Genetic Engineering: Theory and Applications
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Module 5
Recombinant DNA Technology (Part I)
Lecture 14
DNA Delivery in Host (Part 2)

Hello everybody this is Dr. Vishal Trivedi from department of Biosciences and Bioengineering, IIT, Guwahati and what we were discussing, we were discussing about the delivery of the host or the recombinant DNA into the host cells and in this discussion we were, what we were discussing that you can successfully transfer or deliver the DNA into the host cell by adopting two strategies.

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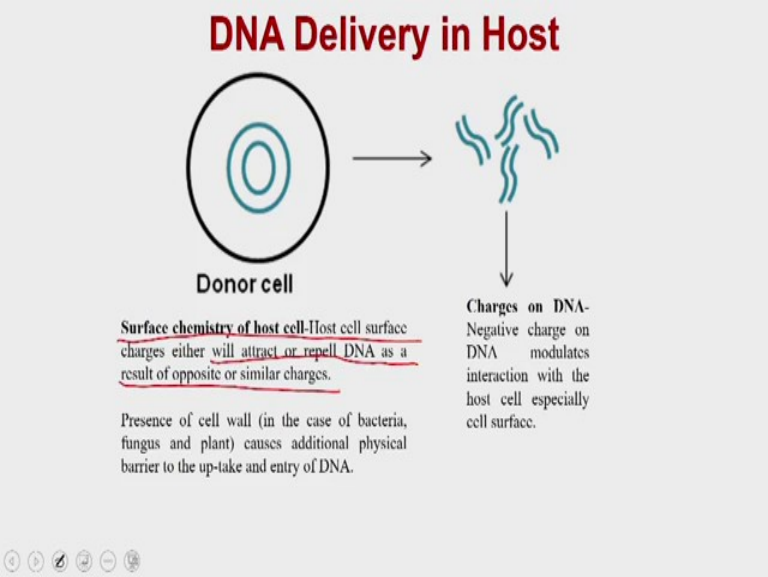

In strategy number 1, you have to work on the surface chemistry of the host cells and according to the surface chemistry you can devise the mechanism so that you will be able to attract the DNA and as a result the DNA will be get entered into the host cells. We have discussed about the transformation into the bacterial cell as well as we have discussed about the transformation into the yeast cells. But there is an alternate strategies and why this alternate strategy is required?

Because in the case of mammalian cells the surface chemistry of a mammalian cell is very-very complicated compared to the bacterial cell or the yeast cells. For example, a mammalian cell of the liver origin, a mammalian cell of the pancreas origin or a liver or a mammalian cell of brain origin all these (thin cell) three different cells from the three different origins are of going to have the very-very diversified surface chemistry and because of that you cannot devise the mechanisms which are going to work on the surface chemistry to deliver the recombinant DNA.

In those cases what you have to devise is, you have to work on the basic principle of delivery of a molecule into the mammalian cell and you have to change (the) DNA in such a way so that the DNA will be accepted by the host cells. So in the today's lecture we are going to discuss about the different strategies what people utilized to deliver the recombinant DNA into the mammalian expression system or mammalian cells.

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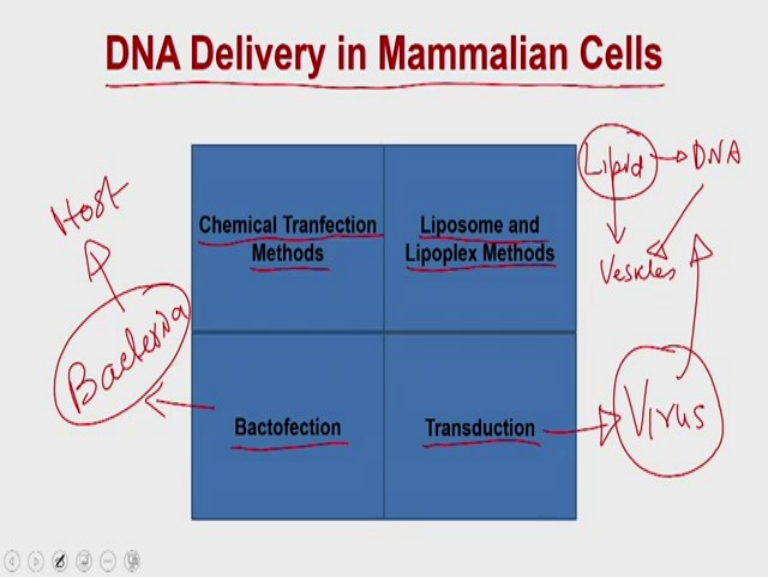

