Enzyme Science and Technology
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Module - IV
Enzyme Production (Part 2: Over-expression)
Lecture - 22
Over-expression of Enzyme in host (Part-III)

Hello everyone, this is Dr. Vishal Trivedi from Department of Bio Sciences and Bioengineering IIT Guwahati. And what we were discussing? We were discussing about the different properties of the enzyme in the course, Enzyme-Science and Technology. And in the current module, we are discussing about the different types of host, what is available for the protein production and how you can be able to utilize these host in the for the protein production.

And so far what we have discussed? We have discussed about the prokaryotic expression system. So, where we have discussed about how you can be able to transform the bacterial cells with the help of the different types of vectors. And then we have also discussed about the how you can be able to induce those cells and then you can collect, break opens and then how you can be able to analyse the protein production with the help of the SDS page.

In the subsequent to that, in the previous lecture, we have discussed about the yeast and as well as the insects aligned as an expression system and we have discussed about the protocols and the procedures, what you have to follow to express the protein into some of these expression system. Now, in today's lecture, we are going to discuss about the mammalian system as an expression system. So, let us start the lecture today.

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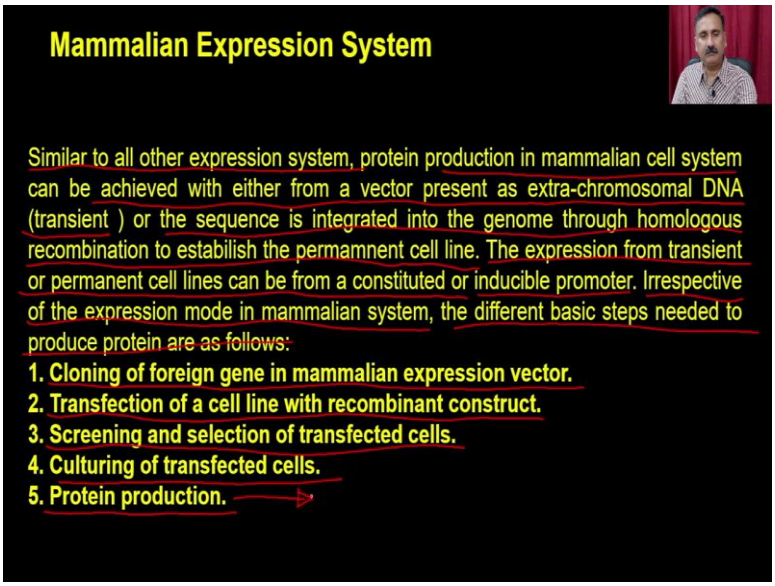

So, mammalian expression system as the name suggests, it is actually going to utilize the different types of mammalian cells for the protein production. So, similar to the other expression system, the protein production in the mammalian expression system can be achieved with either from a vector present and a extra chromosomal DNA, which is going to be a transient expression or the sequence is integrated into the genome to the homologous recombinations to establish the permanent cell lines.

The expression from the transient or the permanent cell line can be from the constituted or the inducible promoter. Just like as we have discussed about the yeast expression system here also you can have the choice of either you want the constitutive expression or the inducible expressions.

Irrespective of the expression mode in the mammalian system, the different the basic topics need to produce the protein are as follows. In the step 1, you are going to clone the foreign gene in the mammalian expression vector in the step 2, you are going to transfect the cell lines with a recombinant construct.

In the step 3, you are going to screen and select the transformed cells. In the step 4, you are going to culture the transfected cells and in the step 5, you are going to do the protein production. If it is a inducible expression system, you are going to add the, if it is a constitutive expression system, then you are going to culture the cells for several

generation and if the expression is going to be correlated to the amount of protein what you are going to express.

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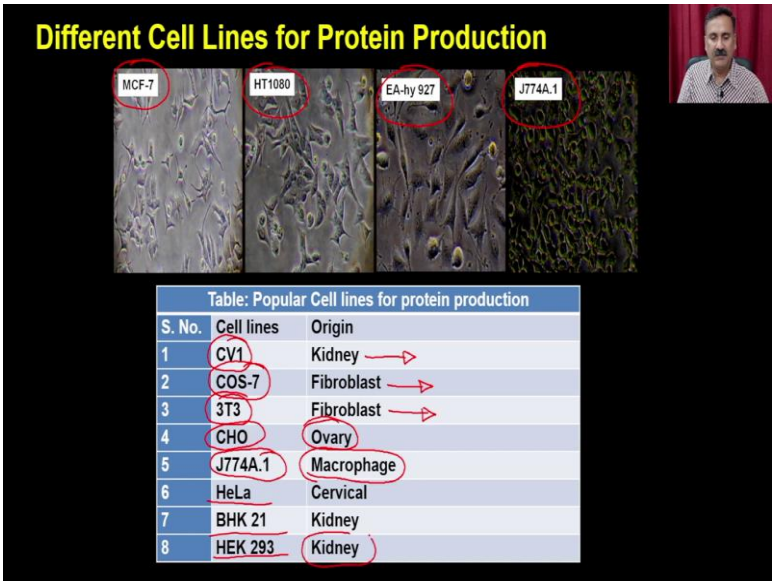

So, these are the some of the cell lines what you are going to use you can use the MCF 7, HT1080, EAH927 and you can use the macro phase cell line J774 and all of these cell lines have their own origins. So, you can actually be able to use the cells as per the origin of the protein as well.

For example, you if you are trying to express in enzyme which is mostly being found in the kidney, then in that case, you should use the CB1 cells ok. Similarly, you can use the cells like fibroblast cells for protein production or the enzyme production where you can use the COS7 or NI3T3 cells. Then you can use the J774, A.1 which are actually the macrophage cell lines. And then you can use the CHO K1 which is actually a ovarian cell lines and HeLa, BHK1 and HEK293, and HEK 293 is a kidney cells.

So, depending on the source of the enzyme, you can actually be able to choose the different options and sometime the rate of the cells and as well as the maintenance is also another parameter, what you can also be keep into the considerations.

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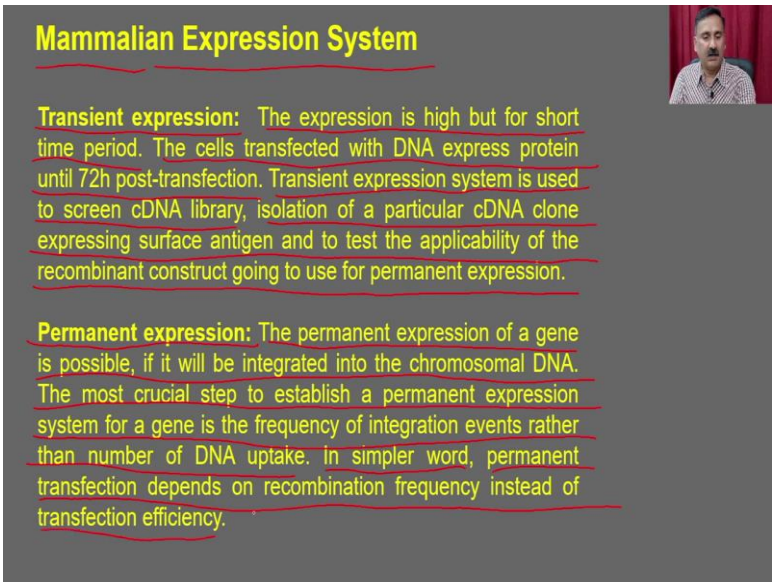

Mammalian expression system, you can actually have the two options either you can have the transient expressions or you can have the permanent expressions. In the transient expression, the expression is high, but for the short period of time because the vector is not going to integrate the DNA into the genome. So, it is actually going to be remain as the extra chromosomal DNA.

So, the cells, transfected with DNA expressions are protein until the 72 hour post-transfections. Transient expression system is used for screening the cDNA library's, isolation of a particular cDNA, clone expressing surface antigen and to test the applicability of the recombinant construct going to use for the permanent expression.

So, transient expression is mostly being used to test that your construct is correct or not. Then you can have the permanent expression, the permanent expression of a gene is possible if it will be integrated into the chromosomal DNA. The most crucial step to establish a permanent expression system for a gene is the frequency of integration events rather than the number of gene uptake.

In simpler words, the permanent transduction depends on the recombination frequency instead of the transfection efficiency.

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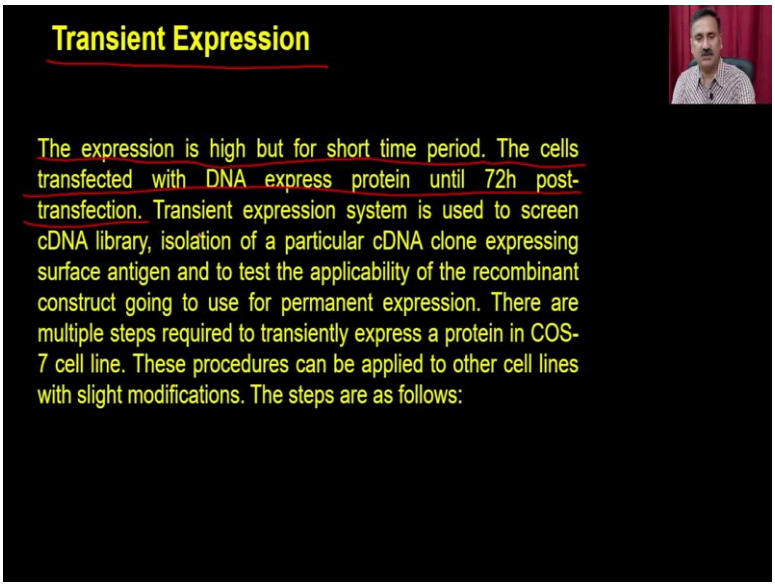

So, transient expression system. So, the expression is very high, but for the short period of time. The cells, transfected with the DNA expressions protein until the 72 hour post-transfections. So, how are you going to do the transient transfections? You can have the multiple steps.

