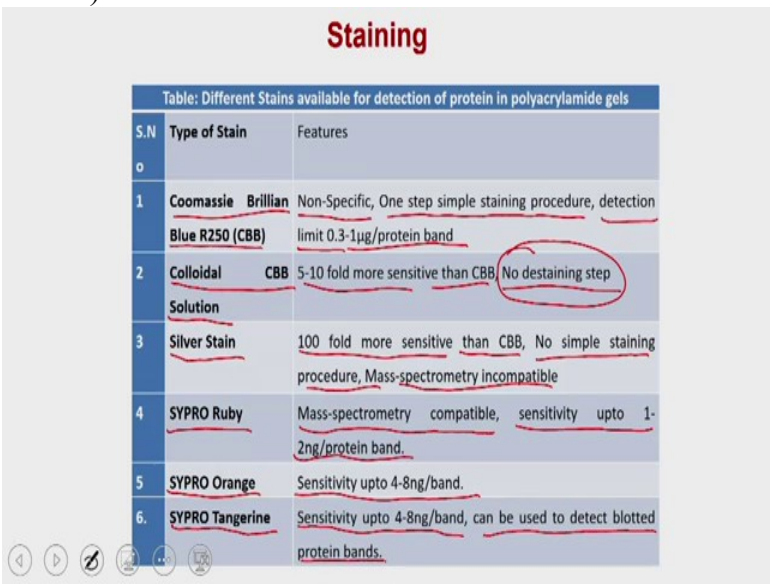
Genetic Engineering: Theory and Applications
Professor Vishal Trivedi
Department of Biosciences and Bioengineering
Indian Institute of Technology Guwahati
Module 9 - Characterization of Isolated Product Part-1
Lecture 30 – Electrophoresis Part-2

Hello everybody! This is Dr. Vishal Trivedi from Department of Biosciences and Bioengineering IIT Guwahati and in the previous lecture what we have discussed? We have discussed about the electrophoresis and how you can dissolve the samples onto the electrophoretic gels and now once you have dissolved the electrophoretic gel, the protein are going to be separated as per their molecular weight and now they are going to give you a pattern.

But the protein itself does not have their intrinsic color, so these protein bands or these protein samples are going to be stained with different types of stain. And in a polyacrylamide gel electrophoresis you have the different choices of different staining dyes which you can use.

(Refer Slide Time: 1:25)



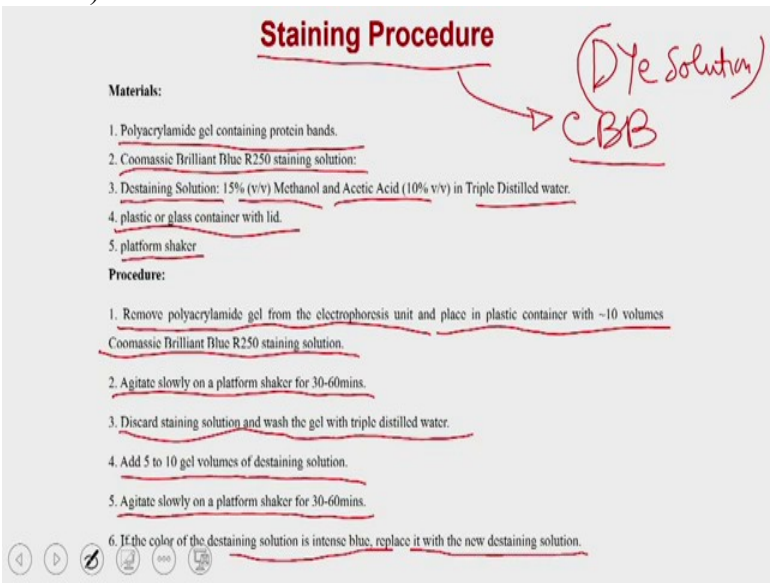
So you can use the Coomassie Brilliant Blue R-250, the Coomassie R Brilliant Blue is a nonspecific dye, it is only specific for the protein but it is not specific for a particular type of protein. But the advantage is that it is a simple step procedure, detection limit is within the range of 0.1 to 1 microgram per protein band. Then we have the Colloidal Coomassie Brilliant Blue R-250 solutions.

The colloidal solution has the 5 to 10 times more sensitive than the normal Coomassie Brilliant Blue solution. And other advantage is that it does not require any destaining step.

Then we have the silver staining. The silver stain is 100 times more sensitive than the Coomassie Brilliant Blue staining. It is not a simple staining procedure, but and as on the other hand it is incompatible with the downstream mass spectrometry applications.

Then you have the SYPRO Ruby. So SYPRO Ruby is a mass spectrometry compatible dye, it is sensitive up to the 1 to 2 Nano grams per protein band. So it is very sensitive and it also is compatible with the mass (spect), so suppose you are resolving a complex biological sample and you would like to identify some of the proteins using the MALDI or some other kind of downstream applications. So you can use the SYPRO Ruby. Then we have the SYPRO Orange, it is sensitive up to the 4 to 8 Nano gram per band. And then you have the SYPRO Tangerine, it is also sensitive up to 4 to 8 Nano gram, it can be used to detect the blotted protein bands.