So for DNA delivery in the mammalian cell people are mostly utilizing the 4 different strategies. The strategy number 1 is called as the chemical transfection methods. In the chemical transfection method people are using different types of chemicals and these chemicals are making the complexes with the DNA and as a result they will be taken up by the cells. Whereas in the other approach where people are using the Liposome or the Lipocomplex methods, in these cases they are using the lipids and these lipids are making a complex with DNA or in some cases these lipids are forming the vesicles and this vesicles are engulfing the DNA and then these

vesicles are fusing with the host cell and as a result the DNA, the recombinant DNA is being delivered to the mammalian cells and then it is going it will reach to the nucleus and it expresses its recombinant gene what you are putting into this recombinant DNA.

The third approach is which is called as the Bactofectin, in the bactofection you are using the bacteria as a vector or bacteria as a source to deliver the DNA into the mammalian host. Similarly in the fourth approach is called as the Transduction. In the transduction instead of bacteria you are using the virus and this virus is also delivering the recombinant DNA which is present inside the virus to the mammalian cells. So let us start with the first approach which is called as the chemical transfection methods.

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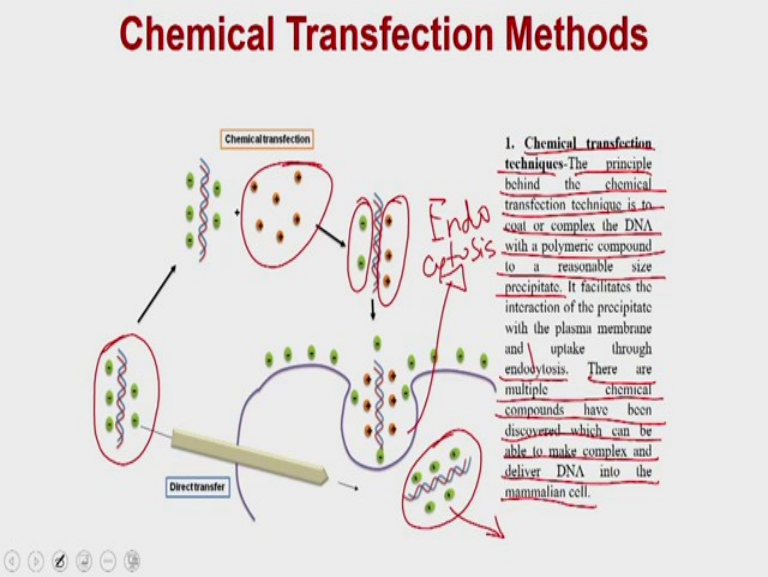

So in the chemical transfection method as you know that the DNA is negatively charged okay, so what you in one of the approach what you can do is you can take a syringe or something and directly transfer this negatively charged DNA into the cell but you know that this kind of thing is not possible or it is also required the extra ordinary infrastructure so that you have this kind of microinjections which can transfer the DNA into the (host) into the mammalian cells that is why and this negatively charged DNA is problematic because it can repel from the host negative (DNA) host, the surface charge present on to the mammalian cells.

Because of that what you are going to do is, you are going to add the chemical molecules which are mostly being cationic in nature or positive in nature, because of that the DNA is going to have the negative charge on one side and it is going to have the positive charge on other side and as a result it is going to make a complex and these complexes are being taken up by the mammalian cells in a process known as the endocytosis and that is how they will going to reach to the nucleus and then in the nucleus this chemical molecule what you are using is going to be released and the DNA is going to be free for downstream expression processes.

So as the as it says, the chemical transfection methods or techniques the principle behind the chemical transfection method is to quote or complex the DNA with a polymeric compound to a reasonable size to precipitate which means, you are going to generate a macro-size particles and once you generate a macro-size particles, these particles are being going to be taken up by the plasma membrane which is a process known as the endocytosis. There are several chemicals or several multiple chemical compound have been discovered which can be able to make complex and deliver DNA into the mammalian cell.