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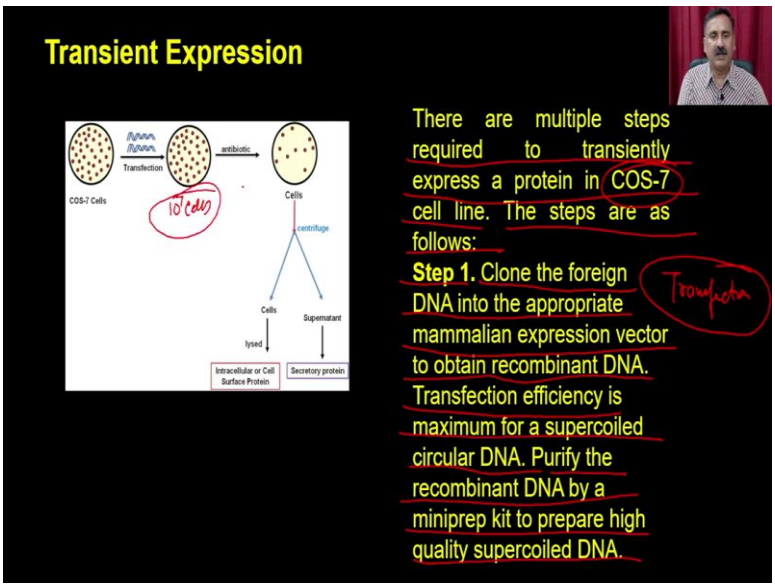

So, there are multiple steps required for the transiently express a protein in COS 7 cell line. So, I have taken an example of the COS 7 cell. The steps are as follows. In the step

1, you are going to clone the foreign DNA into an appropriate mammalian expression vector to obtain the recombinant DNA.

Transfection efficiency is maximum for a supercoiled DNA. So, you can verify the recombinant DNA by a miniprep kit to prepare the high-quality supercoiled DNA. And the in the step 1, you are going to do the delivery of the, you are going to do the cloning and as well as you are going to do the transfection. So, in the step 1, you are going to first plate the uniform cells right, you can plate 10 to the power 7 cells and you can actually be able to do the transfection.

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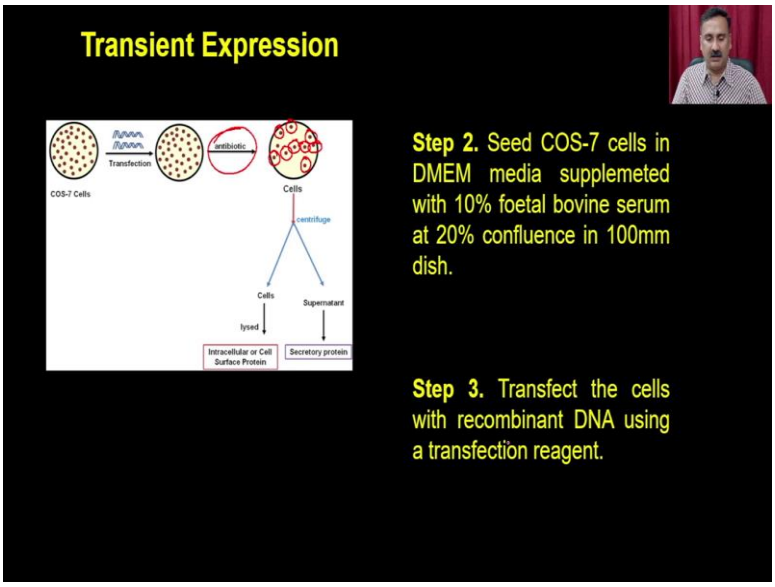

And in the step 2, you are going to do the screening. So, you are going to do the, you know, transfect the cells. So, in the step 1, you are going to do the screening of the cells with the help of the appropriate antibiotics. And when you do so, you are going to have the most the death of the most of the cells except the transfected cells, right?

So, for example, if you use the puromycin or others antibiotics, which are going to kill the non-transfected cell, then you can actually be able to select these cells with the help of the, you know, string the cells with the help of the antibiotics.

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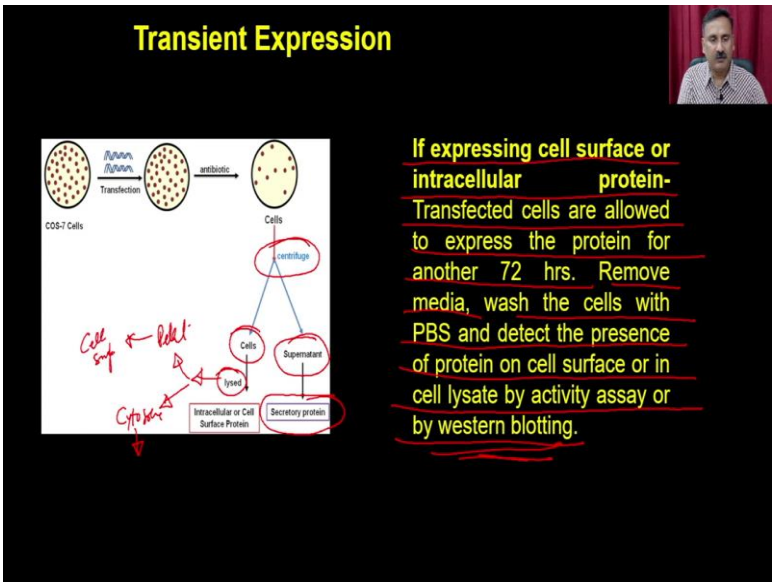

Then the steps 4, you can have the two choices either if the cells, if the expressing cell surface or intracellular proteins. So, you can have two choices either the protein is expressing on the cell surface or it would be, it is going to be a intracellular protein. So, transfected cells are allowed to express the protein for another 72 hours.

You remove the media, wash the cells with PBS and detect the presence of protein on the cell surface or in the cell, lysate by an activity assay or by the western blotting. That anyway, we are going to discuss in the later part of this lecture how you can be able to use the Western blotting to detect the proteins.

But what you are going to do is you are going to collect these cells by the centrifugations, so that will give you the two fractions either you are going to have the cell fractions or the supernatant. So, if you have put the cells under the secretory pathway, it is going to be in the secretory into the supernatant. And if it is being present in the, either with the cell membrane or it will be intracellular protein, it is going to be present on the cell surface.

So, in that case, you like the cells with the help of the appropriate lysed solution and that will actually going to release the content. So, it is going to give you two fractions, it is going to give you the pellet fractions and it is going to give you the cytosolic fractions. And cytosolic fractions is going to be, you know, it is going to be the soluble proteins,

the pellet fraction is going to be the cells which are actually going to be expressed on the cell surface because that is going to be a membrane fractions.

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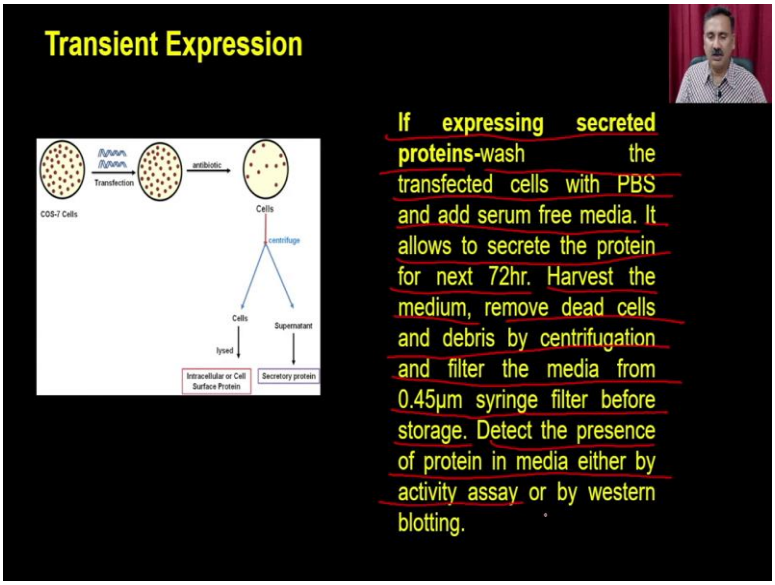

So, in the steps, if the cell, if you are expressing the secretory proteins, the wash the transfected cells with PBS and add the serum free media, it allows to secrete the proteins for next 72 hours, harvest the media, remove the dead cells and debris by the centrifugations and filter the media with a 0.45 micron syringe filter before storage. Detect the presence of the protein in media either by the activity assay or by the western blotting.

