it psi 2 while psi 3 and psi 5 are used for other sample there are multiple psi dyes cyanine dyes we can use any one of them we should use one unique to stain the internal standard. So, what are the benefits of internal standard as you already know by now because I am just repeating myself so that the information is engraved into your brains. Number 1 accurate correct quantization ok very important we want to quantify we need to predict the fold increase we need to say the experimental variation is not due to chance. And if we put a same internal standard across multiple gels and since the internal standard appears on all gels if we normalize that experimental variation will be ruled out and this will help us to confidently say that the difference is due to up regulator down regulated proteins under specific condition or may be due to the either experiment or due to the sample really is happening due to the difference in the sample quality. So, we see that and this also helps us to gain much more confidence statistical confidence when you are comparing the gels because system and inherent biological variation can be ruled out alright if you are to comparing two different samples and we for example, two different biological samples are compared if we pull the proteins of both the samples as standard by normalizing inherent biological variation will also be ruled out.

So, coming to the advantages definitely quantitative profiling we can say the amount of protein multiple samples can be analyzed high throughput detection alright differential expression very important two different proteins altered expression of two different protein under different condition in a single gel can be achieved right. It has got very high sensitivity and resolution already two dimensional g-electrophoresis has got a very high resolution, but combining the technique of fluorescence leads to increased sensitivity. Again quantitative analysis the randomization of sample very important the best practices can be done with the help of software it will produce unbiased result. For example, in the next cycle we can then tag the sample one with the second dye and sample two with the third dye and standard with the first dye and we can again see what are the results right. So, this variation and then final analysis can help us to have a some idea about the inter sample to sample variation can be ruled out.

So, quantitative analysis so, it can be done both I mean by looking at the amount of spot

alright or by comparing the spot to the standard. So, absolute and relative quantitation can be done relative means we are comparing it with something absolute means we are comparing the intensity of the spots directly alright and no gel replicates of the same sample are needed. And lastly there are there has to be some disadvantage because no technique is perfect otherwise technical science advancement will stop right. Main disadvantage as I it was very evident while I was teaching that post staining is necessary. So, there has to be something where we do not need to stain the gels there has to be a procedure that needs to come up that we can directly now quantitate the sample by the by get hold of the protein accessing the protein from the fluorescent gels alright.

So, additional post staining validation for clear protein visualization is a bit need some can be some scope of improvement. Again labor intensive very important the workload conventional from two dimensional conventional electrophoresis is low, but still for I mean good researchers will still use multiple runs to just validate the samples alright. So, when we are running multiple replicates of the same sample for statistical significance it does become labor intensive. And the last thing that I told that since it requires thiol based reductants such as DTT alright which is also a part of dilute sample buffer lysis buffer that can lead to some interference or challenges alright specially when you are using cysteine reactive labels for example, cyanine dyes. So, definitely if you are using other dyes that do not require DTT or thiol based analysis or labeling then this challenge can also be overcome.

So, this is the summary for today's class we discussed conventional two dimensional electrophoresis what are the limitation of conventional 2D electrophoresis, what is the principle of DIG and why it is useful, what is the work flow, what is the importance of internal standard standard and normalization in DIGE and we also discussed the advantages and pitfalls of differential in gel electrophoresis. So, these are my references for today's class and I thank you everyone for your kind attention.