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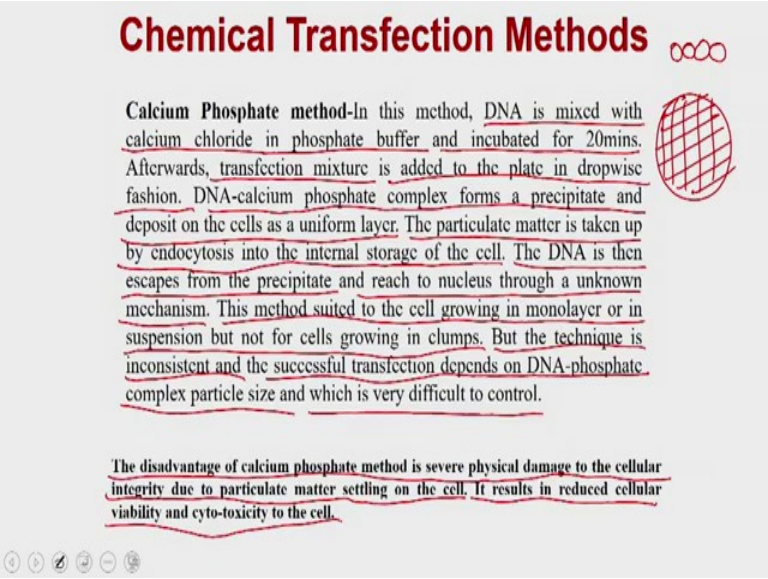

Let us see what are these methods, so the first method which people have discovered is called as the Calcium Phosphate method and in the calcium phosphate method DNA is mixed with the calcium chloride in a phosphate buffer and you incubate this for 20 minutes. Afterwards the transfection mixture is added to the plate in a drop-wise fashion, so what will happen is, the

calcium chloride in the phosphate buffer is going to generate the calcium phosphate and these calcium phosphates are going to make the complex with DNA and then you will spread this particular transfection mixture drop-wise onto the cell, this complex is going to be settled down onto the cells.

So DNA, calcium phosphate complex forms a precipitate which means it is going to form a macro-structure or macro-size particles and these particles will slowly settle down onto the uni-layer of mammalian cells which you are going to have on the plates and they will deposit onto cell as a uniform layer. So it will be like you have the cells on the petri dishes and when you plot it is actually going to make a cover or curtain kind of situation. So the whole cell is, if you see this cells under the microscope what you will see that the cells are covered with a very-very fine powder like structures okay and these powders are nothing but the calcium phosphate particles which are already engulfing the DNA.

So once this particle will settle onto the mammalian cells, the mammalian cells have a tendency that they will take up these particles through a process known as the endocytosis, this is exactly the same process if you are providing the food to the mammalian cell to eat. So in those cases if the food is macro-structured which means it is of beyond a certain size these food is going to be taken up by the process known as the phagocytosis. So if the (process) if the cell will take up these particles, these particles will enter into the cell under the active mode where the cell is going to take up the particle which are being spread on it as a powder.

So then what will happen is the particulate matter, which is actually the powder which you have put onto these cells are being taken up by the endocytosis into the internal storage of the cell. The DNA is and you know that the internal storage environment after the endocytosis is not very, is not going to keep the calcium phosphate to form the complexes. So once it enters into the cell and it is going to the storage area the calcium phosphate is going to be released and the free DNA is going to be available for to escape from the precipitate and to reach the nucleus and although the mechanism how the DNA is escaping from the calcium phosphate precipitate and how it is reaching to the nucleus is still not known.

This method suits to the cells which are growing in a mono-layer which means if the cells are making a mono-layer of the or the thin sheets or thin layer or the cells which are present in the

suspension which means the cells which are growing in the suspension just like the bacteria, so they are not making the cell to cell contacts or they will be good for the mono-layer, the cell which are forming the mono-layer. But the technique is inconsistent and the successful transfection depends on the DNA phosphate complex particle size and which is very difficult to control. So in one of the major drawback of this particular technique is that the, first of all it is only suitable for the cells which are adherent or which are suspension.

For the adherent cells, the cells which are forming the mono-layer, so if you can imagine that if you are working with the cancer cells and they are making the multi-merse because you know that the cancer cells will stick to each other and then they form the foci and all that kind of multilayered cells then the calcium phosphate method is not very suitable or it may not give you the optimal transfections. Same is true for the suspension cells also, if the cells are present in the suspension they should be the single layer suspension not the cells (which) are present in the suspension with the multiple cells adhere to each other or forming the complexes.

The second thing is, when you are adding the calcium phosphate to the DNA, the calcium phosphate is making an aggregate with the DNA and these aggregates actually you have to optimize in which the calcium phosphate aggregates are forming because once if start forming the aggregates initially the size of this aggregate are going to be very small and but as the time will pass these aggregates are going to be bigger and bigger and bigger because these are non-specific aggregates they are not forming by through a ordered fashion or something like symmetry related rearrangement of the molecules.