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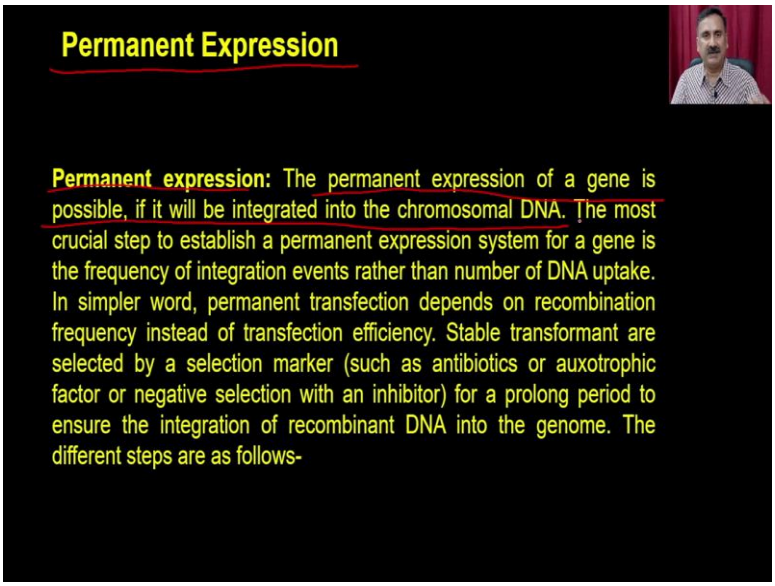

The second option is so, you can also be able to do the permanent expression. So, permanent expression is the permanent expression of a gene is possible if it will be integrated into the chromosomal DNA and the permanent expression also has the multiple steps which you have to follow.

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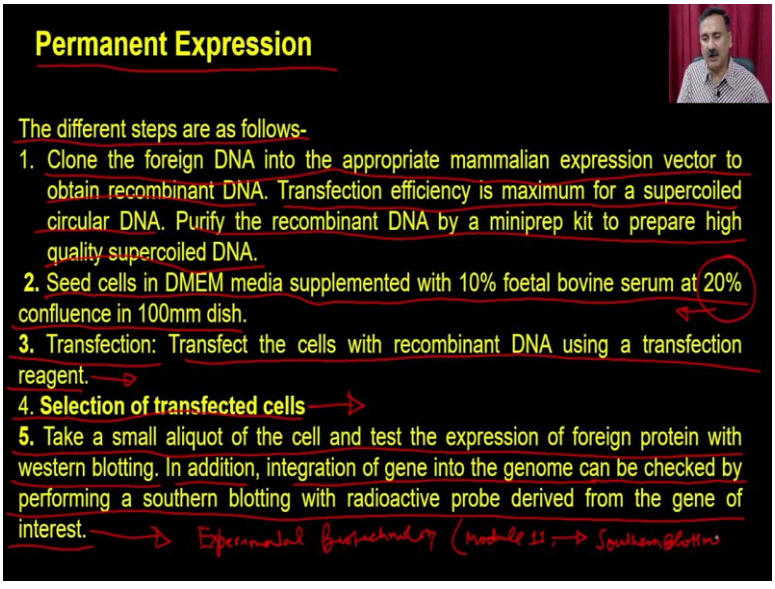

So, in the permanent expression, you have the different steps. You can have the clone the foreign DNA into the appropriate mammalian expression factor to obtain the recombinant DNA. Transfection efficiency is maximum for a supercoiled DNA. So, you

can purify the recombinant DNA by a miniprep kit to prepare the high quality supercoiled DNA ok.

Because the transfection efficiency will actually increases the chances of the DNA to be get integrated into the genome. You seed the cells in a DMEM media supplemented with 10 percent FBS at 20 percent confluence in a 100 mm dish, I am sure many of you do not know that what is mean by 20 percent confluence. So, 20 percent confluence in is the number of cells which are actually going to cover the 20 percent area of that particular dish.

Then you can do the transfections, so transfect the cells with their recombinant DNA using a transfection reagents. You can use the lipofectamine, you can use the BE-based transfections and you can do the other methods what we have discussed in the previous lecture. Then in the step 4, you are going to do the selection of the transfected cells you can have multiple options after selecting the transfected cells.

You can take the small aliquot of the cell and test the expression of the foreign protein with the western blotting. In addition, the integration of the genome in integration of the gene into the genome can be checked by performing a southern blotting with a radioactive probe derived from the gene of interest.

So, I have not discussed about the southern blotting, but if you are interested, you can actually be able to see one of my another MOOCs course where I have discussed. So, this is called as experimental biotechnology and if you go under the experimental biotechnology, the I think the module 11 is actually going to be discussed or going to be deal with the southern blotting.

So, you can actually be able to understand the different steps and as well as the technical details how you can be able to perform the southern blotting to check the presence of Gn Gene. Basically what you are doing is you are taking the genome, digesting it into the different with the help of the suitable restriction enzyme, you are absorbing it onto a agarose gel, then transferring those DNA into the into a nitro cellulose membrane.

And then you are probing that with the small probe, redo-level probe, then that probe is going to be the corresponding sequences what you are going to, you know, related to that

particular gene what you have cloned. And using this, you can be able to say whether the gene of your gene what you have cloned is actually being present in the genome or not.

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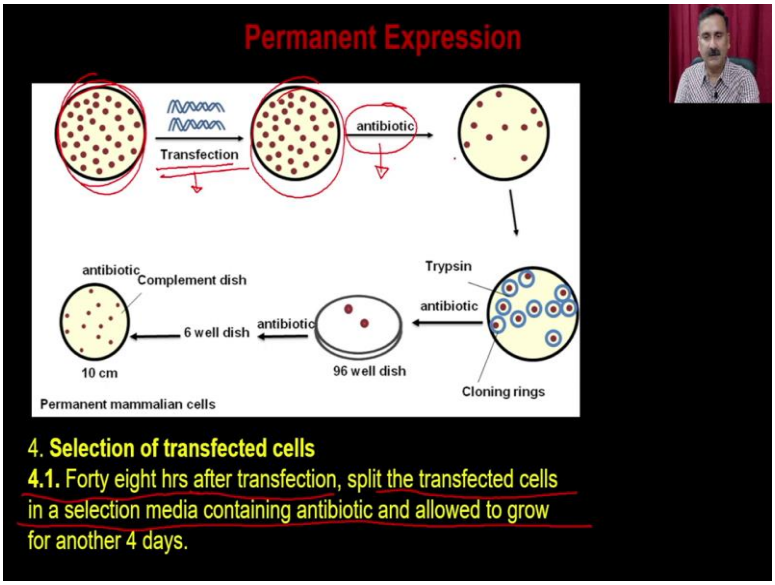

So, that you can actually be able to follow if you are interested to read more about this and blotting, so this is what we are going to do. So, we are going to do first the first you're going to do a plating of the cells, then you are going to do a transfection with the help of the suitable transfection reagents.

Once you have got the transfection, you are going to do the antibiotic screening, you when you use the puromycin, vancomycin, you have lot of options of different types of antibiotics what you can use. And then you are going to do the selection of the transfected cells.

So, in the selection of the transfected cell, you are going to do the two options. So, forty eight hours after transfection, the split the transfected cell in a selection media containing antibiotic and allow the allow it to grow for another 4 days. When you do that, it is actually going to kill most of the non-transfected cell, but you are going to maintain the transfected, going to allow the proliferation of the transfected cells.

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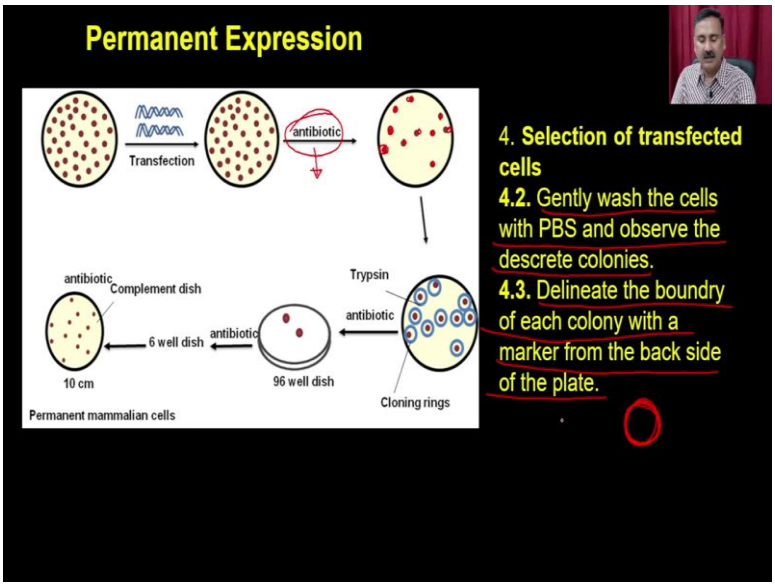

Then you are going to do is you are going to gently, you are gently wash the cells with PBS and observe the discrete colonies. So, what you have see is you are going to see a discrete you know colonies of the cell, right. So, these cells are not, these are transfected cells and they are, you know, not going to be die with the help of even with the antibiotic because the vector is providing them the resistance.