(Refer Slide Time: 3:12)



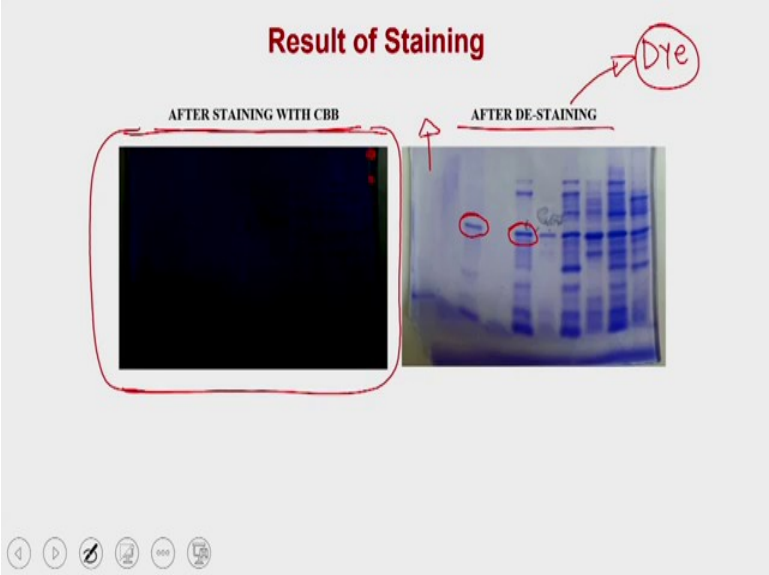
Now in a standard staining procedure what you have is you have a dye solution and this dye solution can be used to stain the dye. So in this particular staining, to explain you the staining procedure what we have done is we have taken a staining procedure with the Coomassie Brilliant Blue. So for that what you need is you need a gel which contain protein band. Then you have, you need a Coomassie Brilliant Blue R-250 stain solutions. Then you need destaining solutions. Destaining solution is methanol, acetic acid and water and then you need plastic or the glass container with the lids and you need shaking platforms.

What you are supposed to do is you remove the polyacrylamide gel from the electrophoresis unit and place in a plastic container with 10 volumes of the Coomassie Brilliant Blue R-250 solutions. Then you mix them slowly onto a platform for half an hour to 1 hour. After that

you discard the staining solution and wash the gel with the triple distilled water and then you add 8 to 10 volume of destaining solutions, you mix them or you mix them slowly onto a shaking platforms for another half an hour to 1 hour.

And as a result what will happen is the destaining solution is going to remove the dye and that is how it is going to give you the staining of the protein. And if the color of the destaining solution is intense blue, replace it with the new staining solutions. So you can do this destaining procedure on multiple times and that is how you are going to see the appearance of the bands.

(Refer Slide Time: 4:59)



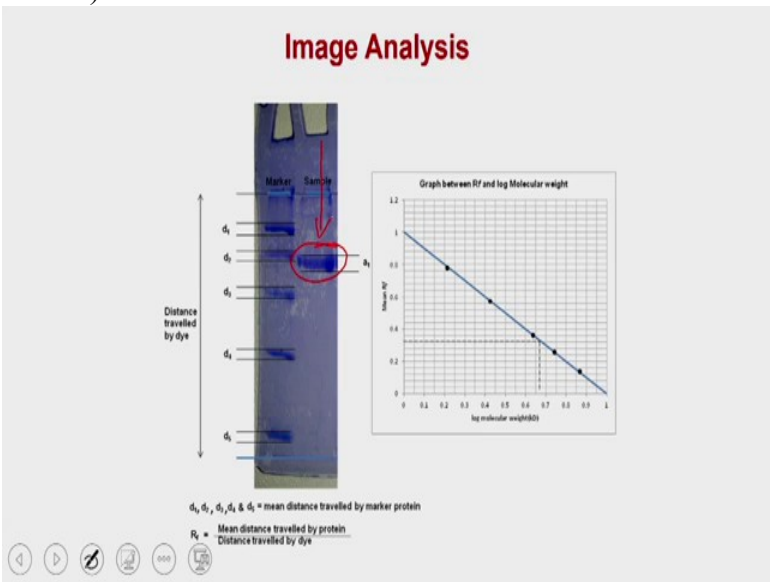
Now once you do the staining, what will happen is after you stain with the Coomassie Brilliant Blue, what will happen is you will see a black colored film. Your gel is going to be converted into this black colored film. But once you do the destaining, what will happen is the dye is a nonspecific dye, so it starts absorbing everywhere onto your gel and this absorption is very very weak compared to its adsorption with the or its binding with the protein molecule.

So when you put it into the destaining solution, the destaining solution is going to start removing the dye from the gel solution, gel block but it will not remove the gel from the places where the protein is present because the affinity of the dye for the protein is more compared to the affinity of this particular dye for the gel monomers or polyacrylamide monomers. So as a result when you destain the dye which is present in the gel will come out and as a result you will see a pattern.

(Refer Slide Time: 6:01)

Once you see a pattern, you are going to see a image. This image is actually having lot of informations, it have information in terms of how many bands are present, what is the position of these bands, what is the concentration of or what is the amount of this protein is present. But this image analysis is also very very complicated and that is why the image analysis requires different types of softwares because every image has the lot of informations and all this information, the processing of this information requires the sophisticated softwares.