So these going to be a smaller at the beginning but they will grow as the time will pause and that is why it is it requires a significant amount of time to optimize the time as well as the temperature as well as the media in which you are going to develop these aggregates and that is why it is very-very cumbersome. Apart from that you have also multiple disadvantages, (one of) what is the disadvantages? The calcium phosphate method is causing the severe damage to the cellular integrity due to the particulate matter settling onto the cell. So you can imagine that you have the heavy particles which are settling onto the cell and these mono-layer mammalian cells, sometime the mono-layer mammalian cell for example if you are working with the endothelial cells or the epithelial cells these cells are very delicate.

So if you are putting a very huge size particulate matter onto them, it actually causes the physical damage to the cell and once it causes the physical damage it is actually not going to allow the Z-cells to recover post subsection and that is how your overall transfection efficiency is going to be very-very low. Which means the calcium phosphate method is having the two disadvantages, one it is inconsistent because you cannot control the aggregation of those particles and the size of those aggregates because the size itself decides what is the transfection efficiency you are going to achieve at the end.

The second, since you are working with aggregates, these aggregates are also going to cause the physical damage to the cell and that may compromise the survival of these cells post transfection. The third method, so at the end it reduces it is a cellular viability and it causes the significant cyto-toxicity to the mammalian cells and that is why the calcium phosphate method is not very popular for the cells.

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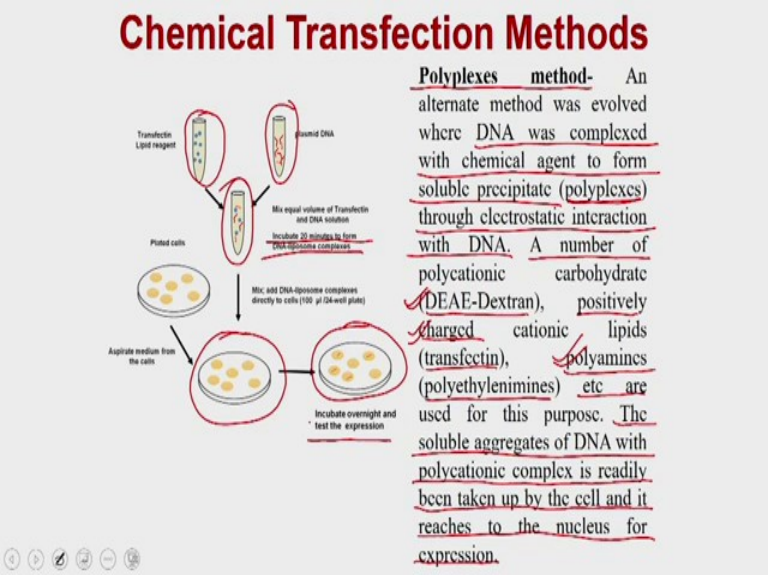

The third, the other method is called as the Polyplexes method, so in the polyplexes method what you do is you make the DNA complex with a chemical agent to form the soluble precipitate or the polyplexes. So this method is different from the calcium phosphate method because in the calcium phosphate method the aggregate what you are forming is the aggregating agent like calcium chloride and the phosphate buffer is non-specific in nature, that is why you cannot

control the size of those aggregates and that is why you need an optimization in the case of calcium phosphate method.

Whereas in the polyplexes method you will use the polymeric substances which are going to form the aggregates of the defined size. So this polyplexes they will make the complex with the DNA through electrostatic interaction which means the DNA is going to be negatively charged and this material what you use for making the precipitate is going to interact with the DNA with a positive charge. Example, in the case of polyplexes are DEAE-Dextran, positively charged cationic lipids such as transfectin and the polymeric amines such as the polyethylenimines these are the (use) all these three different categories of particles such as the carbohydrates, you have the lipids such as which are called as the transfectin or you have the amines. All these three categories are suitable for this purpose, the soluble aggregates of DNA with the polycationic complex is readily being taken up by the cell and it reaches to the nucleus for expression.

So what you are going to do in this polyplexes method is that, what you will do is you take the polyplexes in the transfection reagent in one of the eppendorf then you will take the plasmid DNA in that other transfection then what you do is you take the equal amount of both the transfection reagent as well as the plasmid DNA and you mix them together and incubate for half an hour and or the (30 minutes) 20 minutes to form the DNA liposome complexes and once this is formed then you plate it onto to the cell and you put it onto the cell in a drop-wise and then you allow them to incubate with the cell for overnight and once you do that and you allow them to recover from the stress which is being exerted by the transfection. What you will see is within the 24 hours the DNA what you have put into this plasmids will reach to the nucleus and they will going to show you the expression.