Delineate the boundary of each colony with a marker from the backside of the plate. So, what you are going to do is you are going to, you know, monitor your, and see if the plate is, you know, you can be able to just put a marker and back from the backside of the plate, you are going to say, , this is the colony I have, right. So, from the backside of the plate, you can just put the marker.

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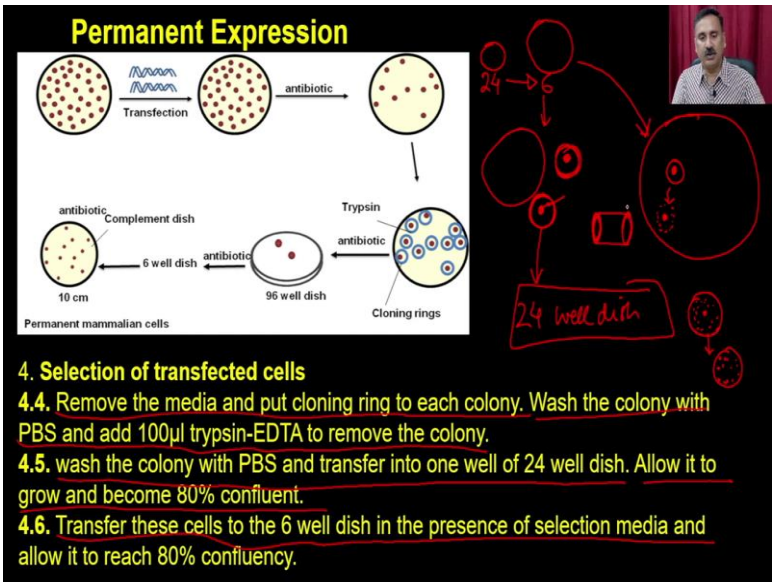

Then what you are going to do is you are going to put the, you will, so remove the media and put the cloning rings into the each colony. So, for example, if you have, this is the colony, you have marked actually, so you can just put a cloning ring actually. Cloning ring is actually a small tube-light structure. So, it is actually going to be like this, ok, small, ok and this side is going to have, you know, the glue actually.

So, when you remove it and you are keep it on this, it is actually going to remain strict. And then you can actually be able to do whatever you like to do with this. It is going to be get isolated from the other colony. For example, this is also another colony, you can put another colony reactions.

So, whatever you want to do with this will not going to affect this because it is going to be sealed from the bottom. So, wash the colony with the PBS and add 100 micro-litre of trypsin EDTA? So, in this one, you are going to add the trypsin EDTA. So, what will happen? This colony is going to be detached from the lead surface and that you can be able to collect. So, wash the colony with PBS and transfer it into a 24 well dish allow it to grow and become anti confluent.

So, what you are going to do is you are going to take out this particular colony and transfer it into a 24 well dish, right. So, that it actually should grow. Because you know that every cell has a tendency that it is looking for the similar type of cell in the closed vicinity. So, if you have the 10 centimetre dish, it is actually going to be so big that this

colony will be actually not going to get the micro-environment, but it is required for its to grow, right.

Because every cell is looking for some minor micro-environment, some kind of growth signal from the other colony also, other cells actually. So, if the plate is very big, this signal is going to be missing. So, what you are going to do? Is you just take put a cloning ring on this, you take out this cell and put it into a small well, like the 24 well.

So, that case, it is actually going to the concentration of these micro-environment or the growth factor, but it actually secreting is very high and that is how it is actually going to grow and it becomes confluent. So, it is going to grow in large quantity and it is going to be confluent ok. So, allow to grow and become 80 confluent. Transfer the these cells to a 6 well dish in the presence of selection media and allow it to grow.

So, what you are doing is you are actually, you have taken out from this, you are first putting into a 24 well dish, which is actually a small area. Then you are putting it into a 6 well dish, so it is going to be even bigger. And from the 6 well you are going to you can put it into 12 well or even like large well like this ok, then you can transfer it onto a big dish and that is why it is actually going to grow.

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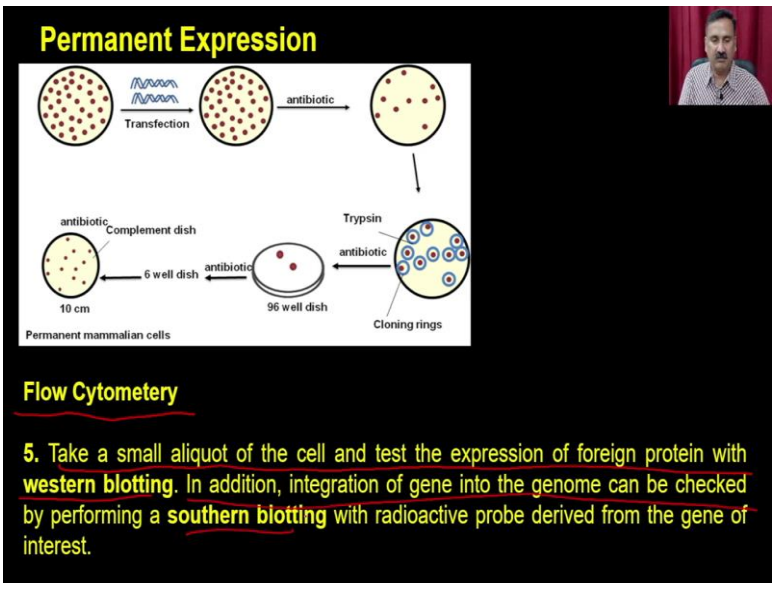

The other method is you can actually be able to use the flow cytometry ok. So, take a small aliquot of the cell and test the expression of foreign DNA foreign protein with the

help of the western blotting. In addition, the integration of genome into genome can be checked by a southern blotting and you can also be able to do the flow cytometry.

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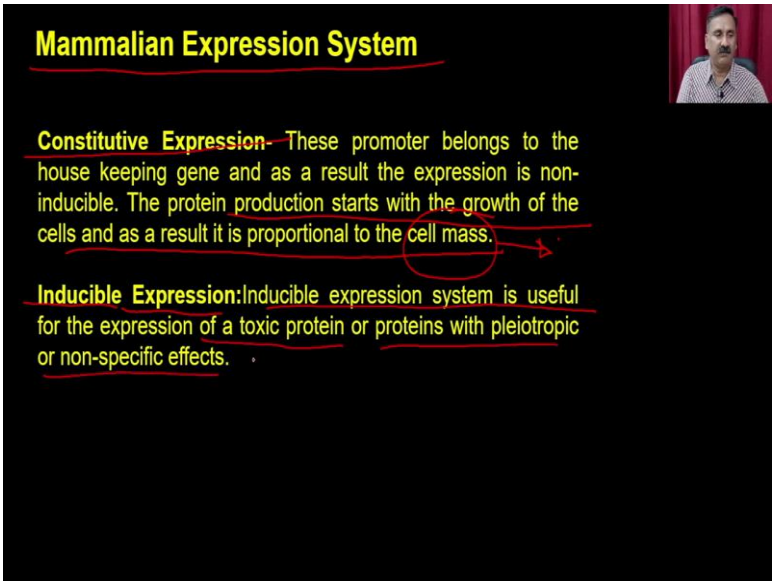

As far as the expression is concerned, within the mammalian expression system you have the two option you can use the constitutive expression or you can use the inducible expression. Constitutive expression is the place where you are going to use the house keeping genes and it the protein production is going to be proportional to the cell mass. So, higher the cell mass it is actually going to have the more number of proteins, but the proportion of your protein will be related to the cell growth.

So, recovery of the protein is going to be a very difficult task when you are you rely on the constitutive expression. Whereas in the inducible expression system, you can actually be able to you know use that for the expression of a toxic protein or proteins with a pleiotropic or non-specific effects.

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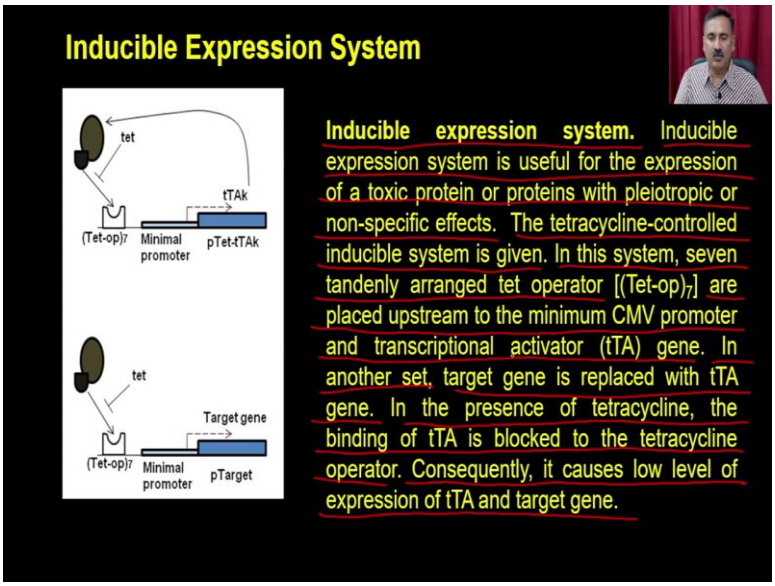

In the inducible expression system, you can use the tet on and tet off system. So, inducible expression system, inducible expression system is useful for the expression of a toxic protein or protein with the pleiotropic or the non-specific effects. The tetracycline controlled inducible system is given.