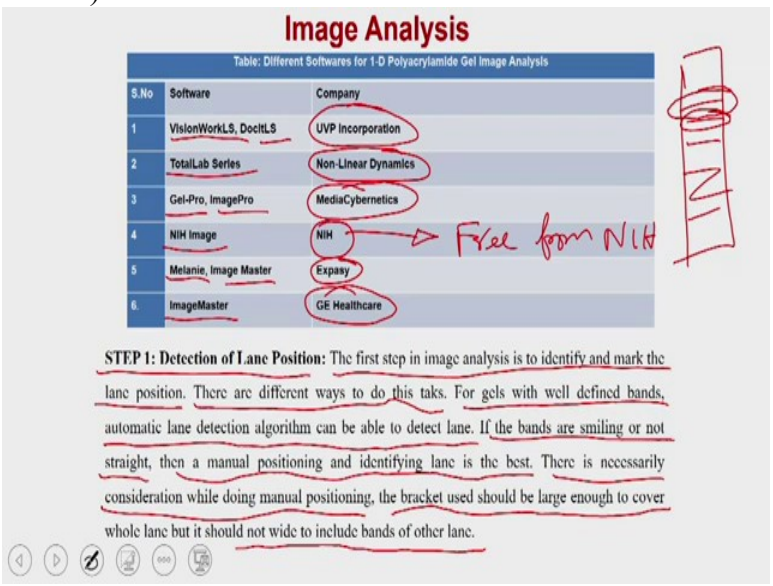
(Refer Slide Time: 6:44)



You can see that if you have a gel image like this, what you have to see is that what is the distance of this particular protein from the well and what is the amount of the protein present.

And all this information is you have to gather from the different lane and that is why people have developed different types of software to analyze this.

(Refer Slide Time: 7:04)



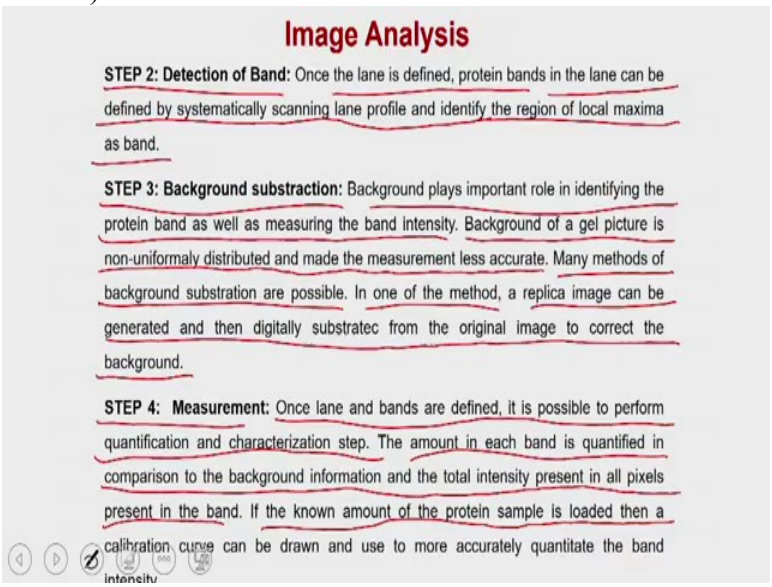
What you have is you have the VisionWorkLS or DocItLS which is actually from the UVP corporations. Then you have the TotalLab Series which is from the non-linear dynamics. Then you have the Gel-Pro or imagePro which is from the Media Cybernetics or you have the NIH image which is actually from the National Institute of Health. And so this is actually a free software which is available from NIH, NIH is a funding agency which is present in the US. So you can actually download the NIH image and that is actually will be useful for analyzing the substances.

Then you have the Melanie or the Image Master which is from the Expasy. And then you have the Image Master which is also from the GE Healthcare. So when you would like to analyze image for (the) and you would like to deduce lot of information, you also have to follow a definite steps and these steps could be different for different softwares. But the basic principle remains the same.

What you have to do is in the step 1 you have to detect the lane position. The first step is to identify and mark the lane positions which means you have to select the lane and then there are different ways to do this task. For gel with the well-defined bands, automatic lane detection algorithm can be used to detect the lane. If the bands are smiling or not straight, then a manual positioning and identifying lane is the best.

There is necessarily consideration while doing manual positioning, the bracket used should be large enough to cover the whole lane, but it should not wide enough to include the bands of the other lane, which means if you have one lane and you have the different bands, so if you have the discrete bands, you can use the automatic mode to select the lanes. But if these lanes are merged or you have the smiley bands or some kind of resolution problem, then you have to manually select the bands. But when you do manual selections, you have to ensure that you select the bands with the proper width but it should not be so big that you are selecting the bands of the, you are selecting the separate band also.

(Refer Slide Time: 9:32)



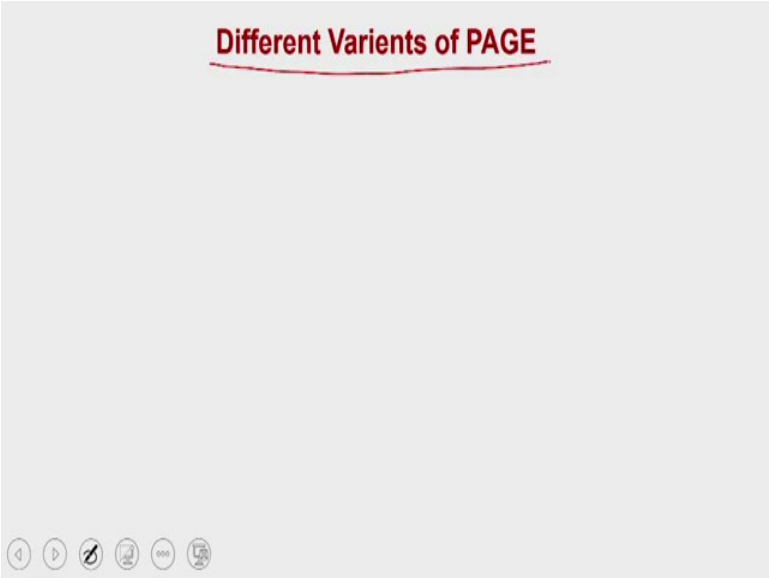
Once you selected the band, then you have to have the detection of the band. Once the lane is defined, the protein band in the lane can be defined by systematically scanning lane profiling and identify the region of the local maxima as band. Then the step 3, you have to have the background subtractions. The background play important role in identifying the protein band as well as the measuring the band intensity. The background of a gel picture is non-uniformly distributed and made the measurement less accurate.