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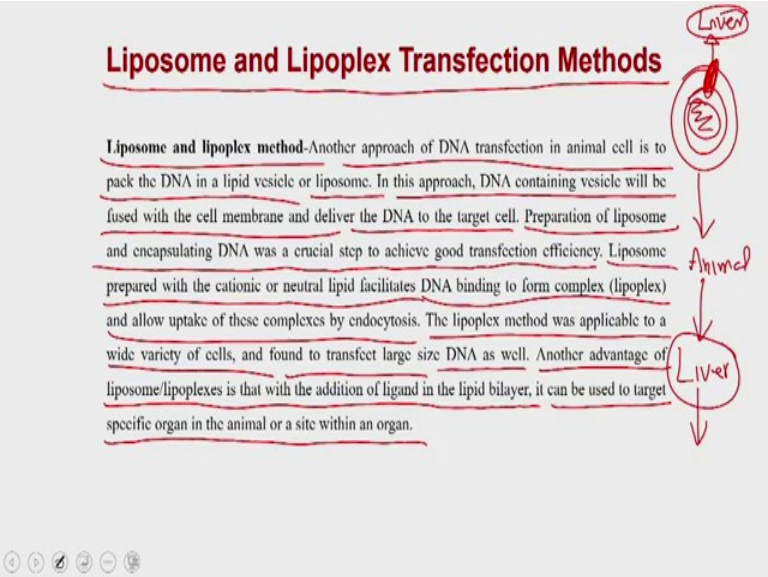

Now the second approach is called Liposome and Lipoplex Transfection method, so the liposome and the liposome transfection method is an alternate approach where you are going to use the DNA transfection in animal to pack the DNA in a lipid vesicle or the liposome. You know that if you take the lipid vesicle and sonicate these lipid vesicles will rearrange and they will form the liposome. The liposomes are the kind of the bi-layer vesicles and inside they have a (hole) they have the space where the material can be stored. So the DNA containing vesicle will be fused with the cell membrane and deliver the DNA to the target cells.

The preparation of liposomes and encapsulation of DNA was a crucial step to achieve the good transfection efficiency. The liposome prepared with the cationic or neutral lipids facilitates the DNA binding to form the complexes. So what will happen is if you take the cationic lipids which means if you take the positively charged lipids while you are making the liposomes these cationic lipids are going to attract the DNA which is negatively charged and that is how the DNA will aggregate onto these vesicles and these vesicles are going to be taken up by the cell very easily, through a process known as the endocytosis.

The liposome method was applicable to a wide variety of cell and found to transfect large size of DNA, so that is the advantage that the liposome mediated transfection is as universally accepted for many mammalian cells and in addition you can use this method to transfect the large size of DNA as well. Another advantage of the liposome or lipoplex is that the addition of ligand in the

lipid bi-layer it can be used to target the specific organ in the animal or a site within the organ. Which means, what it is saying is, that if suppose you have prepared the liposomes and suppose you add ligand which means suppose you add a antigen which is for the liver okay.

Then and suppose you load this with the plasmids then what will happen is, if you take this transfection mixture and inject it to the animal then what will happen is that within the animal it will not only going to transfect the cells, it will reach to the liver because of this antigen which is going to be recognized by the liver cell and then it is going to deliver the particular type of plasmid to the liver cell and you can express the protein which is being encoded by this plasmids. So that is how you can actually modulate or you can actually re-engineer the lipoplexes or the liposome vesicles to facilitate the targeted delivery of your recombinant DNA to a specific cells within the organ or within the animal.

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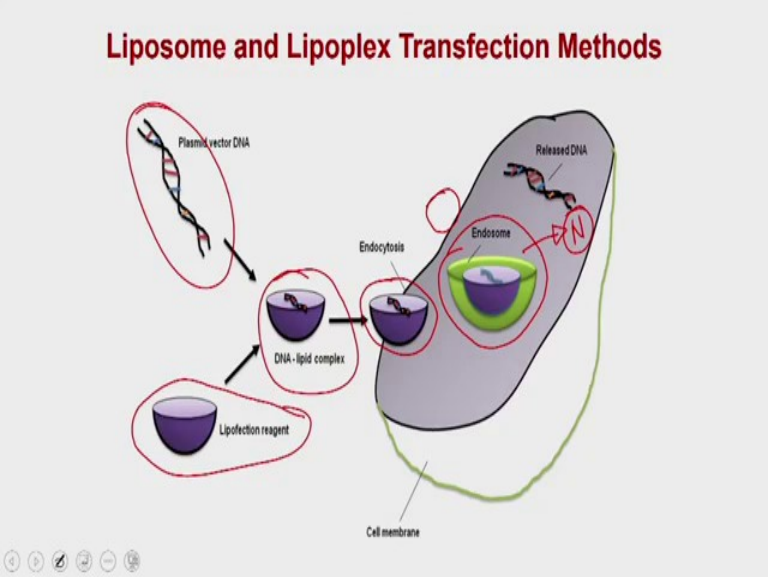

