Genetic Engineering: Theory & Applications
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Module 6: Recombinant DNA Technology (Part-ll)
Lec18: Protein Production In Host (Part-III)

Hello everybody this is Doctor Vishal Trivedi from Department of Biosciences and Bioengineering IIT Guwahati. And what we were discussing, we were discussing about the protein production strategies in different types of host. In this module what we have discussed so far, we discussed about the protein production strategies in the E coli as a system, then subsequently we have discussed about the yeast as an host; which is actually the basic Eukaryotic host and unicellular Eukaryotic host.

So the manipulation as well as the protein production in the Eukaryotic system such as yeast is the simplest system. Then subsequently we have discussed about what is the disadvantages of using the yeast as an expression system. And then subsequently we have talk about Insect Cell line as an Expression System, in that we have discussed about how to clone the proteins into the Baculo Expression Systems, how to screen them, how to select and then ultimately how to generate the recombinant viruses which you can use to infect the insect cells so that the insect cells will over express the proteins.

And at the ends we have also discussed about how to retrieve these cells whether the cells would be cytosolic in nature or the protein what you are going to express (in the) is going to secrete into the media of the insect cells. And both of these strategies have the different requirements in terms of the media what you are going to use for protein production. So now, continuing the discussion about the different types of host. Today we are going to discuss about the Mammalian cells as an expression system.

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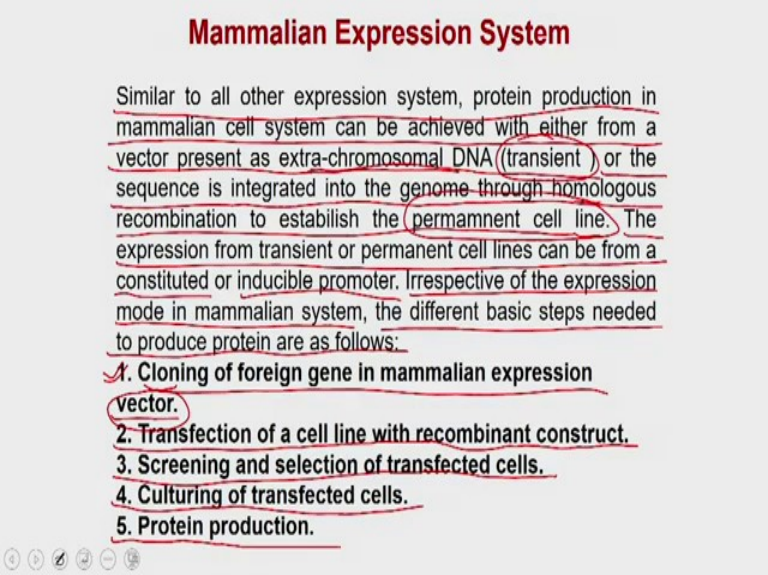

So Mammalian Cells there are different types of Mammalian Cells which are being used for protein productions. So Mammalian Expression system is the most complicated Eukaryotic expression system which people are using for protein production as well as for asking the questions or studying the transcription as well as translation of the foreign genes or foreign proteins. Sometimes they are also looking for what will be the impact of or what will be the functional role of a particular protein in those cases, they over express that particular protein into a cell.

And then ask what will be the physiological as well as the phenotypic changes occurs in that cell. So similar to all other expression systems, the protein production in the Mammalian cell system can be achieved with either from a vector based such as the extra-chromosomal DNA and once you produce from the extra chromosomal DNA, it is going to be transient because the extra chromosomal DNA is going to be degrade in due cause of time, or some cases you can actually sequence you can integrate into the genome through homologous recombination to establish the permanent cell line.

So the expression from the permanent cell line will be intact. It will going to express as long as you grow these cells. The expression from a transient or the permanent cell line can be from a constitutive as well as the inducible promoter. As we discussed in the yeast as in the case of

yeast, you have the choices of either using the constitutive promoters or the inducible promoters in the case of Mammalian Cells as well. Irrespective of the expression mode in Mammalian system, the different basic steps which you are required to produce the protein are as follows.

The first step is that you have to clone the foreign gene in Mammalian Expression vector. We in our previous lecture, we have discussed different types of Mammalian Expression vector which are available for cloning the foreign gene into the vector. Then once you generated the foreign this recombinant clone, then the second step is that you have to transfect the (cells) cell line with the recombinant construct.

This one also, the transfection of the Mammalian cells also we have discussed in detail; where you can use either the chemical method or the lipofectamine or there are three to four different approaches what we have discussed in the case of (Mammalian expression) Mammalian cells and the transfection of Mammalian cells. Then once you transfected the cells you have to screen and select the transfected cells and ultimately you have to culture these transfected cells and then you have to do the protein production. So let us continue with the Mammalian expression system and understand all these steps.

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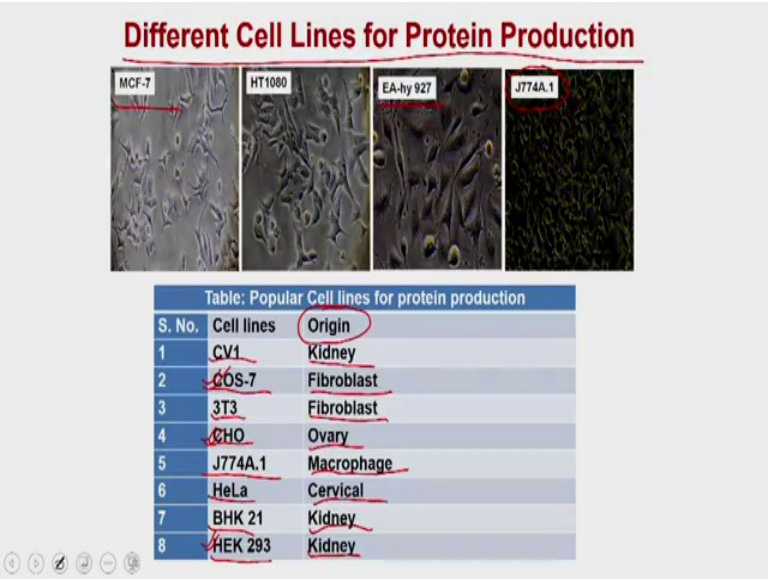

So in the Mammalian expression system you can use different types of cell lines for protein production. These are not the exhaustive lists of cell line what you can use. But these are the

different cell lines which are very, very popular in terms of protein production as well as performing the experiments into the Labs. So you have the different types of cell line and they are been derived from the different origins, for example you have the MCF-7 which is a breast cancer cell line, you have the HT 1080, you have EA-hy 927 which is the endothelial cell line and then you have the J774A.1 which is a macrophage line.