Many methods of background subtraction are possible. In one of the method, a replica image can be generated and then digitally subtract from the original image to correct the background. So the background is very important. If the background is very high or background is very low, that actually is going to decide what is the shape of this particular band and what is the intensity of these bands because all the intensities are going to be calculated based on the background.

So, if your background is very high, the image analysis is going to be less and less accurate. More important is if the background is non-uniform which means in some lane it is lower on a lower side, in some lane it is on the higher side, so because of that you will be having a difficulty to choose what background I should subtract. And in those cases if you go with the higher background, you are actually going to cut the signal from the protein which are closer to the lower backgrounds signal and so on.

And then you have to do the measurements. So once lane and bands are defined, it is possible to perform the quantification and characterization steps. The amount in each band is quantified in comparison to the background information and the total intensity present in all pixels present in the band. If the known amount of the protein sample is loaded then a calibration curve also can be drawn and that calibration curve can be used to calculate the intensity in your band.

(Refer Slide Time: 11:46)



Explaining you the different steps of image analysis using a separate software, we have prepared a small clip which actually will allow you to see all these steps which we have just now discussed where we are selecting the lane, we are selecting the bands, then we are telling you how to measure the intensity, how to measure the position of this particular band. So all these we have prepared in a small demo. In this particular clip what we have, we have discussed the technical detail as well as the steps what you have to follow.

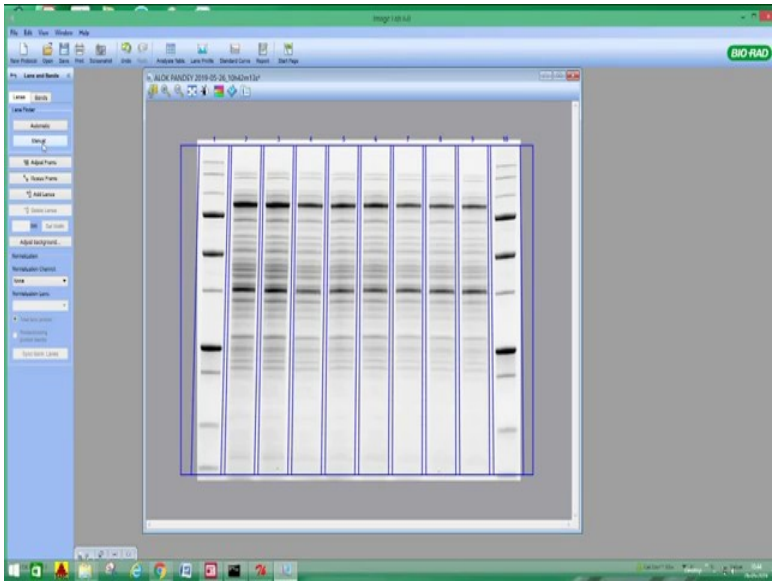
[Video Presentation Starts]

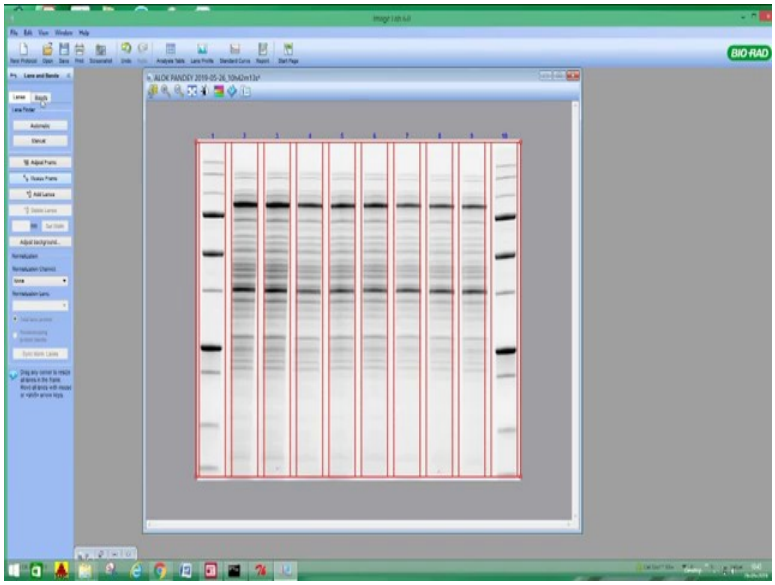
(Refer Slide Time: 13:00)

In this video we will show you how to analyze a particular band of interest from SDS (()) (12:30) electrophoresis gels using image lab software. So here I will show you how to open the image lab software and how to analyze different components. So you have to go to Start and type Image Lab. So it will open. You need not to do anything. Just, this is we are using new protocol, so just have to, first I will show you how to analyze the protein gels.

So this is our gel image. So this is the molecular weight ladder, first row and last lane. And these are the fractions. So we want to analyze what are, how many different bands are there, first component and what is the intensity of these bands and what is the molecular weight. So I will try to show one by one. First, after getting the image or you can get from, you can open any image lab dot scn file from instrument. So go to here on left side top panel. You can see image tools. So if you go there, there are different data.

