Experimental Biotechnology
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Module-I
Basics of Laboratory Research

Lecture-08
Basics of Electrophoresis (Part-2)

(Video Starts: 00:23) (Video Ends: 01:00) Hello everybody, this is Dr. Vishal Trivedi from department of biosciences and bioengineering IIT Guwahati. And we were discussing about the electrophoresis, so in the previous lecture, we have discussed about the basics of the electrophoresis. And how the electrophoresis is actually separating the different types of molecules and how the technique is being evolved. So, initially the people were using the movement bounding electrophoresis.

And then they have overcome the deficiencies in that particular technique by the introduction of the solid support media. For example the acrylamide or the agarose and as a result they have developed the zone electrophoresis. So, the gel electrophoresis is one of the form of the zone electrophoresis and then within that we have discussed about the vertical gel electrophoresis, how to cast the gels, how to perform the gels.

And what are the different mechanisms of the stacking of the sample and as well as the other kind of minor details. And at the end of the previous lecture we have also discussed about the how to perform these electrophoresis in the gel. So, in that I have taken you to my lab and the students have shown you how to cast the gels and run the gels. So, in the today's lecture, we are going to discuss about the staining of the gel.

And as well as how once you got the stained a gel, how you can be able to do the image analysis. We have the multiple options in terms of the different types of a stain and we have the advantages as well as the different types of options available with the different stains.

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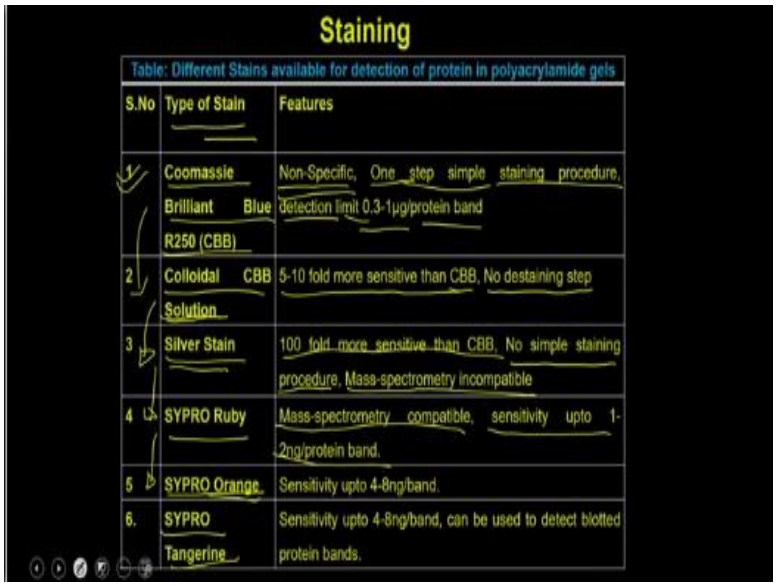

So, we have the coomassie brilliant blue R250 as an stain and the coomassie brilliant blue R250 is the one of the most popular stain what people are using for the acrylamide gels. And it is nonspecific, so it does not stains the any particular type of protein. For example it cannot stain the phosphorylated protein or acetylated protein but it will stain all the protein what is present in the gel.

But one of the major advantage of the coomassie brilliant blue mediated staining is that it is the one step simple staining procedure. And the major disadvantage is that the detection limit is in the range of the microgram per protein band or the microgram per protein present in the lane. Then to improve this people have developed the colloidal coomassie brilliant blue solutions. So, the colloidal coomassie brilliant solution has the 5 to 10 fold more sensitivity than the normal coomassie brilliant blue R250.

And added advantage is that you do not need to do these staining steps. Then compared to that people have come up with the silver staining, the silver staining is even 100 folds more sensitive than to the coomassie brilliant R250. But the disadvantage is that it is not a simple staining procedure, it requires multiple steps and it requires the different types of reagents. And on the other hand it is also not mass spectrometry incompatible which means if you stain your gel with the silver stain you cannot use the protein from directly for the mass spectrometry.