I have given the list of cell line here also in which you have the CV 1 cell line, which is actually from the kidney. Then you have the COS-7 cell line which is from the fibroblast origin. Then you have the NIH 3T3 which is a fibroblast. Then you have the CHO-k1 which is actually the ovarian origin. Then you have the J774A.1 which is of the macrophage origin. HeLa which is a cervical cancer cell line. Then BHK 21 which is a kidney cell line an HEK 293 which is a kidney cell line. And among these cell line, why we are talking about these cell line because you have to choose these cell line as according to the origin of the foreign gene.

For example, if I have to over express a cytokine gene into the for protein production, I would prefer to use the immune cells. In those cases I may prefer to use the J774 which is a macrophage cell line. Similarly if I have a protein production I would like to just use some Mammalian cells. Then I will go for those cell line which are easy to propagate easy to grow and the manipulation of the cells in the cell culture would be easy.

In those context you can use the either the COS-7 cells or the CHO cells because these cells are very easy to grow in the Mammalian (ex) into the Mammalian media. And these cells are easy to manipulate, compared to all other cells and the other third cell line what you can also use is HEK 293 which is actually very, very easy to manipulate very, very easy to transfect and very easy to propagate within the media.

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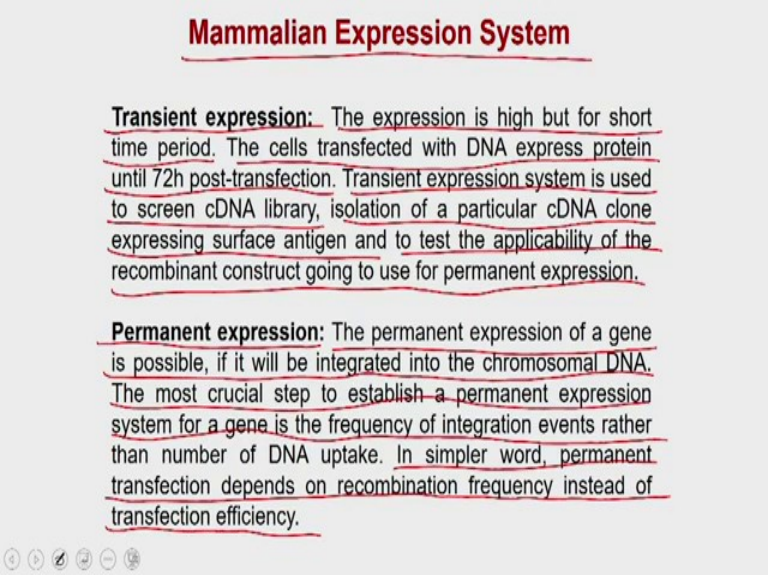

As we discussed you can have the two different mode in which you can get the protein expression either it would a transient expression or it would be a permanent expression. So transient expression would be expression were the expression is going to be very high but it would be a very short period. Which means if you transfect the cells with the DNA expressing the protein it will go into give you the protein production until the seventy two after the post transfection. After seventy two hours the extra somal DNA or extra chromosomal construct which you are going to transfect into the cell is going to be degrade by the cellular machinery and eventually the protein production is going to stop.

Transient expression system is used to screen the cDNA library, isolation of a particular cDNA clone expressing surface antigen. Or to test the applicability of recombinant construct going to be used for permanent (trans) permanent expression. So before you go for the exhaustive exercise of permanent expressions you also want to test whether the construct what you have prepared is going to express and give you the protein or not. For those kind of expression as well you always used the transient expression. But in general the transient expression system does, is not been used very extensively for protein production, it is always been used to answer the research related questions.

For example if I over express this particular protein what how and what way it is going to change the morphology as well as the phenotype of that particular cell. For those kind of experiments you use the transient expression. But if you want to do the protein production the transient expression system is going to be very, very costly because you are not going to get the protein production post seventy two hours. This means after every seventy two hours you have to do another round of transfection which means you have to isolate the DNA, you have to make the super coiled high quality DNA which will going to transfect into the Mammalian cells and then you also have to use the transfection regent.

These transfection reagents are going to be costly so if you do multiple rounds transfections and try to use those cells for protein production. It is not going to be economically viable options for protein production. For that purpose people always used to prefer making the permanent transfections or the permanent expression system. So in permanent expression system you are going to use and you will use the gene and integrate into the chromosomal DNA.

Once you integrate that into the chromosomal DNA the protein will going to be expressed along with the or the number of protein or gene or once as soon as the cell will divide you are going to have the more number of copies of that particular gene. So it is actually going to continue along with the cell division. So that is why even if the cells will divide it is, you are not going to lose the DNA and that is why called as the permanent expressions.

The most crucial step in the permanent transfection is the establishment of permanent expression system for a gene is to integration of that particular gene and that depends on the number of times or number of frequency of integration into the genome. So you have to achieve that by simply screening the cells on multiple rounds just to see that it is getting integrated into genome. And that is why the permanent expression system or development of the cells which have the permanent expression is a time consuming as well as the labour intensive exercise.