(Refer Slide Time: 14:25)

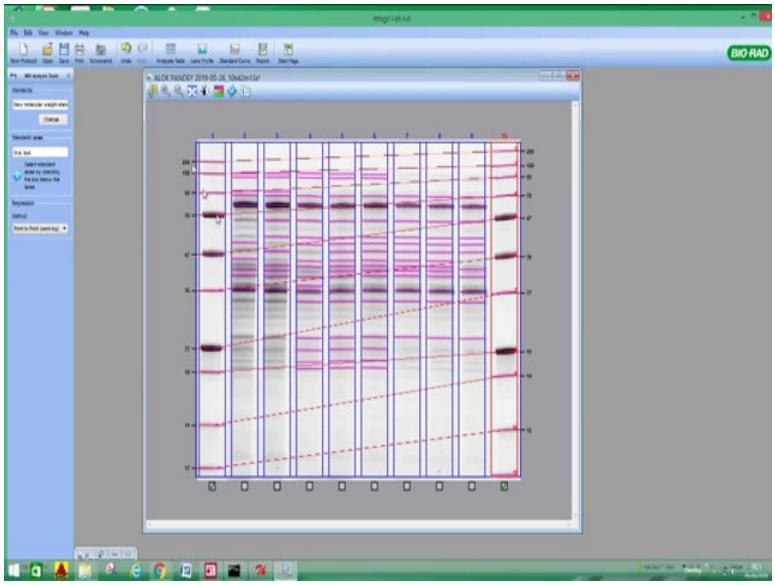


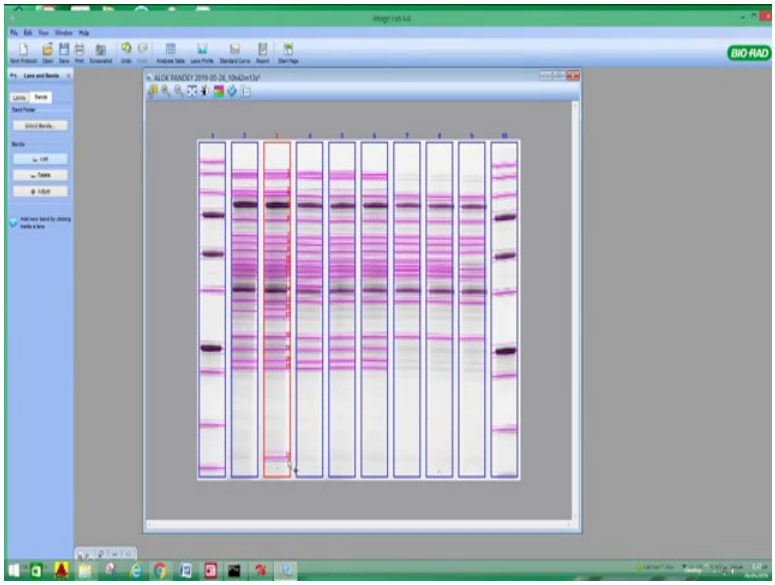


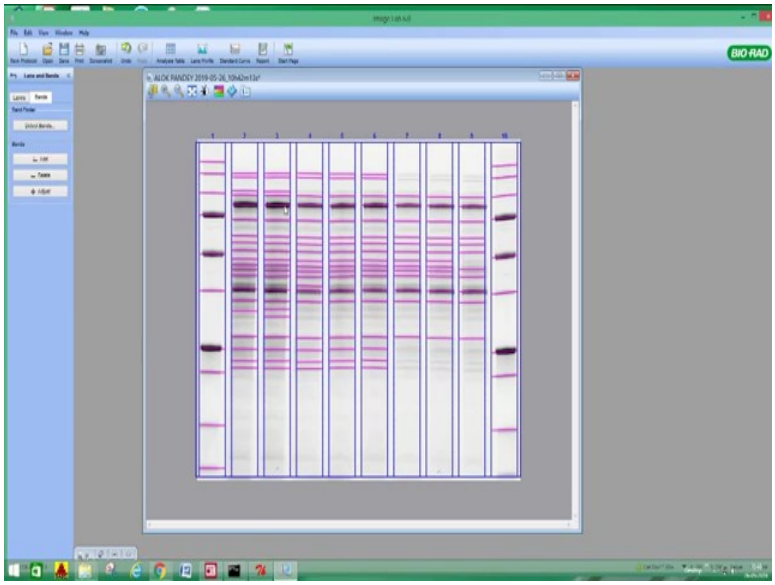
You do not want to see complete picture, so just you can crop, crop the image and like this. Just say crop, so this is the complete image and go back here. After that you have to identify how many bands are there, how many lanes are there. Just leave these two software, it will do automatically or in another way you can select manually also. So let us see how it will perform. So click on lanes and bands. So lane finder, here lanes either allow the software to detect or you can do it manually.