To overcome these people have developed the three more the stains like the SYPRO ruby, SYPRO orange and the SYPRO tangerine. And all these are actually the mass spectrometry compatible dyes and they are sensitive up to the 1 to 2 nanograms per protein bands. And for example the SYPRO orange it is a orange color dye and then SYPRO tangerine is also is a very, very sensitive and it is also compatible with the mass spectrometry.

So, even after the staining once you got the band you can be able to use the downstream applications like the mass spectrometer you can identify the protein with the help of the different proteomics approaches. So, in a typical staining procedure what you are supposed to do is you have to first take the dye and then you have to prepare the staining solutions. And once you prepare the standing solution then you incubate the gel into the staining solution and then you have to perform the destaining steps or in some cases that destaining steps is not required.

So, to explain you the staining procedure we have taken an example of the coomassie brilliant blue and how to perform the staining procedure.

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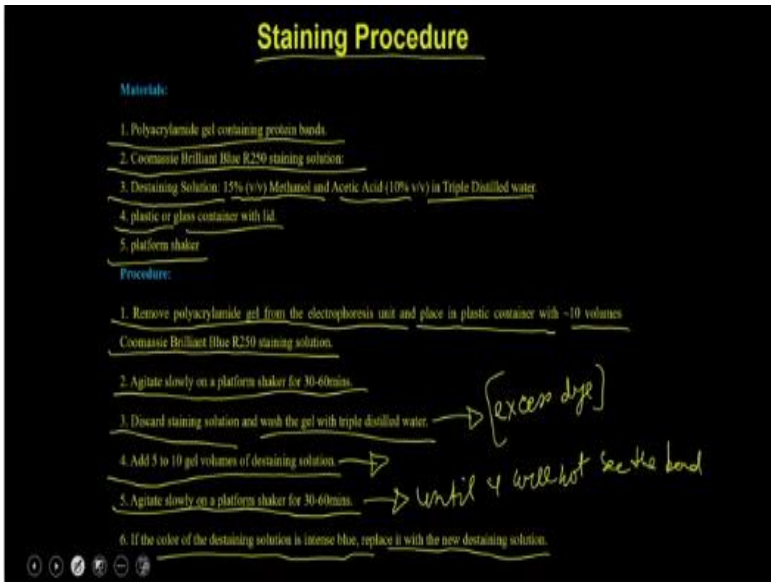

So, for the staining of the gel with the coomassie brilliant blue the material what you require is the polyacrylamide gel which contains the protein bands. Then you require a coomassie brilliant glue R250 staining solutions then you require a destaining solutions. So, the destaining solution which contains 15% methanol, acetic acid 10% and that all you are going to prepare in the triple

distilled water and you require a plastic or the glass container with the lid and then you need a shaker.

To perform the coomassie brilliant glue mediated staining, first what you have to do is you remove the polyacrylamide gel from the electrophoresis unit. And place it in a plastic container with 10 volume of the coomassie brilliant blue R2 staining solutions, you agitate the slowly on a platform shaker for 30 to 60 minutes. Then you discard the staining solution and wash the gel with the triple distilled water. So, that actually is going to remove the excess dye what is been attached to the gel.

And then what you can do is, you can add 5 to 10 volume of destaining solutions. And as a result what will happen is it is actually going to remove the dye from the nonspecific bound places and that destaining solution, you have to keep the gel in the destaining solution for 30 to 60 minutes or until you will not see the bands. If the color of the destaining solution is intense blue you can replace it with the new destaining solution. So, that it can be able to utilize or it can be start removing more and more dye from the (()) (07:51) weakly bound places.