But once you are done you are sure that this particular protein is going to be expressed from these cells lifelong. You don't need to do another round of transfection or selections and so initial steps are time consuming but subsequently it will, it is going to pay you in terms of reducing the cost of the transfection reagents or making the DNA as well as it is going to reduce the

manpower requirement to do these jobs. In simpler words the permanent transfections depends on the recombination frequency instead of transfection efficiency.

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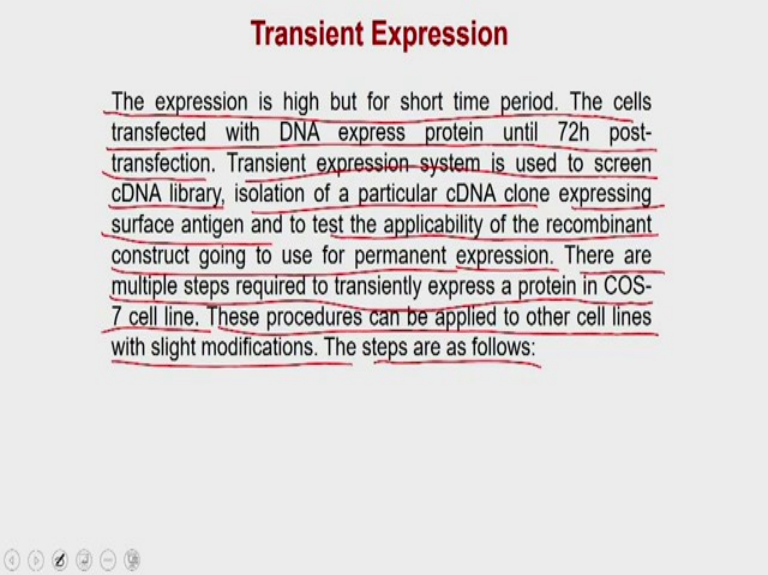

So let's start with the transient expression and understand what are the different steps you have to perform to make the protein under the transient expression systems? So as I said the transient expression system the expression is high but it is short lived. The cells transfected with DNA express protein until seventy two post transfection. Transient expression system is used to screen cDNA library, isolation of a particular cDNA clones expressing surface antigen or to test the applicability of the recombinant construct going to use for opponent expressions. There are multiple steps required to transiently express a protein in COS-7 cell line.

So in this particular example we have taken the COS-7 as a COS-7 cell line to show you that how to do the transient expressions. But for other cell lines also the steps are going to be remain constant, except that you might have to optimise or standardise these steps depending on what cell line you are using. Suppose some cell lines which you are using which are not adherent cell line but non adherent cell line then you have to change little bit. But more or less the steps are going to be remain constant. So the procedure can be applied to other cell line but with slight modification. The steps which you are going to use for making a transient expression are as follows.

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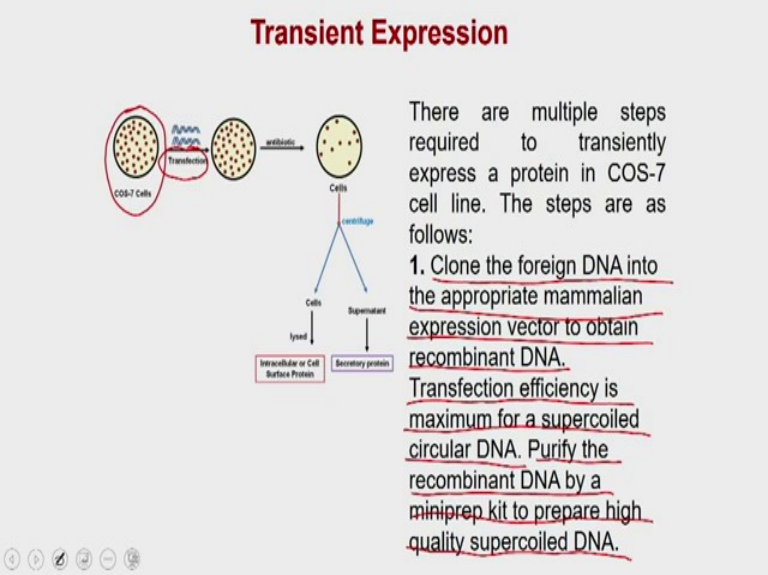

So in the first step what you are going to do is, you first plate the COS-7 cells into a plate, okay and then what you have to do is the first step is, you take the super coiled high quality DNA and do the transfections. So First is clone the foreign DNA into the appropriate Mammalian expression vector to obtain the recombinant DNA and then the as we discussed in the past also the transfection efficiency is maximum for a super coiled circular DNA. So you have to purify the recombinant DNA by a miniprep to prepare the high quality supercoiled DNA.

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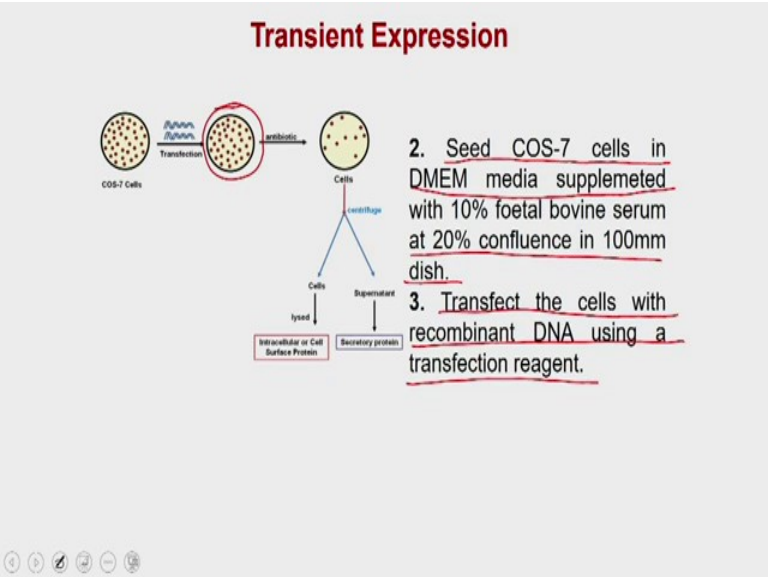

Once that is done, then the second step you have to do is the transfection and once you are done with the transfection, so in the second step what you do is you seed the COS-7 cells in DMEM media, which is actually the standard media recommended for growing the COS-7 cells at a confluency of twenty percent in 100 mm dish. Once the cells are adhered and they are ready then you do the transfection of these cells with the recombinant DNA which you are going to use with the transfection reagent.