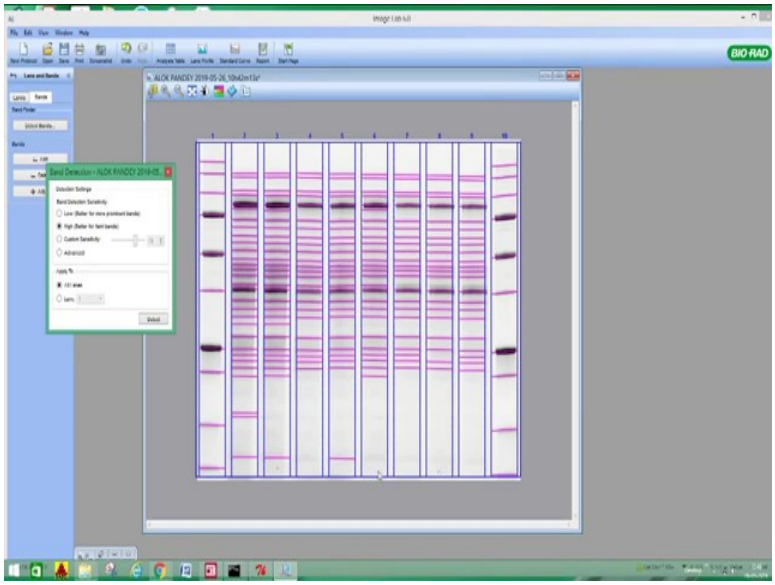
So if I ask for automatic, so it will see, you can see there are lanes given on the top of the gel; 1, 2, 3, 4, 5, 6 up to 10. So this is something different or if you want to do it manually also you can do. Suppose, if you want to do, enter number of lanes, how many lanes are there? So 10. So here you can adjust lanes see this is not fitting in the completely. So you have to adjust like this to get complete lanes. Now you can see the lanes are completely adjusted, fitted in the lane completely. So this is another way, manual way.

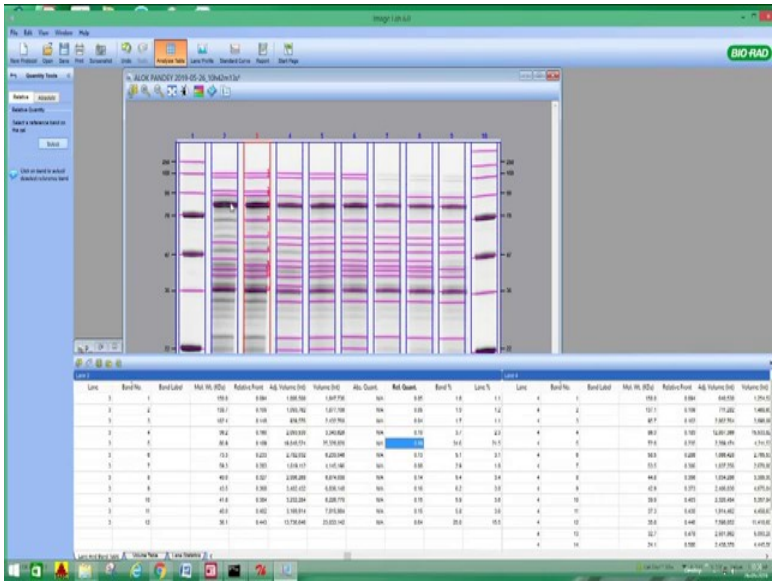
(Refer Slide Time: 16:33)











So next, now after identifying the lanes, you have to go to bands, how many bands are there. So you allow the software to detect the bands. So here few options are there. Band detection sensitivity, 1 is low, low means better for most prominent ones. So detection sensitivity low, if you are keeping it low sensitivity, then it will detect only prominent bands which the software can observe based on intensity. Otherwise you can do another thing. Select high, high sensitivity means it will select a faint bands also, as you can see it has recently that.

Or you can manually select sensitivity. So I am asking high, better for faint bands. See how it will do. See these many bands are there. Each and every single band will be detected. Although some here are left out, but you can still manually add the band. So, if you want only prominent bands, so in that case you just select low which is better for prominent bands. So these many bands.

So now question comes, you do not want to calculate molecular weight or intensity values, that is quantification for all these bands. Either you can keep these bands selected by software or you can remove. So for deleting suppose if you want to add some particular band, suppose here I want to add this band, bottom of this lane. So just go, add, just it added another extra band.

So, if you want to delete some bands, you do not want these many bands, you want to only calculate for these prominent bands. We just go to delete, we just keep on doing till you get satisfactory bands. This way you can do. So you can add also. So after being done this, we have first detected the lanes and in second we have detected the bands. Now it is time for calculation of, or molecular weight analysis. So here the tricky part is you have to select the lane, which lane you have loaded the molecular weight marker. And selecting the first lane, and the last lane, so it automatically came.

Automatically it came. So, if you are using your gel of interest, so in the case what you can do is you need not to worry about, you just have to give your molecular weights. Suppose this is a randomly taken. By default it has taken Bio-Rad Precision Plus. You can change this one also, this pattern you can change also. Suppose, you want to add some new protein molecular weight standards, you do not have this Bio-Rad Precision Plus. You have loaded some other thing, but you know what are the first band appears on top to lower bottom band. So, if you know, you can add new, just go new standard or you can give the name.

Suppose I am giving new molecular weight standards something x, x, y, okay, so company's name. So after that you have to add each and every band. Suppose first one, I am just giving these values only for convenience purpose, 250, and second band is 150. It need not to be these values, whatever your choice of marker is you can give those values, those k d values. You need not to worry about what it has written. It will automatically disappears.

And third band is just say 99, and fourth band is 78, and fifth band is 47. Sixth band is 36, seventh band is 22, eighth band is 19, ninth band is 14, this is 12 and last one is you can keep 8. This is just for example purpose only. This is not actually any standard molecular weight maker. Just for your understanding I am giving this one.

After giving the molecular weight, you just click okay, and you select okay. Yes, you want to, it does ask, you want to apply. Yeah, yes, I want to apply, so scene, here this is the molecular weight what we have selected. See, here new molecular weight marker is x, x, y, so here you can see what we have given is it is coming as it is.