In this system, seven tandemly relate tet operator placed upstream to a minimal CMV promoter and the transcriptional activator gene. In another set, the target gene is replaced with a tTA gene. In the presence of tetracycline, the binding of tTA is blocked to the tetracycline operator. Consequently, it causes the low level of expression of tTA and the target gene.

So, this is the schematic diagram of explaining how the teti promoter is working. So, you have actually a tet operators which actually has the tet, which has the you know tandemly relate tet operators and that is being released, that is being placed upstream to the minimum CMV promoter and then you have the transcriptional activator gene.

So, in another set, the target gene is placed with the tTA gene and in the presence of tetracycline, the binding of the tetracycline into this is actually going to block the tetracycline operator and consequently it is actually going to induce the expression of the tTA gene and as well as the target gene.

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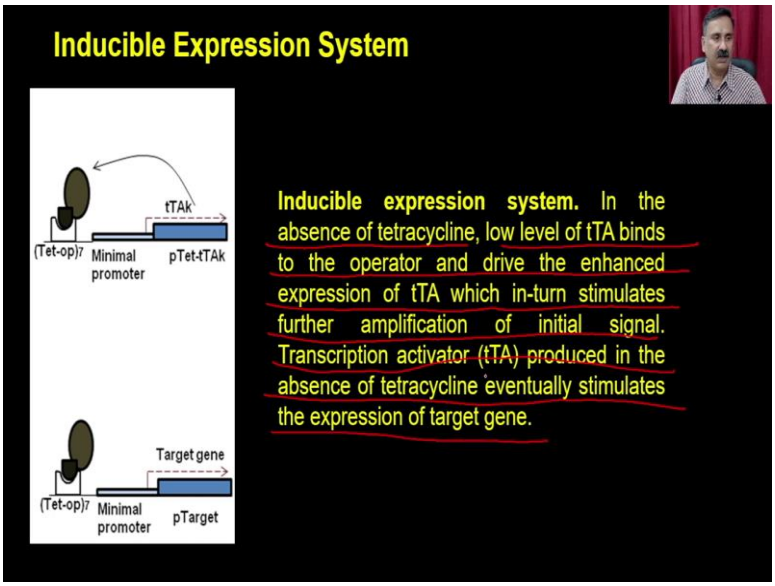

So, in the absence of tetracycline, the low level of tTA binds to the operator and drive the enhanced expression of tTA which in turn stimulate the further amplification of the initial signal and transcriptional activator dropped in the presence produced in the absence of tetracycline eventually stimulate the expression of the target gene.

So, this is all about the expression machinery, what we have discussed for the protein production or the enzyme production. We have discussed about the E.coli expression system, we have discussed about the yeast expression system insect cell line expression system and as well as lastly we have also discussed about the mammalian expression system.

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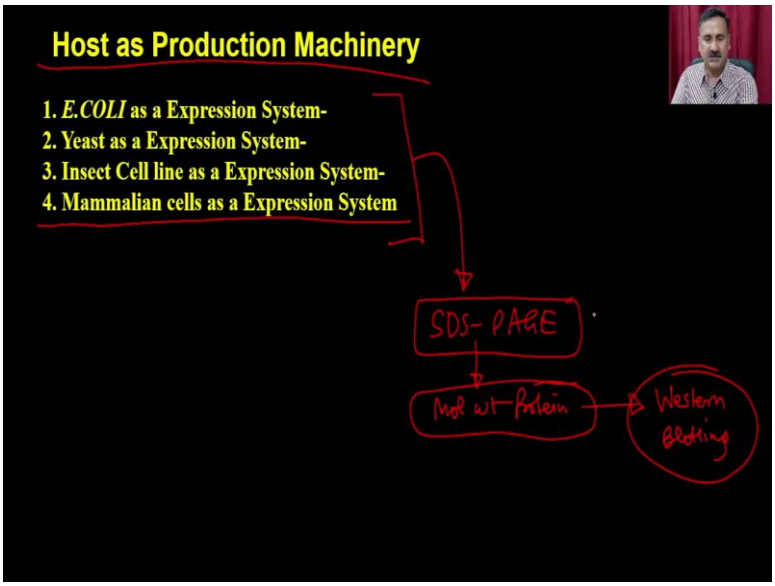

Irrespective of the expression system, you can be able to check the over expression on to the SDS, but SDS page is only going to say you that there is a over expression of the particular molecular weight of protein ok. But it will not going to give you the identity of this protein and this identity of this protein is very important to know that protein of your interest is actually going to be expressed and that you are actually going to do with the help of a technique which is called as the western blotting.

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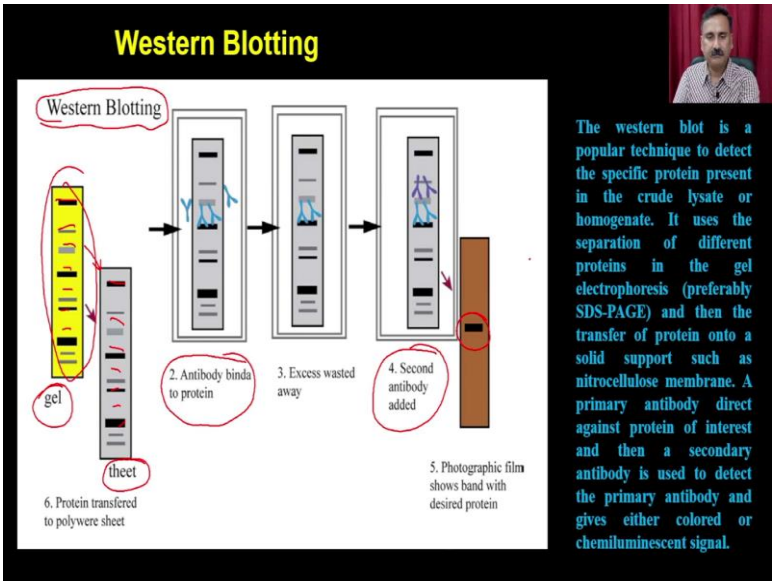

So, let us discuss about the western blotting. So, western blotting can be done in a is a multiple step reactions. So, what you are going to do is you are going to first, you know. So, in the western blotting what you are going to do is first you are going to run the SDS page ok. So, you are going to get the pattern of the bands on to the gel like SDL gel.

You are going to transfer that on to our nitro cellulose membrane and that pattern is going to be maintained right. And then you are going to treat it with a primary antibody. So, primary antibody will go and bind to your target protein and ultimately you are going to do a washing step.

So, that washing is going to remove non-specifically bound protein and it is going to remain, allow the specific proteins to be bind and then you are going to add the secondary antibodies and secondary antibodies will go and bind to the primary antibody and then you are going to add the substrate and that substrate is going to give you the signal.

So, there are multiple steps what you have to follow and there is a discrete recipes and protocol what you have to follow to perform the western blotting.

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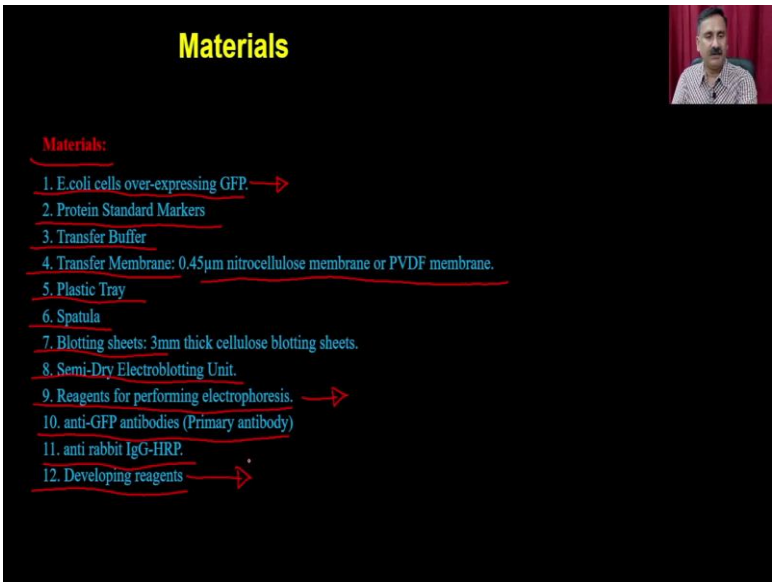

What about the material you require? So, in this case we are taking an example of E. Coli which are expressing GFP proteins or GFP protein site. Then you require the protein

standard marker, you require transfer buffer, you require the transfer membrane like the nitro cellulose membrane or the PVDF membrane.

You require a plastic trays, spatula, blotting sheets, electro blotting units, you require the reagent for performing the DS page that anyway we have discussed in the previous lecture right, you require the primary antibody which is the anti GFP antibody and you also require the secondary antibodies and then you also require the developing reagents.