You have an option to use the different types of transfection reagent which we have discussed in the past. You can also use the virus mediated or the bacteria mediated DNA delivery as well. So these are the different methods. Once you are done the transaction these cells are now been transfected. Then what you have to do is since all of this recombinant DNA is going to have the selection pressure you have to select these cells.

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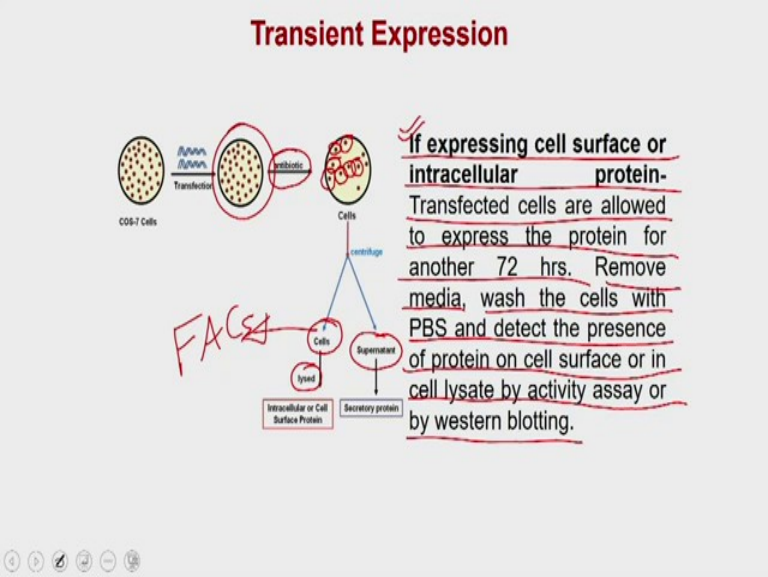

So that is the third step, you have to select these cells and suppose in this case we have taken an antibiotic so what you have to do is you grow these cells in the presence of antibiotic. As soon as you put the antibiotics the most of the cells which are not being transfected are going to die and then you are going to have the cells which are been transiently transfected.

So these cells are expressing your protein and along with that these cells are also expressing the antibiotic resistant genes as well. Now what you have to do is, these cells are ready to use for

transient expressions. In the transient expressions you have the option whether you want to have the protein onto the surface or the cytosol. Or you want the protein to be expressed or secreted out into the media.

Depending on the conditions you can use the different types of media. So imagine that if the protein is present on the cell surface or the intracellular protein which means that the protein is associated with the Cellular fractions. Then in that case you, so transfected cells are allowed to express the protein for another seventy two hours. Remove media and then wash the cells with PBS and detect the presence of protein on cell surface or in cell lysate by activity assay or the western blotting.

So once you have these transfected cells you centrifuge them that will give you the cellular component as well as the supernatant which is actually the media. The cellular component you can lysed or suppose the cells are present on the cell surface then you can take these cells and use the FACs to detect the presence of antigen on the cells surface. Otherwise you can lyse the cells and detect the protein into the intracellular or cell surface protein using the western blotting or the activity assay.

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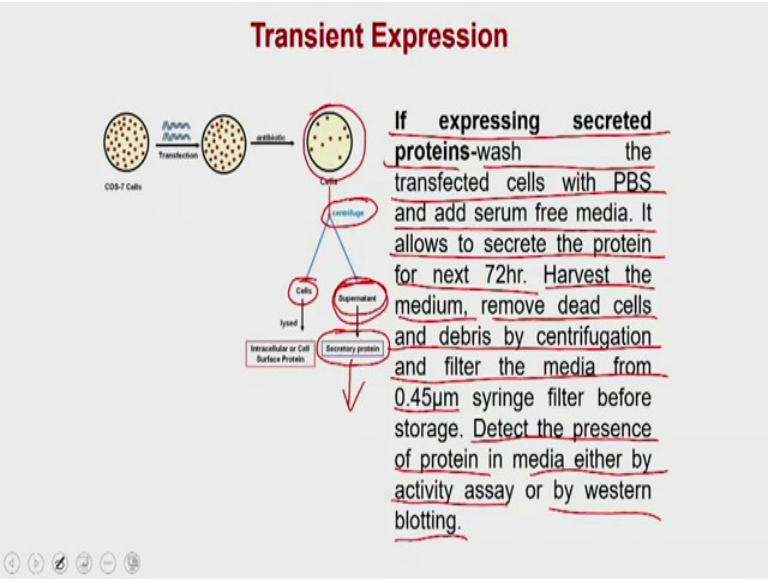

But if the protein is expressed and secreted into the external media, then what you have to do is, if the protein is expressing secreted out then you wash the cells with PBS and add serum free

media, okay? It will allow to secrete the protein for another seventy two hours. Then what you do is harvest the media, remove the dead cells and debris by centrifugation and filter the media by a 0.45 micron meter.

So if the cells, if you are putting the protein into the secretory pathway the cell, the protein will present into the supernatant in that case what you do is you wash these cells with the serum free media and then incubate them into the serum free media. These cells then will secrete the protein into the supernatant, you centrifuge you recover the cell you centrifuge and that will give you the cellular component as well as the supernatant. This supernatant you can pass through it to the 0.4 micron metre so that you can remove all the dead and damaged cells and you will only go into get the clear supernatant.