So the next thing is you have to calculate molecular weight of these things. So you just need not to worry about anything and you just go to analysis table. So here analysis table it will give for each and every lane details with proper band percentage and lane percentage. Suppose, in first lane it corresponds to, first lane, lane 1 it corresponds to molecular weight C, from lower weight higher one. So what are the values? Different values? And absolute quantification, this we will see in next part. And percentage, how much percentage it is, these things we can see.

So, if you want to calculate suppose in lane 2. You have lane 2. Lane 2, this is the fifth band I think, so it will give what are the molecular weight. So this is starting from bottom, band number, this is 1, 2, fifth band I think. So fifth band means on top fifth band is 87.4 kDa. This is 78, we are calculating for this one. This is almost comes 87.4 kDa. And the relative front is 0.187. So in this way you can calculate molecular weight of the, your band of interest.

So this is all about molecular weight. You can save this thing and export this result to a new Excel sheet. So after being done this now we are moving to quantity tools. So quantity means you know, you have loaded something, what amount you have loaded in this lane. If you know that thing, we can calculate remaining lane's quantity how much we have. So there is two parts; one is relative quantity, select a reference band and the gel. So you have to select

one reference band. Based on that it will calculate all the bands' relative quantity. But it does not give any value.

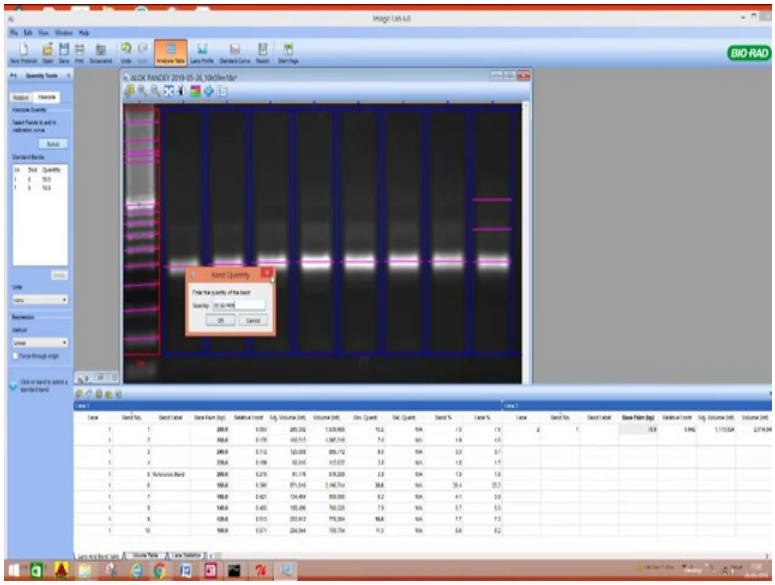
Suppose I am selecting this one, this is the reference one. So automatically it will calculate the remaining proteins, lane 3, this is lane 2. So it will calculate automatically relative quantity, it is taking 1 for this reference one. So based on this it will calculate all the band's relative intensity. Suppose if you say this is 1, what could be this for band 1? It is 0.59 and band 2, 0.15, 0.15, 0.06, 0.07. So in other lane 3 also the band is this is fifth band.

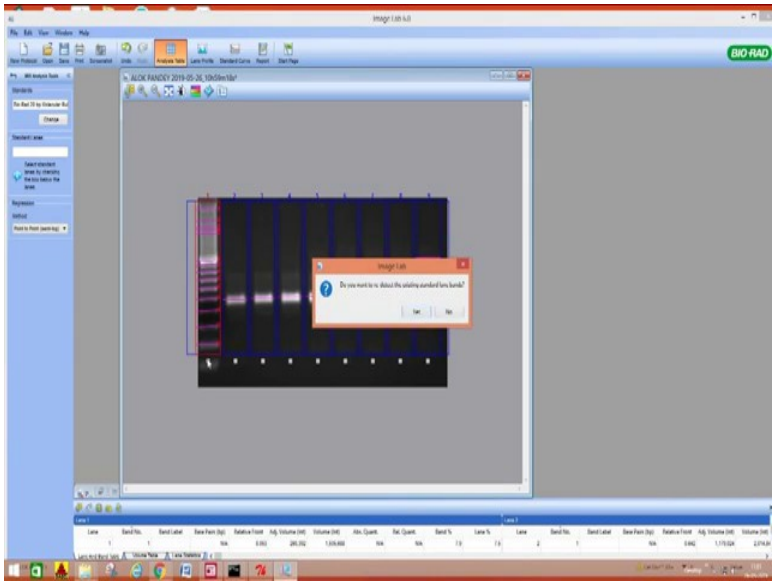
So lane 3 band 5 that is molecular weight 7. The relative quantity is 0.89, so this is 1. If you are saying this is 1, this one is 0.89. So in this way you can calculate relative quantity. And you do not want this. You know, some number, suppose how much protein it is present in this one, so in that case you can go to absolute quantity. At least you need 2 to 3 bands. So see, I am selecting this one. Okay, this is, I am saying this is the quantity of 50 Nano gram. And I am selecting this quantity of 65 Nano gram. And I am selecting another one, this is 25, I mean automatically it gives.