In a typical liposome mediated transfection what you do is, you take the plasmid DNA okay and then you take the lipofectamine reagent, so lipofectamine is a patented transfection reagent which is available from the company then you take the plasmid DNA and the lipofectamine you mixed them together to form the DNA liposome complexes then these DNA liposome complexes once they reach and they docked to the cell the cell will engulf these vesicles through a process known as the endocytosis and once they will be engulfed by the endocytosis they will reach to a vesicular structure which is called as the endosome, so within the endosome the DNA is being

released from the vesicles and then it will reach to the nucleus and within the nucleus it is going to express this protein.

So in a typical lipofectamine mediated transfection reagent or the PI based transfection reagent you have the multiple steps to perform, first you split the cells put it onto a single layer, let the cells to be adhered then you do the washing and then you perform the incubations of the plasmid DNA along with the lipofectamine or the transfection reagents whether it is lipofectamine or the PI based and then you can actually add that to the mammalian cells and you can perform the transfection. So we have in a typical experiment of transfection you have to perform multiple steps and you have to take the multiple precautions to perform this transfection experiments. So in a typical transfection experiments, we are having the following steps.

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Hello everyone, in this video we will show how to sub-culture the cells and count the cells and see for the transfection study. First we have to remove the remaining media then trypsinize the cells then we will count the cells and see it.

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Now I will show how to do Trypsinization. Now I am going to add the trypsin to detach the cells.

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After cells are detached we have taken into clean falcon then we have to centrifuge the cells. As the cells are very delicate we have to centrifuge it 1500 rpm for 2 minutes.

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Now we have to remove the super latent and re-suspend cells in fresh media.

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After re-suspension we have to count the cells, so I am going to take 20 micro liter of this cell suspension and mix with the 20 micro liter of trypan blue and count under neubauer chamber.

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Before counting we have to see how a counting chamber or hemocytometer look like.

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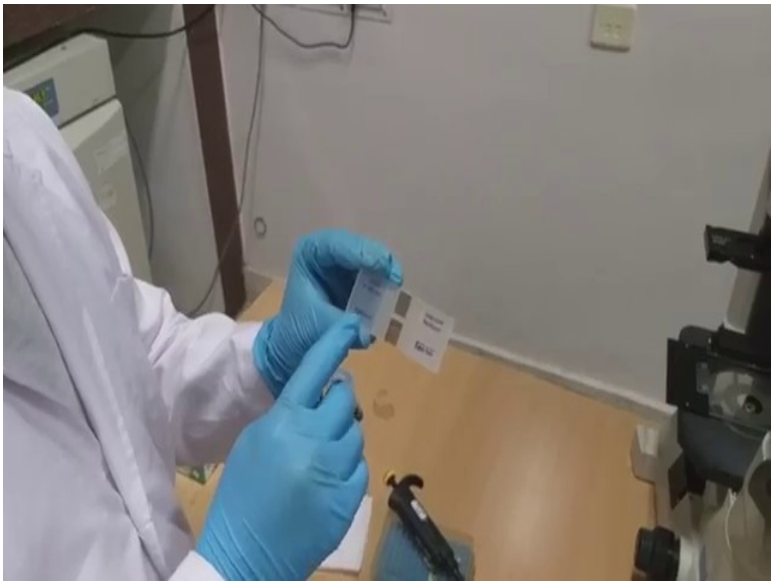

This is the typical hemocytometer also called as neubauer chamber which contains this squares in the upper side and lower side. With each square having the trough point 1 mm and area of 0.0025 millimeter square.

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Now I am going to put a cover slip on this chamber then I will add slowly self-suspension through capillary action it will spread all over the squares.