Once you have done that then you can detect the presence of your protein in the media either by activity assay on the western blotting. So then you have the secretary protein and you can do the western blotting or the activity assay for presence of low protein. So this is all about the expressing the protein into the Mammalian expression system under the transient expression. But if suppose you want to make the permanent expression system then you have to you first generate the cells under the permanent transfectant and then once your cells are ready then you can use them for protein production.

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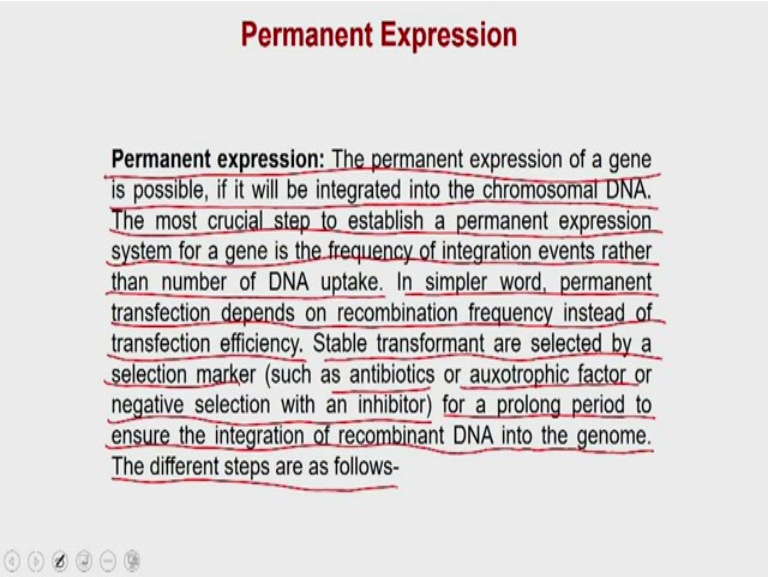

So under the permanent expression, the permanent expression is of a gene is possible if it will be integrated into the chromosomal DNA. The most crucial step to establish a permanent expression system for a gene is the frequency of integration event rather than the number of DNA uptake. In simpler words the permanent transfection depend on the recombination frequency instead of the transfection efficiency. The stable transformants are selected by a selection marker either as an antibiotic or the auxotrophic factor or a negative selection with an inhibitor.

All these selection of the cells we have discussed in the past, where either you use the antibiotic which is going to be a positive selection or the auxotrophic method where the media is not going to have the particular type of nutrients so the cell will not be able to grow but once your recombinant DNA will be there, if that recombinant DNA is going to provide the nutrients and that you can use even on the nutrient deficient media.

And the third is negative selection where you are going to add the chemicals and that chemical is going to kill the cells but if the protein is the chemical is being inactivated by the presence of your particular recombinant DNA then the cell will survive. But the question is this screening has to be done for a prolonged period of time to ensure that the recombinant DNA is getting integrated. You have the different steps which you have to follow for making the permanent expressions.

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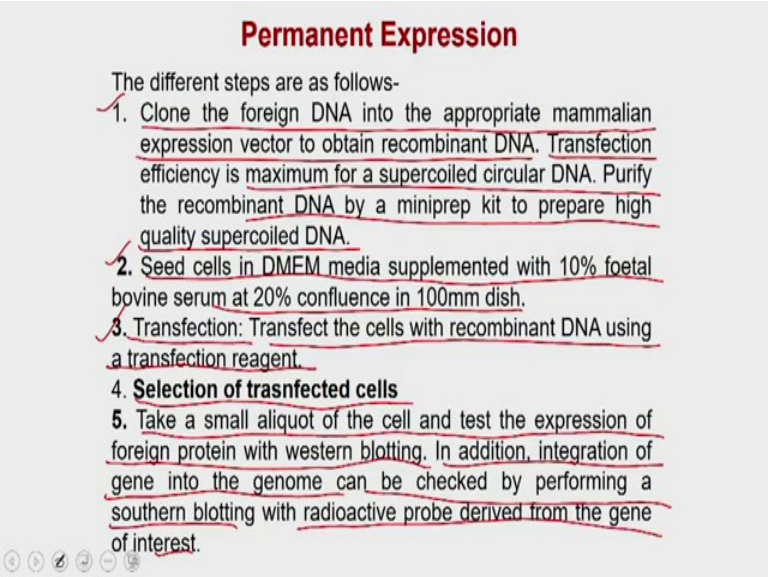

What are the different steps? So in the step one you first clone the foreign DNA into the appropriate expression vector to obtain the recombinant DNA, this step is exactly the same as we have discussed for the transient expression. You know that the transfection efficiency is going to be maximum for supercoiled circular DNA. Then you purify the recombinant DNA either by the miniprep or in the high-quality supercoiled DNA.

Then what you have to do is, seed the cells in DMEM media supplemented with ten percent BS at a twenty percent confluence in 100 mm dish. Then the third step you have to do a transfection. So transfect these cells with a recombinant DNA using a transfection reagent. This step number 1, 2, and 3 are going to be the identical what we have just discussed for the transient expression system.

Now in the step number four you have to do a selection of the transfected cells. And once you have selected the transfected cells then you can take a small aliquot of these cells and test the expression of foreign protein with the western blotting. In addition, since we are looking for the permanent expression you also have to see that the gene is getting integrated into the genome. That you can check simply by performing a southern blotting with a radioactive probe derived from the gene of interest.

If you remember we have discussed in the past how to generate, how to prepare the radioactive probes. Either you can use different types of method or different types of approach which we have which we have discussed while we were discussing about screening of genomic library as well as the cDNA library. So the similar kind of approach you can use to prepare the radioactive probe of the sequence which is from the foreign gene and then that you can use in a standard Southern blotting techniques to detect the genome.

And if it is going to give you the signal that will indicate that the, your gene of interest is been integrated into the genome. So these are the five steps you are supposed to do. We are not going to discuss about step number one two and three because these are we have already discussed in the past in terms of the transient expression. We are going to discuss about the step number four about the selection of the transfected cells in the case of permanent expression.