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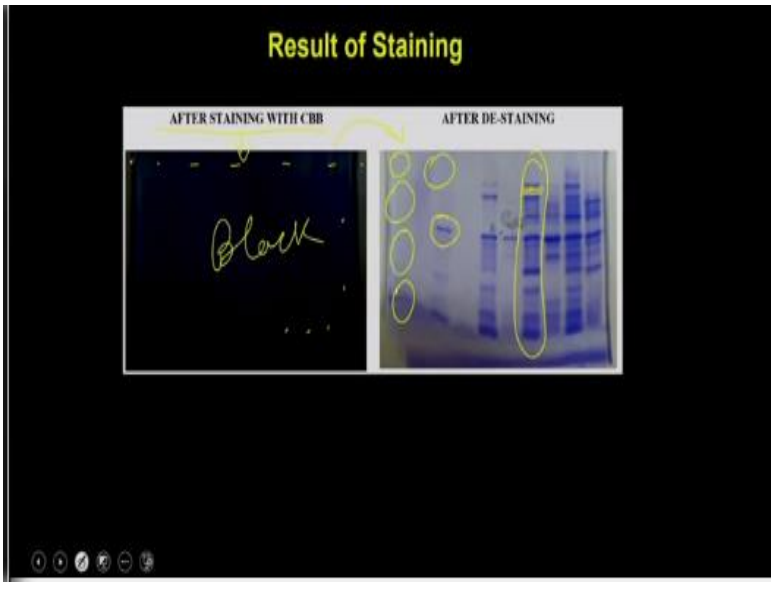

And if you do so, let us see if you stain the gel with the coomassie brilliant blue in the beginning, the gel will be look like as the black firm. And this is all because the coomassie brilliant blue is going to bind every places irrespective of whether you have the protein or not which means it is

actually going to bind to the polyacrylamide as well as the proteins and then but the affinity of the coomassie brilliant blue for the polyacrylamide as well as for the protein is going to be different.

So, once you do the destaining step, so what will happen is the coomassie million blue is going to be removed from all the nonspecific polyacrylamide gel. But the place where you have the protein it is not going to be removed because it has a more affinity for the coomassie brilliant blue. So, as the result, you are going to start seeing the protein bands, so this is the typical pattern of the protein band what you are going to get after their destaining steps what you can see is that even between the bands.

Also the dye is been removed by the destaining solutions. And this is what you have to achieve simply by going into the destaining solution. So, after the every round of destaining solution you have to observe the gel whether you are getting the bands or not. And accordingly you can optimize that particular step to see that whatever you are seeing is actually the adequate and it is actually going to give you the appearances of all the protein bands present in the lane.

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Now, once you got this band, once you got this image what you can see is and what you can be able to analyze the two things. One is the position of the band, the second is if distance from the origin. And based on this and apart from that you can also be able to see what is the intensity of

this particular band. So, these are the 3 information you can be able to infer when you will actually going to do the image analysis after the staining procedures.

So, image analysis is a very, very complicated procedure because it requires the understanding of not only the band of the protein band but also the neighboring backgrounds. So, that is why it image analysis requires the some kind of assistance from the softwares which actually is going to integrate. And which is actually going to change the image into a binary format. And as a result all these spots are going to be converted into the binary format of 010101 like that.

And as a result what you can see is that it actually is going to change the image into a kind of a 01 format. And as a result what you will going to see at the end is, if for example if suppose this is your band ok. So, what will happen is every spot is going to be converted into 01 approach which means wherever you do not have the intensity that will be considered as 0. Whereas wherever you have the protein band it is going to be considered as 1 or 10 or 50 or so that depends on the calibration of the softwares.

So, if you can calibrate the software and it will actually going to analyze your image it can actually and assigns a random number to particular intensity. And as a result you can be able to convert this whole image into that particular format and that actually helps the softwares to understand each and every pixel which is present in the image. And that is how you can be able to identify the position of this band, you can be able to measure the intensity of this band. So, for this purpose we have a couple of softwares which can be used to perform this function