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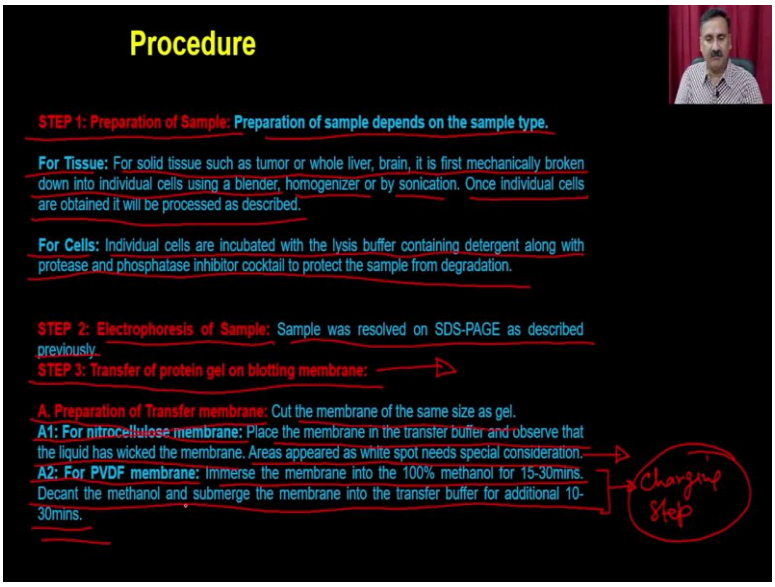

So, in the step 1 you are going to prepare the sample right. So, preparation of the sample depends on the sample type for the tissue like for example, solid tissue such as tumor or whole cell or brain it is first mechanically being broken down into individual cell using the blender homogenizer or by sonication's. Once the individual cells are obtained it will be processed as described.

So, first you are going to have the you know mechanically you can homogenize and break down the tissue into individual cells and then these once the individual cells individual cells are incubated with the lysis buffer containing the detergent along with the protease and phosphate buffer cocktail. Then the step 2 you are going to do the electrophoresis of the sample that anyway we have discussed. The samples are resolved onto the SDS page and we discussed previously.

Then the step 3 you are going to do the transfer of the gel onto the blotting membrane. So, that is a very very important and crucial event because the how good you are doing the transfer that actually is going to tell you the that is actually going to decide the quality of the western blot.

So, first you have to prepare the transfer membrane. So, cut the membrane of the same size as gel and then you are going to have these events. For nitrocellulose membrane the place the membrane in the transfer buffer and observe that the liquids are has wicked the membrane. Areas appeared at white spot needs white special considerations.

Then for the PVDF membrane the immerse the membrane into 100 percent methanol for 15 to 30 minutes decant the methanol and submerge the membrane into a transfer buffer for additional 10 30 minutes. So, for the PVDF membrane you are actually going to have a charging step.

Charging step means where you are going to treat the membrane with the 100 percent methanol and once you decant the methanol you can merge the membrane into a transfer buffer for additional 10 to 30 minutes ok. So, that is how you can be able to you know make the membrane. So, that it should be able to bind the proteins.

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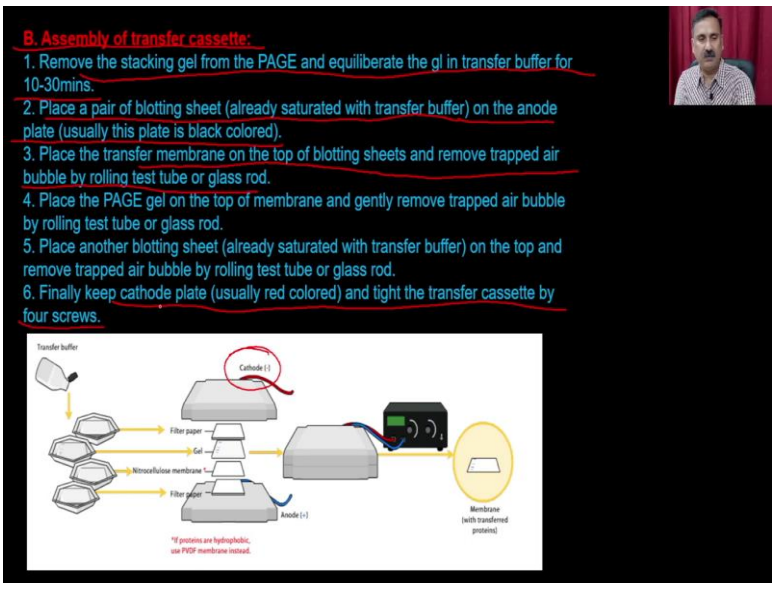

Then in the step 3 you are going to do the assembly of the transfer cassettes. So, this is the assembly of the transfer cassettes. You can remove the stacking gel from the page

and equilibrate the gel into the transfer buffer for 10 to 30 minutes. Place a pair of blotting sheets already saturated with the transfer buffer onto the anode plate. Usually it is a black color plate.

So, first you are going to keep the filter paper onto the anode plates. Then you place the membrane sheets, transfer membrane onto the blotting sheets and remove the trapped air by the rolling bulb, test tube or the glass bottles. So, first you are going to put the filter papers then you are going to put the net of schedule membrane, then you are going to put the gel, then again you are going to put another sheet of filter paper which is dipped in the transfer buffer and then you are going to put the cathodes.

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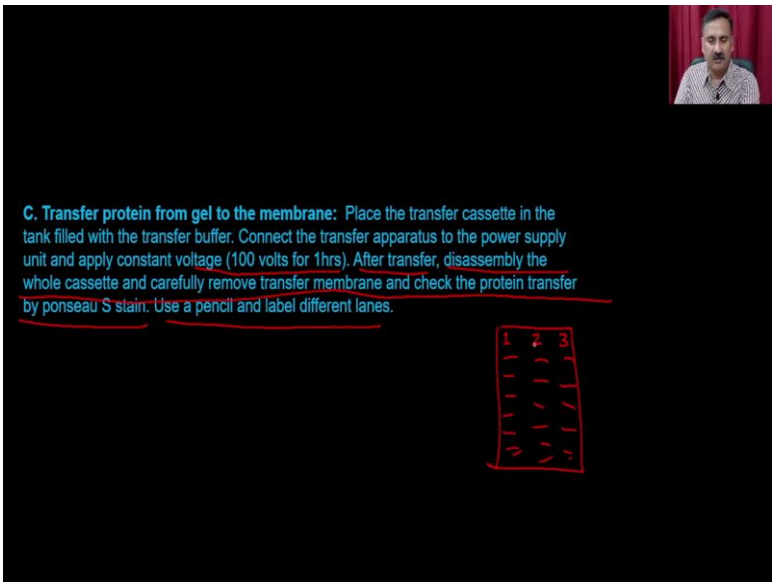

And by and after this you are going to tie this up and you are going to put the turn on the electrophoresis. So, finally, you are going to keep the cathode plates and tighten the transfer cassette by the four screws and after this step you are going to add, you are going to turn on the electrophoresis.

So, you are going to apply the constant voltage like for 1 hour. So, after the transfer you disassemble the whole assembly and carefully remove the transfer membrane and check the protein transfer by the ponseau stain. Use a pencil and paper and label that differently. For example this is like. So, initially you will, since you are going to do a ponseau stain you are going to see the bands.

So, what you are going to do is you take the pencil and you write like this is the line number 1, line number 2, line number 3 like this. That this pencils writing is going to remain there. You cannot use the permanent marker because the subsequent steps probably may remove that.

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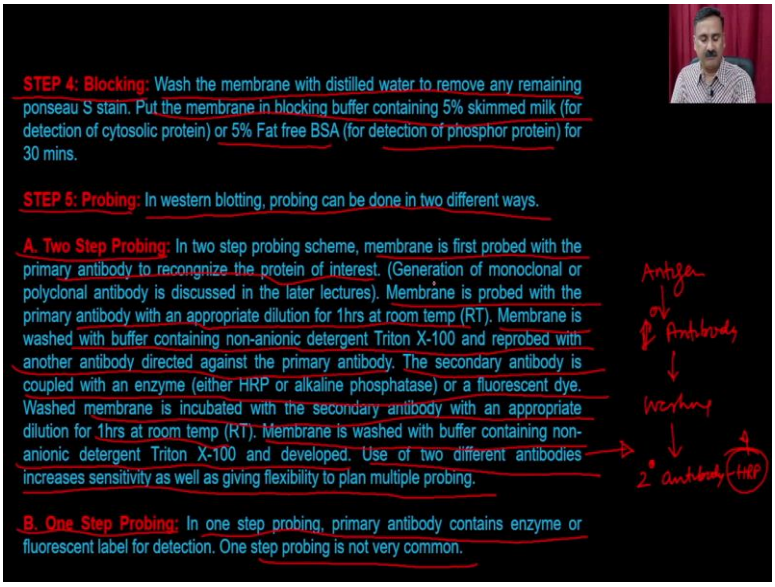

Then in the step 4 you are going to do the blocking. So, wash the membrane with distilled water to remove any remaining ponseau stain. Put the membrane in blocking buffer containing 5 percent skimmed milk. If you are doing the western blotting for the detecting of phosphorylated protein, then you can use the BSA. Then step 5 you are going to do the probing. So, in western blotting probing can be done in two days, two ways, right either you do a two step probing or the one step probing.