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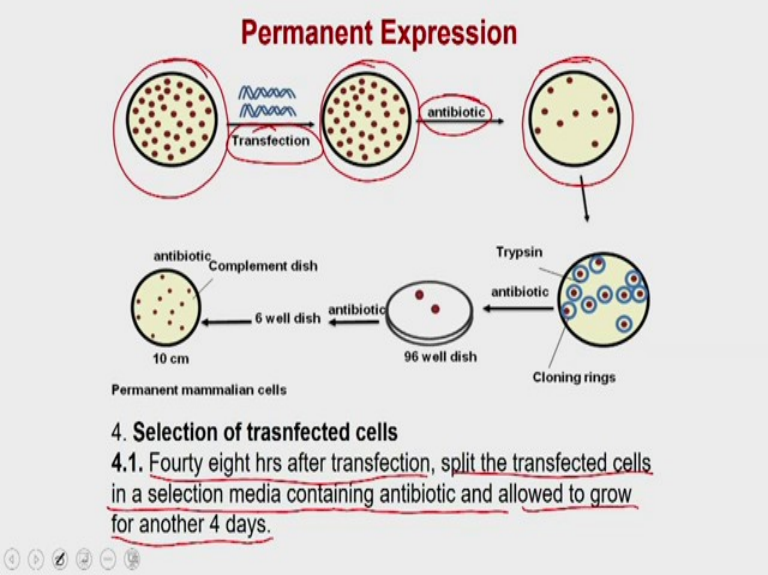

So in the case of permanent expressions the step number one is that you first plate the cells at a twenty percent confluence. Step number two, you do the transfection so the cells are getting transfected. Then you are going to put them under the selection pressure either it is antibiotic, auxotroph or the negative selection. Once you do that you are going to see that some of the cells are going to survive. They will not go into die because these are the cells which are getting transfected and these are the cells you have to select. So in the forty eight hours after the transfection, split the transfected cells in a selection media containing antibiotic and allow to grow for another four days. Which means once you are done the transfection you take out these cells and plate it into another dish which contains the antibiotic and let them to grow for another four days.

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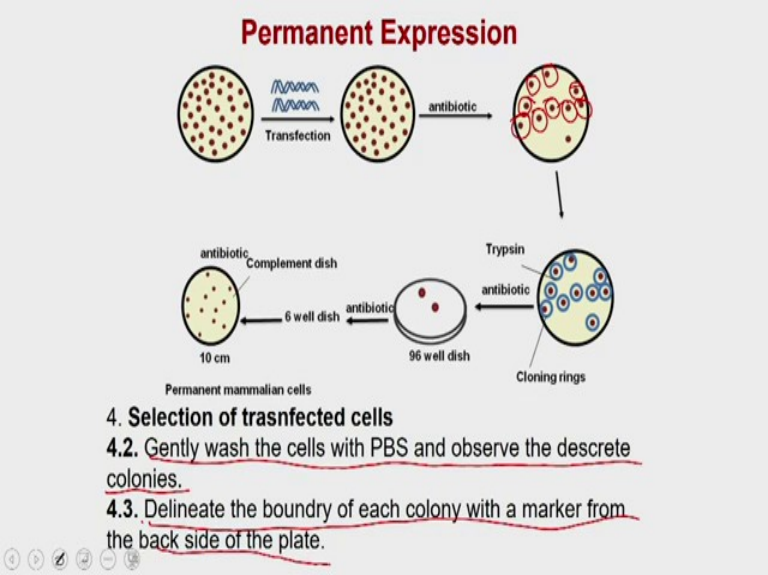

In these four days the lot of cells are going to die and what you have to do is, every day you have to you gently wash the cells with PBS and observe that you are going to have the discrete colonies or not which means this you have to continue by gently washing the cells so that all the dead and the damaged cells you should remove from the dish. Otherwise these dead cells are also going to damage your transfected cell as well. Now what you have to do is, since you got these dishes what you have to do is, a cell culture dish has a plate like thing and from the back side what you have to do is you take the marker and then you and encircle what are the places you have the colonies like as I am doing now, right. So if that you have to do from the now back side. So delineate the boundaries of each colony with a marker from the back side of the plate.

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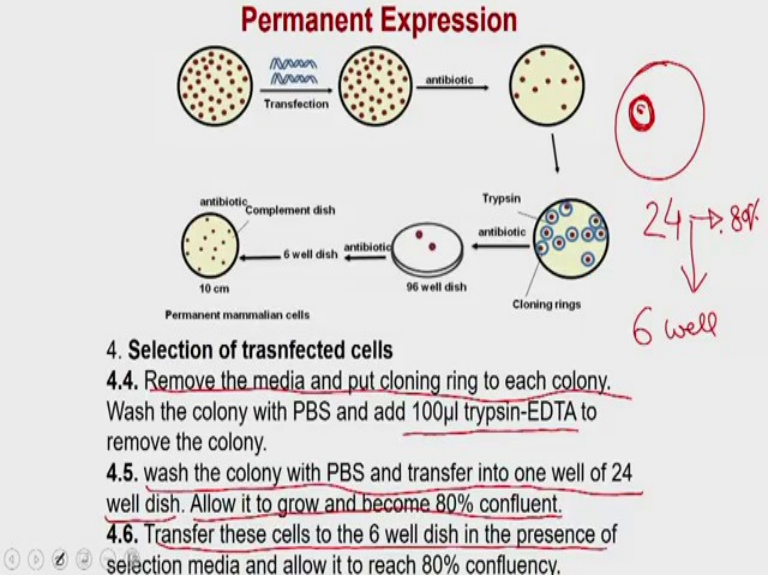

Now remove the media, okay and put the cloning rings. So cloning rings are actually the ring like structures and they are been, once you put them onto a particular plate they will go and get stick, okay. So if suppose this is the plate and you are going to put the cloning ring, the cloning ring is actually going to make this as like a well. So once you put the cloning ring, the whatever you put inside the cloning ring, suppose this is your Colony what you would like to isolate, once you put the cloning rings the cloning ring will make this particular area impermeable or the media will not go into leak out, okay.