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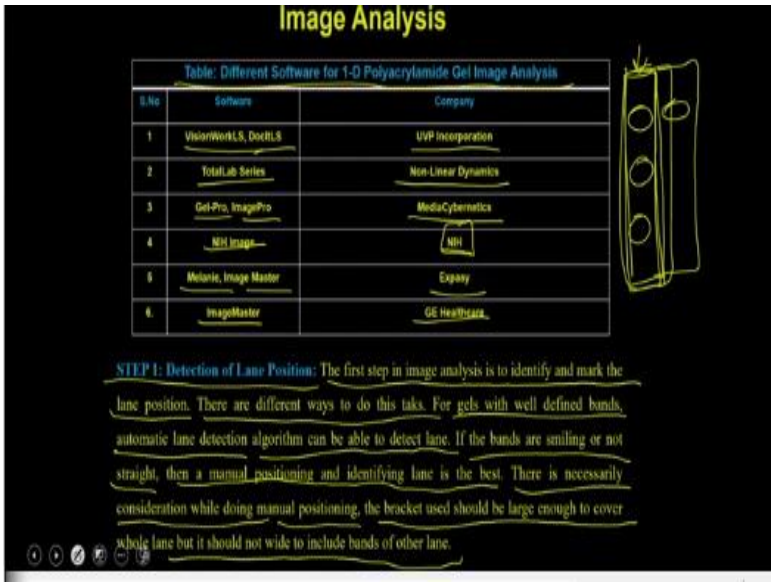

So, these are the different softwares what you can use for the polyacrylamide gel analysis, these are vision works or the doc itself which is from the UVP incorporations, total lab series which is from the nonlinear dynamics, Gel pro or ImagePro which is from the media cybernetics and NIH image which is actually a free software which is available from the American funding agency NIH.

And then you have Melanie or Image Master which is from Expasy and then you have the imageMaster which is from the GE Healthcare. Now, to analyze a image we have the discrete steps. So, what is there in the step 1, the step 1 you have to first detect the lane positions, the lane in which you are interested to do the all the image analysis operations. So, in the first step in the image analysis to identify and mark the land positions which means if you have a lane you have to first mark the lane that I want to analyze this particular protein lane.

There are different ways to do this for gel with the well defined bands automatic lane detection algorithm can be able to detect the lane. If the bands are smiling or not straight then a manual positioning and identifying lane is the best. There is a necessarily consideration while doing manual positioning, the bracket used should be large enough to cover the whole lane but it should not be wide enough to include the lane of the other lane.

So, when you are doing the first step, first step you have to detect the lane when which you are interested to do the measurements. This has to be very precise, so that it is only detect the lane 1, it should not going to take up the band from the neighboring lane which means if you have 2 lanes side by side, you can just say select the lane what you are interested to measure. But it should not be so big that you actually going to take up the band from the neighboring lane as well. So, once you are done the detection of the lane position then you have to go for the step 2.

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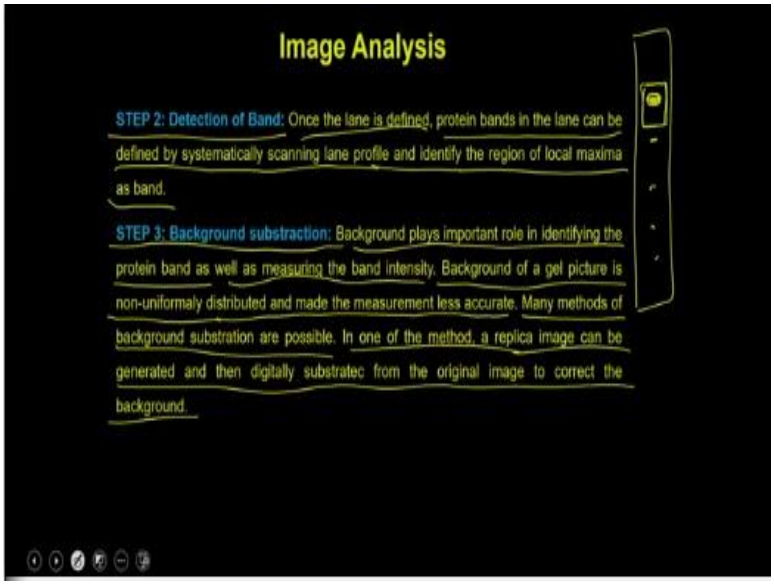

In the step 2 you have to do the bands, in the bandwidth you are interested to do the position, to know the position of that particular band. As well as to know the intensity of the band which means within the lane you can have the multiple bands. And then within this lane you can also select the band which you are interested to measure or which you are interested to analyze. Once the lane is defined your protein band in the lane can be defined by systematically scanning the lane profile and identify the region of the local maxima as a band.