In the two step probing, a membrane is first probe with the primary antibody to recognize the protein of interest and then membrane is probed with the primary antibody with an appropriate dilution for 1 hour. Membrane is washed with buffer containing non-anionic detergent triton X 100 and reprobed with another antibody directed against the primary antibody. The secondary antibody is coupled with an enzyme or a fluorescent dye.

The washed membrane is incubated with the secondary antibody with an appropriate dilution for 1 hour. Membrane is washed with buffer containing non-anionic detergent

and developed. Use of two antibodies increase the sensitivity as well as the giving the flexibility to plan the multiple probes.

So, if you use the two antibodies; first you are going to have the antigen like the that is a protein of your interest and you are going to first probe that with the primary antibodies and then you are going to do the washing. So, that non-specific antibodies are going to remove and then you are going to put the secondary antibody and this secondary antibody is actually coupled to some enzyme. So, either it can be coupled to HRP or alkaline-phosphatase and this HRP is actually going to give you the signal.

Secondary antibody and why we do? so two step probing because the two step probing in actually increasing the sensitivity. In a step one step probing you can actually be able to add the primary antibody which contains the enzyme or you can use the fluorescent label for detection. One step probing is not very common , it is a two step probing, but it is very common.

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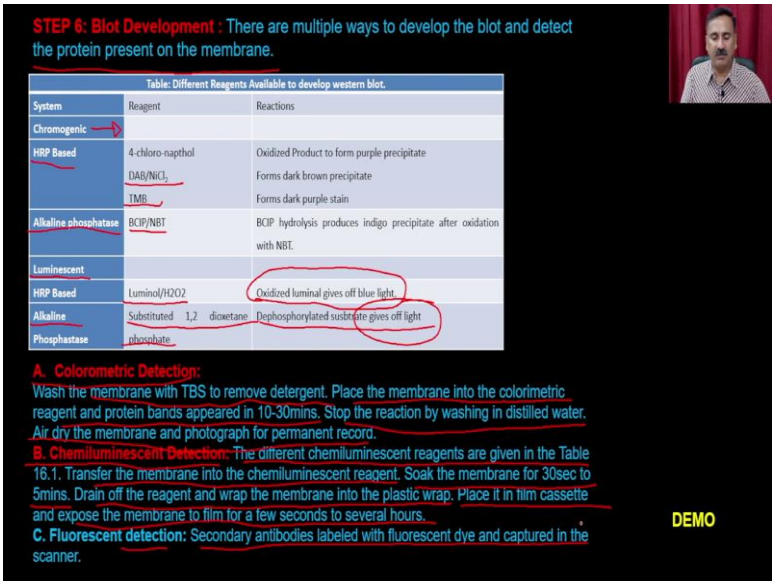

Depending on the enzyme and as well as the different options you can actually be able to use the multiple ways to develop the blot and detect the protein present onto the membrane. You can use the. For example in the HRP based system you can use the chromogenic substrate or you can use the luminescent substrate.

So, for example, if you use one to use the chromogenic substrate which is actually going to give you the color you can use the DAB or TMB. For alkaline phosphatase you can use the BCIP and NBT system. If you want to use the luminescence as the readout then in for the HRP you can use the luminol or H2O2 system.

Whereas for the alkaline phosphatase you can use a substituted 1, 2 dioxetane phosphate and it is actually going to give you dephosphorylated substrate gives the light. Similarly, in the luminol system the luminol oxidation is going to give you the blue color light. So, after that you are going to do the colorometric detection.

So, wash the membrane with TBS to remove the detergent. Place the membrane into the colorimetric reagent and protein band present in appeared in 10 to 30 minute. Stop the reaction by washing and distil buffer, air dry the membrane and photograph for permanent record. If you want to do a chemiluminescent detections the chemiluminescent detections are given in the table.

Transfer the membrane into the chemiluminescent reagents. Soak the membrane for 30 seconds for to 5 minutes. Drain off the reagent and wrap the membrane into plastic wrap. Place it in a cassette and expose that the membrane to a film for a few seconds for 4 hour. Fluorescent detection you can do the secondary antibodies labeled with the fluorescent dye and that also can be used and then you can actually be able to capture the signal in a scanner.

So, to explain all these steps we have prepared a small demo and where the students are actually going to show you how you can be able to you know do the charging of the membrane, how you can be able to assemble the cassettes to transfer the bands from the SDS page to the nitrocellulose membrane and so on.

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Hi everyone, myself Sooram Banesh research scholar at Department of Biosciences and Bioengineering IIT Guwahati. In this video we will demonstrate you how to do a western blot and how to analyse the result using ECL, electrochemiluminescence substrate.

So, here what we will do? We have to run gel first then we will transfer. The transfer method how to do the transfer we will show in this video. In previous video we have already shown that how to run how to prepare a SDS page and how to run proteins samples. So, in this video particularly we are interested in factors associated with the western blotting. For doing western blot we need membrane and transfer buffer and the transfer medium, this one is we use to transfer this gel to membrane.

So, here membrane can be two kind; one is nitrocellulose which has low protein binding efficiency and hydro building nature another membrane is PVDF. This is hydro-phobic membrane and higher protein binding capacity. So, the processing for western blot is different from different for nitrocellulose and PVDF.

If you are using PVDF membrane we have to take we have to cut the part whether if you have ready made pre cut blots then no need, if you have if you are taking from a bundle you have to cut precisely how many wells you want. So, after that you have to label front and the back where the front side can be used for transferring the protein and that can be used in previous step further steps also like antibody incubation.

So, here for if you want to use PVDF membrane you have to charge with the methanol. So, since the PVDF is a hydrophobic membrane you cannot directly transfer the transfer in the aqueous medium. First you have to keep in methanol for at least 20, so after this can be called as charging. So, after this we will use that for transfer.

So, this is pre-soaked in methanol and equilibrated in transfer buffer. So, here while doing transfer we need to consider few things. The buffer always should be in chilled condition. Otherwise during this transfer at high voltage it will generate high temperatures. So, that may degrade your protein or decrease the efficiency of the transfer.

That is why we need to keep the buffer always in chilled condition and let us start the procedure. So, we need a pre-run gel, so we already finished the gel running. In addition to that we also need sponges which will give cushion to the gel, so that gel may not destroyed during the transfer.

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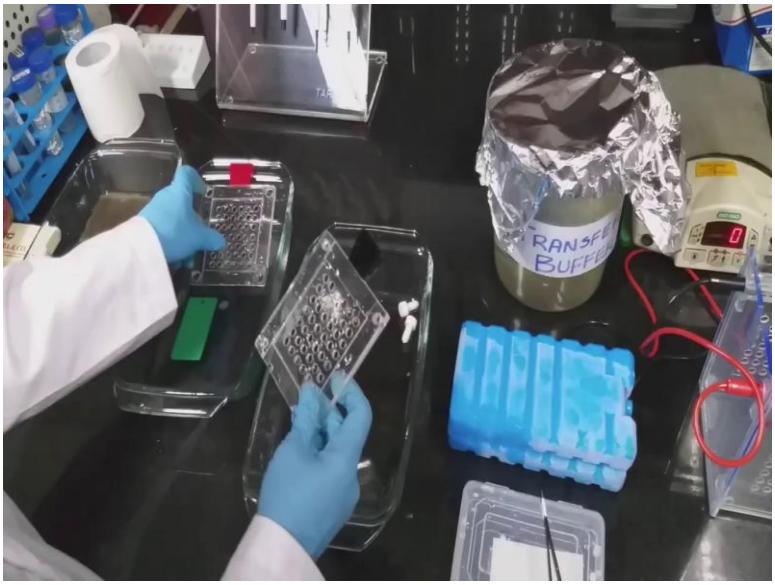

So, this is the cassette we will use for the transfer, so this is negative side of cassette and this is the positive side. So, we are going to keep gel on negative side and positive side the blot membrane. So, when we when we apply voltage from this side to this side the negative protein it will be, it will be moved to positive side and it will be captured in the membrane.

So, first for doing this, these sponges we need to keep and also this may be give some non-specific binding to membrane. So, what we will do? We will put a blotting sheets on top of this. So, after this you have to remove air bubbles if any present. So, once you inserted the blotting sheet, then you have to keep your gel.

So, here we have to remember that gel after finishing the SDS based running you have to keep in transfer buffer. So, that it will give identical condition for equilibration kind of thing during transfer, so that protein transfer may be easy. So, this is the gel I am keeping on the negative side. So, after that we have to overlay with the membrane. Next we have to remove any air bubbles if present.

We have to overlay with another blotting sheet and remove the air bubbles. Each end every time when you introduce something you have to remove air bubbles. So, this is the final sheet. So, this is the positive side of the cassette just have to like this. These are the screws.

We have to tighten it up, then only the contact between the gel and membrane will be sufficient to get transferred. First you do not tight initially you just keep and after that rest the positive side of the cassette then tight the screws. So, all these things should be done in the transfer buffer only unless specified.

So, this is the chilled transfer buffer now we are going to do transfer. Pour sufficient buffer, keep this ice pack also if the chilling is not sufficient then there may be heat generation. So, in order to prevent that we will use this ice pack. So, this will keep the buffer cooled till the transfer end of the transfer.