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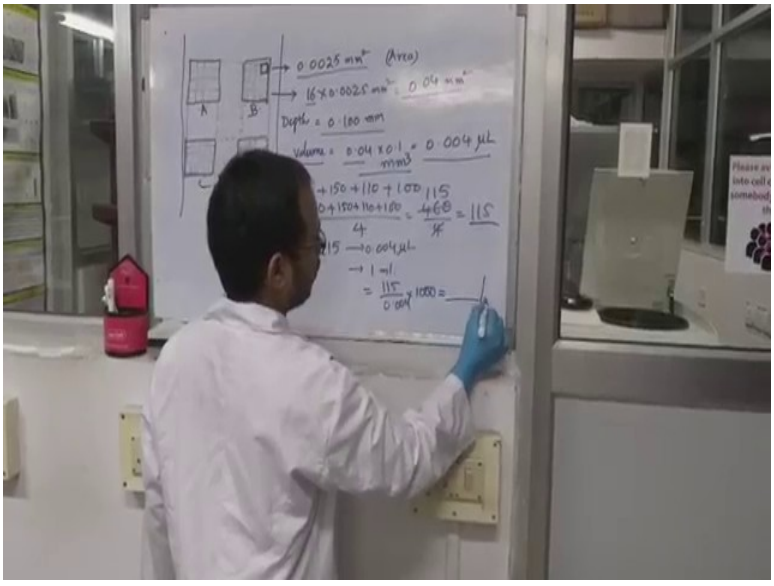

So we checked the how many cells are there in all the squares. Now we have to count, how to count the cells? So here a cubical neubauer chamber which contains squares, 5 squares so, we have to count cells in these squares. So each square is an area of 0.0025 millimeter square and total small squares 16. So total area of this whole squares is 0.04 millimeter-square. So the depth

of the, this each well is 0.1 millimeter, so what is the volume? 0.04 into 0.1 so that is total 0.004 millimeter cube or 0.004 micrometer.

So say, we have combined the cells in each well, say this is A, B, C, D here we have 100, here we have 150, here we have 110, here we have 100 again so the total cells we have to take average that means 100 plus 150 plus 110 plus 100 divided by 4, total 4 squares we are counting. The average is 115, so 115 cells in 0.004 microliter volume, so how many cells per 1 ml? So that we can calculate simply 0.004 into 1000, that will give the volume cells per ml.

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Chemical transfection method there are wide variety of (())(35:37) available like using cationic lipids or peptides or polymers. In this method we are going to show PEI based transfection, for that we have to mix DNA with the incomplete media first. After mixing we have add polyethylenemine directly to the doubling chamber, then we can see a visible white precipitate which means the DNA is complexed with the PEI and ready to. The ratio between DNA and the transfection reagent should be 1 is to 4. Let's start with it.

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We have already encoated incomplete medium, so for this we have to add plasmid DNA, this is the DNA already concentration has divided. The mixing should be proper otherwise there is no (())(37:13). You can tap the tube in order to get mixed.

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This is the PEI transfection reagent so this is 1 mg per ml concentration. We added 10 micro gram of DNA so we have to add 1 is to 4 that means 40 micro grams of PEI. So I am going to

add 40 micro liter of PEI to the… mix properly. Incubate the tube for at least 5 minutes to get the complex form.

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The 5 minutes are over then we can see the visible precipitate, white precipitate inside the tube. So I am going to add this complex directly to the cells here, the cells contains reduced serum medium, reduced serum containing medium so if serum, 10 percent serum if we add 10 percent serum then it may complex with the PEI DNA complex then it may not get internalized itself, so you should take care of that.

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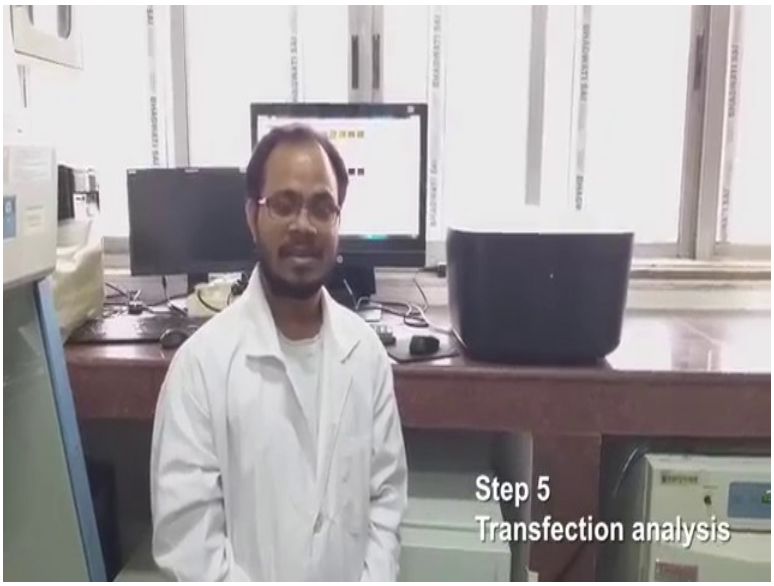

We have successfully transfected the cells, now we will try to analyze whether the transfection is successful or not.