Then the step 3, the step 3 is that you have to do the background subtraction. So, once you select the band you are not going to select the band but you are also going to select the neighboring background. So, that background has to be subtracted from your protein bands, so that you are going to get the absolute intensity of that particular band. So, the background plays the important role in identifying the protein band as well as measuring the protein band intensity.

Background of a gel picture is non-uniformly distributed and made the measurement less accurate, which means if the background is uniform you are going to see the measurement more and more accurate. But if the background is non-uniform, that actually is going to bring more and more trouble because software does not know what is your intensity and what is your background.

So, if it is, it is a uniform background, it is actually going to give you the more uniform numbers which means it is going to assign very comfortably the 0 to the every lower intensity pixel. But if it is a lower high, lower high then it will not be able to judge where your intensity of the spot is starting, where your band is existing within the gel. So, that actually is going to make the measurements more and more erroneous.

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, once you are done with the background corrections, then you can go to the next step and ask the software to give you the intensity.

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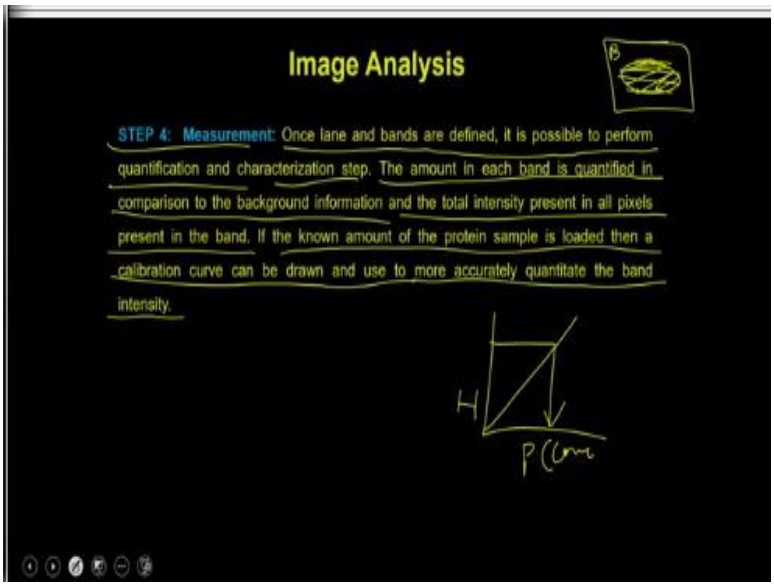

So, the next step is the measurement steps, so once the lane and the bands are defined it is possible to perform the quantification and the characterization steps. The amount in each band is quantified in comparison to the background information. And the total intensity present in all

pixel present in the band, which means if you have a band you are actually going to get deep intensity of each and every pixel of this band.

And that is how and that intensity will be in relation to the background what you have in the neighboring to the surrounding this particular protein band. So, if the known amount of a protein sample is loaded then a calibration curve can be drawn and use to more accurately quantitate the protein band, which means if you have the known amount of the protein band, what you can do is you can measure the intensity versus the protein concentration.

And you can be able to draw a calibration curve between this and once you have that particular type of calibration curve, then you can be able to use this calibration curve to measure the intensity of the protein present in this particular band. So, this is a brief overview of the image analysis just to explain you these steps in more in detail. I will take you to my laboratory for extensive demo on one of the softwares where the students are going to show you each and every steps with the help of the softwares.