So, once that is over you directly take out the cassette and keep. If there is a buffer insufficiency you can add on top of that. Make sure that the cassette completely submerged, so that the transfer will be proper and there is no air bubbles. So, once the setup is over now you can transfer now transfer is going on, so how much voltage we need to give it depends on transfer to transfer it varies.

Generally in our lab we will give at least 2 hours of transfer at 120 volts which is sufficient to transfer even low molecular weight proteins also. But from instrument to instrument also it varies you need it to optimize before doing transfer. After 2 hours we

have to remove the blot and incubate with the blocking buffer. So, I am going to stop here remove the cassette keep it in a tray.

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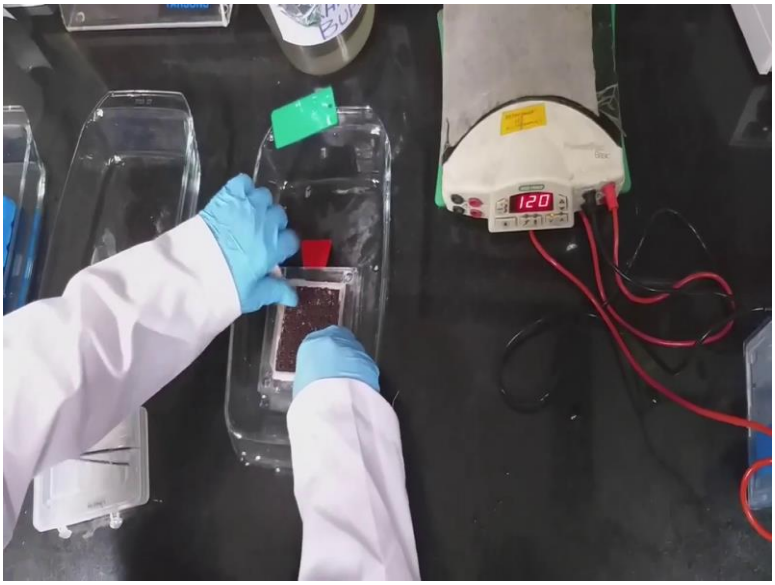

Remove this screws properly and gently remove the sponges take out the blot and keep it in blocking buffer. In this condition we have to keep. If you are keeping it room temperature it is for 2 hours at least, if you are keeping in 4 degree Celsius you can keep over night. The blocking buffer contains skimmed milk or BSA along with (Refer Time: 43:24).

The western blot transfer its all depends on the efficiency how precisely you are doing the transfer. For example you should not use your bare hands while handling the blot or gel. So, whatever the proteins present on your fingers it will transferred into gel or membrane which will give high background during development of the blot. So, always use gloves.

Apart from that while handling the instrument make sure there may be possibility of electricity the shock may happen sometime. So, we have to that time also we need to use gloves. And after finishing the transfer you have to clean all the apparatus properly and dry it for the next time use.

After the blocking of the membrane we have to remove the membrane and incubate with the primary antibody without washing. The main purpose of the blocking is that it will

occupy non specific sites other than the respective protein, so that when antibody comes it will bind to that the specific protein and gives no nice.

So, after this we will incubate with the primary antibody for overnight at 4 degree Celsius then wash 3 times at least 15 minutes each with the TBST buffer or PBST buffer and again treat with incubate with the secondary antibody suitable secondary antibody. For 5 hours at 4 degree Celsius or 2 hours at 3 hours at room temperature. After that we need to wash properly at least 3 times then we will develop with the develop the blot with the electro chemiluminescence substrate.

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In earlier western blot how to do western blot video we showed how to transfer the proteins to membrane. So, we are we incubated with the primary antibody following secondary antibody and washing thoroughly now here we show how to develop a blot. For developing a blot we need chemiluminescence substrate.

In most of the commercially availabilities, luminal is the one of the substrate we used for this purpose. So, luminal in presence of hydrogen peroxide and peroxidase in here which present in the secondary antibody horseradish peroxidase conjugated secondary antibody this horseradish peroxidase converts luminol to excited state luminol by deep protonating and oxidizing it. So, this product this excited state product gradually leaves the energy by releasing luminescence products that light will be detected using this instrument.

So, these are the commercially available chemiluminescence substrate solutions. So, it is available from a wide range of companies we have to mix one is to one ratio. So, we have to pick out the blot, drain the buffer whatever we present properly. So, after that you keep blot in between a plastic paper files, then we will take chemiluminescence substrate. So, after that you have to slowly press and remove air bubbles.

This is the tray we used for developing the blot. So, we have to open the system properly align the tray and then shift blot to the tray.

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Once it is over, you have to just close.

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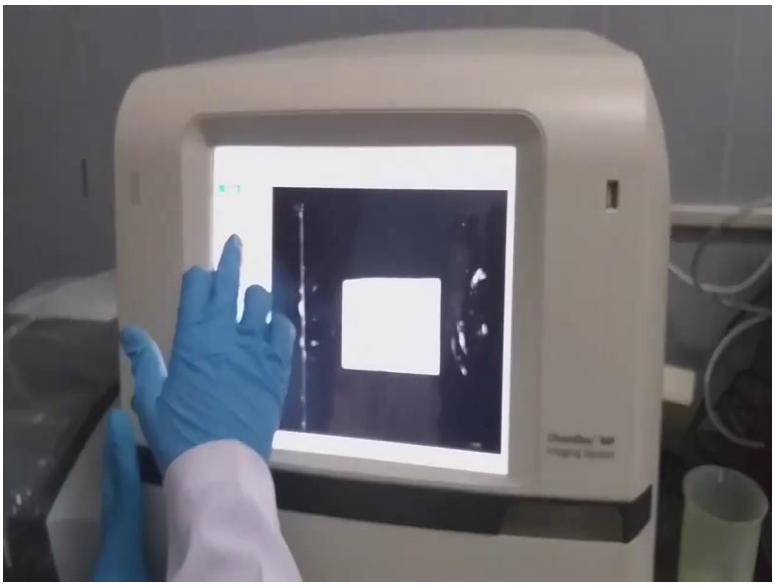

Here we have to select application.

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We want blots that is chemiluminescent.

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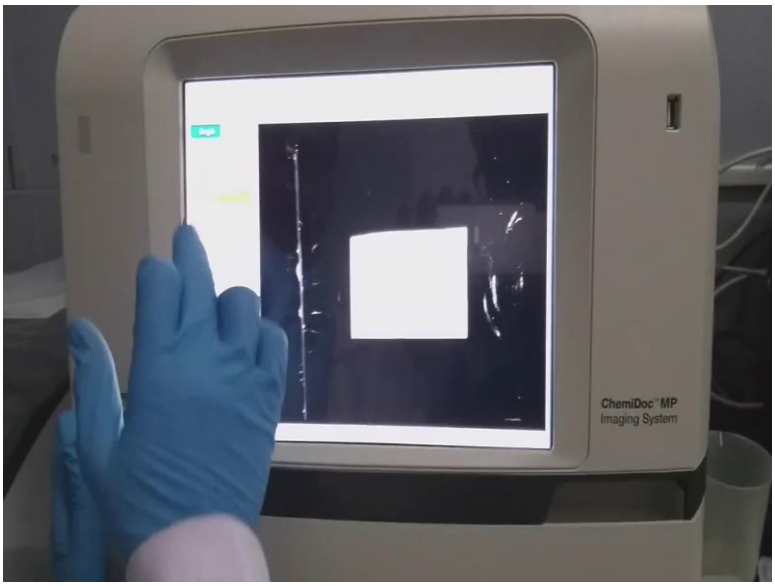

And what exposure want?

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You have two options; manual, auto. Auto, in auto, two options are there. Optimal auto exposure, rapid auto exposure, we will choose optimal auto exposure.

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So, you can enlarge the blot also.

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Once it is over, you just stay. So, this is the developed blot.

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So, as we can see the bands, the bands pattern. So, this is how we develop western blot through electro chemiluminescent substrate. So, in this video we demonstrated how to transfer the proteins to the blot and what are the precautions need to be taken while doing the western blot and also how to develop the blot and what is the lay in principle behind the developing the blot. So, I hope this will help you to understand the basic outlaying mechanism of how western blot works. Thanks for watching.

So, in the demo, students have explained the different steps and I hope the demo video could be helpful for you to understand the practical aspect of the same western blotting and it will help you to perform the experiment in your laboratory. So, what we have discussed? We have discussed about the expression of the protein utilizing the mammalian expression system.

And in that you can have the two options either you can have the transient expression system or the permanent expression system and both of these options have their own positives and negatives. And apart from that you can also have the option of non constitutive expression or the inducible expression system.

And. So, based on the different types of requirements and as well as the criteria you can have the choice of choosing the different conditions for expressing the protein into the mammalian expression system. And once you are going to express the protein of your

interest, you can also should perform the specific test like the western blotting to detect the protein on the nitrocellulose of membrane.

So, with this I would like to conclude my lecture here in our subsequent lectures we are going to discuss about the protein purifications, so that you can be able to isolate the enzyme of your interest from the crude lysate.

Thank you.