Then what you have to do is, you have to add the hundred microlitre trypsin EDTA. So into this you are going to isolate this Colony simply by putting the hundred microlitre of trypsin EDTA. So you fill this cloning ring with the hundred microlitre of trypsin EDTA, once you do that the cells which are been adhered, they will come off and present into the liquid. Then you wash the colony with the PBS and transfer this single colony into another twenty four well dish and let it be growing. So allow it to grow and become eighty percent confluent once it become eighty percent confluent, then you (ru) do another round of selection, okay.

So then what you do is, so you have put it into twenty four well dish, okay? And then you allow that to go for eighty percent confluency. Now what you once it is getting confluent, then you take out these cells and put it into the six well dish. So initially you started with the twenty four

well dish you can and then you to transfer these cells to the six well dish in the presence of selection media which means you can still have the antibiotic pressure and allow that to reach to eighty percent confluency.

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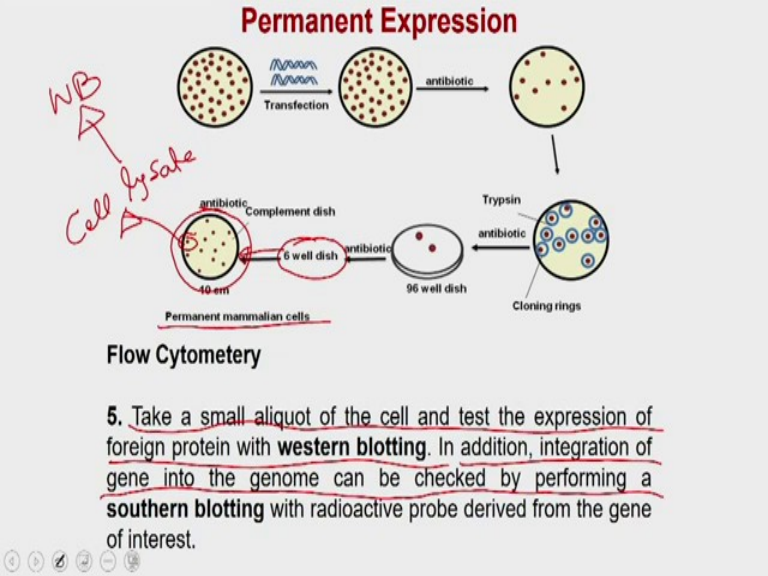

So once it is reaching to the six well dish eighty percent confluency, you can take out from here again and let it grow into the ten centimetre dish in the presence of the selection media and that actually is going to give you the permanent Mammalian cells. Now whether you have the permanent Mammalian cells or not that you have to test into the subsequent step. So in the step number five you take the small aliquot of the cells and test the presence of foreign protein with the western blotting.

So what you have to do is, take the small amount of cells and prepare the cell lysate and then you do the cell lysate and use it for the western blotting and the cell lysate you can do and you do the Western blotting to check whether the foreign protein is present into that western blotting or not. In addition since we are talking about out the permanent transfection you have to also check the integration of these gene into the genome. The standard technique is Southern blotting, so you can prepare a probe for that foreign gene and then use that foreign gene to detect fragments into the southern blotting. It gives you the signals that will confirm that the gene is been integrated into the genome.

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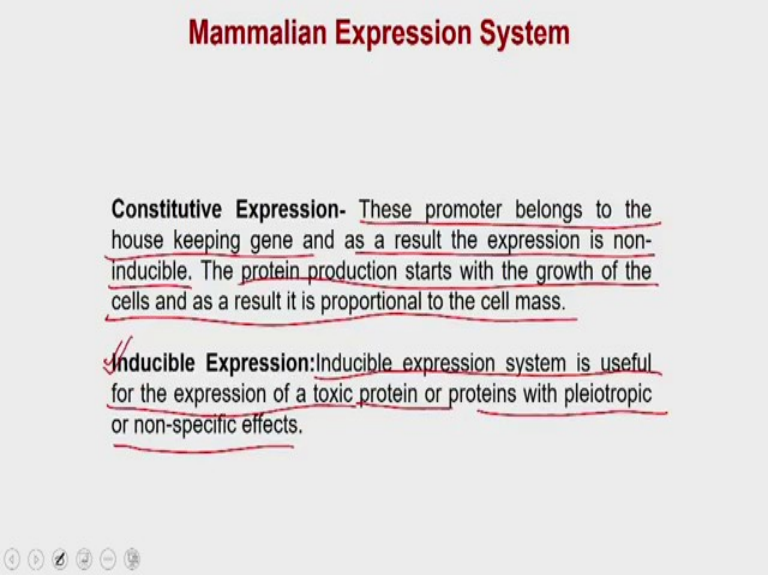

So this is all about the transient or the permanent expressions and within the Mammalian expression system you have the option either to go with the constitutive expression or to the inducible expression. So in the constitutive expression just like as we discussed of the yeast cells, you have the option to use the promoters of the housekeeping genes and as a result the expression is non inducible because it is going to use the house keeping promoters and the protein production would be in proportion to the number of cells which are present. And as a result the expression is going to be non inducible. So the protein production starts with the growth of the cell and as a result it is proportional to the cell mass. Whereas similar to the yeast where we were using the methanol as a inducer you can also have the inducible expression system.

So in the inducible expression system is very useful for the expression of a toxic protein or the protein with the pleiotropic or non specific effects which means if you are over expressing a toxic protein or the protein which have a very, very adverse effect on the cellular physiology in those cases what you are going to do, you can use the inducible promoter system and what will happen is, once the you reach to a cell mass, then you can add the inducer and that actually will allow the cells to over express that particular protein eventually because the protein is toxic in nature. It is going to kill the cells or it is going to adversely affect the growth of the cell. But at that moment, it may not be an issue because you already have a very, very high cellular density

of these cells. And that actually will give you very high production of the proteins. So let us discuss about the inducible expression system.