These softwares are available from the companies, so they may not be freely available to you to for practice. But I hope this demo is going to be helpful for you to understand the whole procedure. **(Video Starts: 18:26)** In this video, we will show you how to analyze a particular band of interest from SDS or Agarose gel 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 go 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 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 SEN file from instrument. So, go to here on a left side top panel you can see image tools, so if you go there,

there are different data, 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 this to 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 you can see on 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 lane, see this is not fitting in the completely, so you have to adjust like this to get complete lane. Now, you can see the lanes are completely adjusted fitted in the lane completely, so this is another way manual way.

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, one is 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 (()) (23:21) 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 of here or 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, see you do not want these many bands you want to only calculate for this prominent bands, just to go to delete, you just keep on doing till you get satisfactory bands ok, 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 or molecular weight analysis. So, here the tricky part is you have to select the lane, which lane you have loaded the molecular rate marker. So, I am selecting the first lane and the last lane ok, so automatically it came. So, if you are using your gel of interest, so in that case what you can do is, you need not to worry about, you just have to give your molecular weights.

Suppose this is the randomly taken by default it has taken Biorad 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 Biorad 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 xxy ok, some 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 markers you can give those values, those KD 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 the last one is you can keep 8. This is just for example purposes only, this is not actually any standard molecular weight marker just

for your understanding I am giving this one. After giving the molecular weight you just click ok and you select ok, yes it is asking you want to apply yeah yes, I want to apply, so seen.

Here this is the molecular weight what we have selected, see here new molecular weight marker xxy. 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 ok. So, you just need not to worry about anything and you just to go to analyze this table. So, here annalistic table it will give for each and every lane details with proper band percentage and lane percentage.

Suppose, in first lane, lane 1 it corresponds to molecular weight. See from lower weight higher one, so what are the values different values and absolute quantity this we will see in next part. And band percentage how much percentage it is, these things we can see. So, if you want to calculate suppose 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 to 5th band I think.

So, fifth band means on top fifth the 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 lanes quantity, how much we have. So, there is two parts one is the relative quantity, selector reference band on 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 1. So, based on this it will calculate all the bands relative intensity. Suppose if you say this is 1, what could be for band 1, it is 0.59 and band 2, 0.15, 0.15 0.06, 0.07. So, in other lane 3 also that band is this is

5th band. So, lane 3 band 5 that is molecular weight 7, the relative quantities 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 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 ok, this is a I am saying this is the quantity of 50 nanogram. And I am selecting this quantity of 65 nanogram 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 nanograms, 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 after 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 bands, you can use this for annotation also. So, you can go to annotation tools, suppose you want to indicate this band ok, 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 a picture with this 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 ok. So, this is the way how the analysis can be done using image lab software. So, this is all about proteins, so now I want to do a the same analysis for the nucleic acids suppose DNA. So, just go now you have DNA, so all the part is 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 lane sand 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 asking like that ok. 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, base pairs. So, in this way you can give or you can use one that has given already given in the software. So, as you can see, this is already this is depicting here what is the base pairs amount of each lane, so here also this is same. So, the next thing is after being completed, 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 in lane 2.

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 (()) (39:14) the DNA also. So, here also same in case of protein analysis, here relative, so you have to select 1 band suppose I am selecting this one. Based on this it will calculate the all bands relative intensity relative quantity or you can select band standard 1 and this is quantity.

This band quantity I am it is as 30 nanogram, so another band at least we need to this is as 10 nanogram. After giving 2 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 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 Ends: 41:39)** So, in this demo the students have explained you how to select the lanes, how you can be able to measure the intensity of every steps.

And how you can be even be able to determine the molecular weight of the particular protein. Because if you have the markers because in the example the student have shown that the if you

have a molecular weight marker run onto the gel on the in one of the lane, you can use that information even to calculate the molecular weight. So, with this we would like to conclude our lecture here, in the subsequent lecture we are going to discuss about the some more aspects related to electrophoresis, thank you.