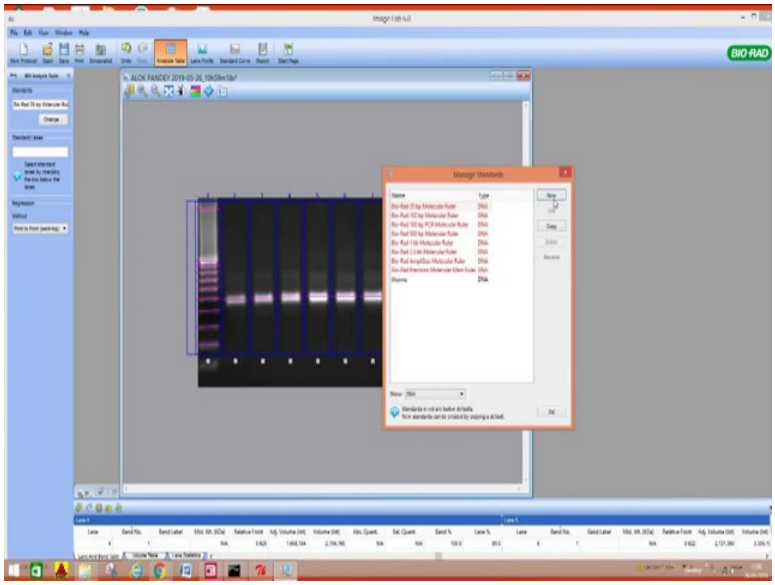
So you just take your mouse pointer to any band you want to calculate the quantity, actual quantity. So you calculate, you can click on that one, it will give automatically 72.214 Nano grams. You want to calculate this band, so you just keep that one, it will give the value. You want to calculate this one, it will give the value. So in this way you can calculate absolute quantity of the any given band in the gel. So you can export these results or you can save from this one also. So after quantification of the protein lanes and the bands you can use this for annotation also. So you can go to annotation tools.

Suppose you want to indicate this band, okay, you just give the arrow mark. Otherwise you want to show this band. So this is also another way. You can take this as screenshot or you can save as picture with these things with annotation tools. You can add text also. Suppose you want to add text here, you just add something, suppose lane 2 like that, lane 2 band 5. So this is the way how the analysis can be done using Image Lab software. So this is about, all about proteins.

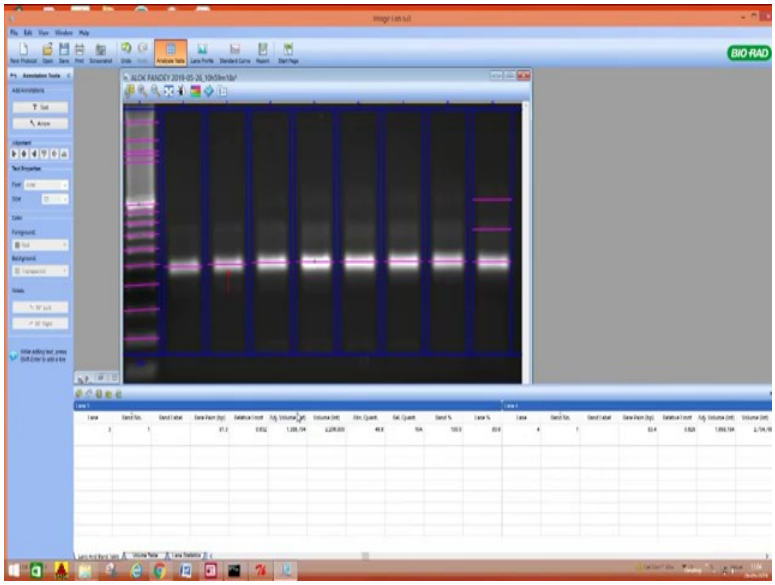
(Refer Slide Time: 30:18)






BIO-RAD
Manage Standards


BIO-RAD


BIO-RAD

So now you, I want to do the same analysis for the nucleic acids, suppose DNA. So just go, so you have, now you have DNA. So all the parties same as compared to proteins. So you can go to Image Tools for cropping, you can crop it. Or whatever the part you want to keep, you can keep that one and say crop. After that lanes and bands. So it is exactly same protocol for proteins and nucleic acid also.

It will detect the bands, low better for prominent bands, I am saying, I am asking like that, okay. So after that it detected lanes and bands also. Now you can go to molecular weight analysis. So here also, either you can give your choice of interest, molecular weights you can give, and you can create new and add the values. This is here nucleic acids molecular weight based pairs. So in this way you can give or you can use 1 that has given in, already given in the software. So as you can see this is already, this is depicting here what is the base pair's amount of each lane. So here also this is same.

So the next thing is after being completed you have to, you need not to specifically look into what this band corresponds to, you just go to lane 2 values. This is the lane 1, this is lane 2. Only single band we are predicting, that belongs to 78.6 base pair value. This is in length. So as you can see this is 80 and here it is coming this is 78.6 base pairs. So in this way you can calculate the molecular weight of the nucleic acids also.

And the other part is quantity tools. You can quantize the DNA also. So here also same in case of protein analysis. Here relative, so you have to select one band. Suppose I am selecting this one. Based on this it will calculate the all band's relative intensity, relative quantity. Or you can select band standard one and this is quantity, this is band quantity. I am saying it is as 30 Nano gram. So another band, at least we need to, this is as 10 Nano gram. After giving two values, you can click any band. Suppose this one, 39.92, this one 55 and this one 63, this one 64.

So in this way you can calculate the intensity, absolute quantity of the any given band. You can use annotation tools also by giving arrow or showing, representing graphically also you can do same thing. And you can export this screenshot also. So you can save these things and you can export. Here the export options are there. You can do exporting also. So I hope this software information part help you to understand the how software can be utilized for analysis of different bands and molecular weight purpose or quantification purpose.

[Video Presentation Ends]

In this clip we have discussed all the steps and I hope this image analysis demo could be useful for you to analyze your protein gel samples as well as it may help you in advancing your work. Thank you.