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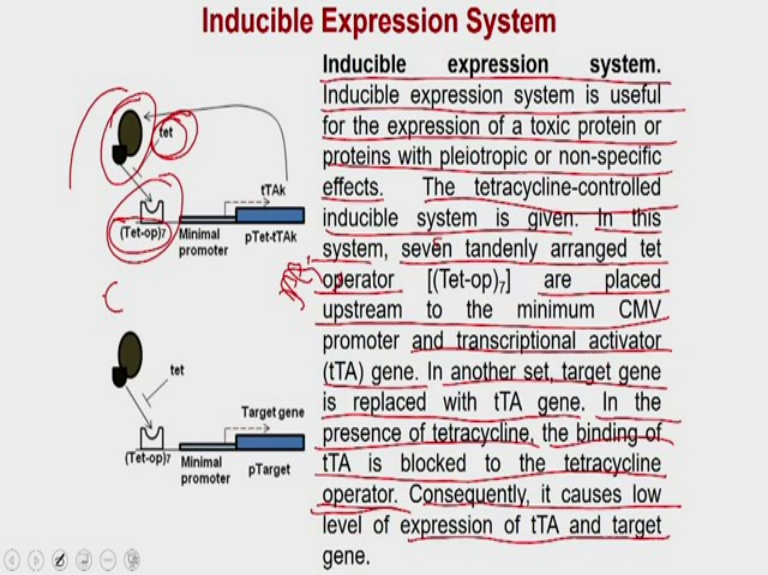

So in the inducible expression system inducible expression system is useful for the expression of a toxic protein or protein with the pleiotropic or non specific effect. The tetracycline-controlled inducible system is given in this diagram. In this particular system you have the seven tandemly arranged tet operators. And these tet operators are being placed upstream to the minimal CMV promoter and the transcriptional activator tTA gene. In the another set what will happen is, the target gene is replaced with the tTA gene.

In the presence of tetracycline, so what will happen in the presence of (Tet); so you grow these cells in the presence of tetracycline. The binding of tTA is blocked to the tetracycline operator which means as long as the tetracycline is present it will not going to allow the operator to bind to the tTA operator, the tetracycline operators. Consequently, what will happen it will cause because your operator will not going to, the tetracycline operator will not bind. That is why it is not going to drive the production of target gene as well as the expression of tTA or target gene.

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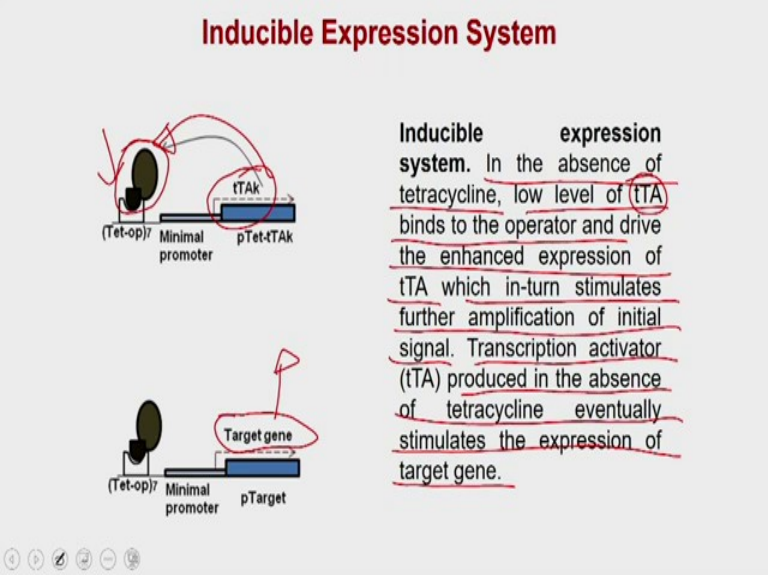

But once you remove the tetracycline, okay so in the absence of tetracycline the low level of tTA binds to the operator and that actually will enhance the expression of the tTA, which in turn will stimulate the further amplification of initial signal. Thus transcription activator tTA produced in the absence of tetracycline eventually stimulate the expression of target gene. Which means once you remove the tetracycline, the low level of tTA will bind to the operator and that actually will stimulate the production of the foreign gene as well as that tTA protein, this tTA will go and bind to the another round of operator.

And since initially there will be a low level of tTA. But once the it will go into to produce more amount of tTA it is going to generate very inducible system and that is how it is going to induce the production of protein from the target gene. So this is called as the Tet-on Tet-off inducible promoter system or inducible expression system in the Mammalian expression system. We have discussed about different types of expression systems, we started with the very simple expression system such as the E-coli expression system.

And we have discussed many aspects or many approaches what you can use to over express the tetracycline expression system. Then subsequently we moved on discussed about the yeast as an expression system, which is actually the simplest expression system in the Eukaryotic system.

And then we have also discussed about the insect cell line as an expression system. And by the end of this lecture we have also discussed about the Mammalian expression system.

We have discussed about how to produce the transient expressions or how to generate the permanent expressions into the Mammalian expression system and at the end we have also discussed about the constitutive as well as inducible expression system in the case of Mammalian expression system.

So with this I would like to conclude our lecture here. In the subsequent lecture we are going to discuss about the recovery of the proteins from the over expressing cells and then subsequently we are going to discuss about how to recover the product, how to verify the product and how to characterise that particular that particular product because as you remember, we have to use this product for downstream biotechnology applications.

So all these products have to be characterized using the standard biochemical as well as biophysical parameter associated with these products and once these product are been verified on these parameters then only we can put them into the application part and at the end of this course we are also going to discuss about the applications of these biotechnology products. And with this we would like to conclude our lecture here. Thank you.