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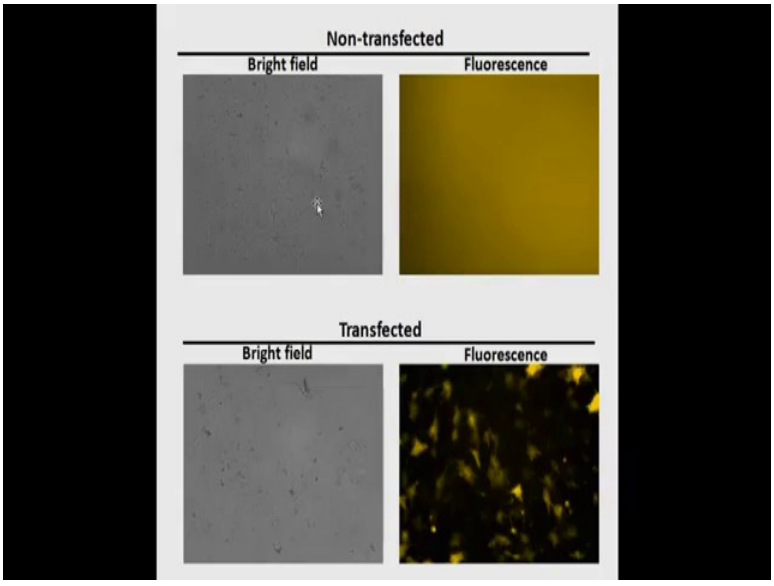

After transfection we have to keep the transfected plate into CO2 incubator for 48 hours. After 48 hours we have to observe for transfection under fluorescence microscope, these are the non-transfected cells and these are the transfected cells. As we can see clearly there is a fluorescence in transfected cells and high background in non-transfected cells. So this confirms the

transfection but further we have to analyze for the specific protein of interest using western blot. So far we have learned that how to transfect the cells, we have used polyethylenemine based transfection reagent which is basically a polymer, which conjugates with the DNA and precipitation will use for the transfection. The most of the transfection processed through endocytosis.

During transfection also we have to make sure the ratio between the transfection reagent and DNA should be optimized, the result what we have shown you is the optimized one. So you have, according to your requirement you have to optimize the transfection reagent versus DNA and also the plasmid DNA need to be contamination free otherwise you can see bacteria gone before the transfection happens. We can do the same transfection through electrophoretic method also but in that case we should not use any salts while preparing plasmid DNA, that should be taken care of otherwise there should be some conduction inside the tube so it will kill the all the cells, so these precautions need to be taken.

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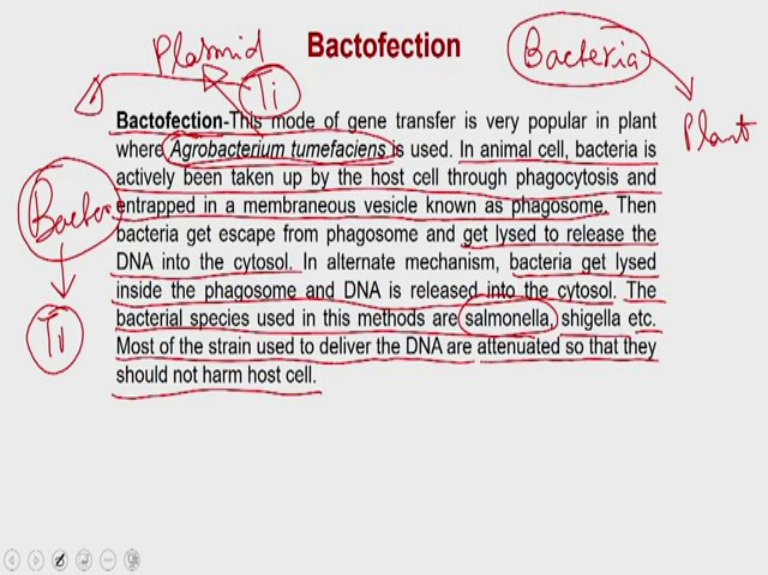

Now let us move on to the next methods and the next method is called as the Bactofection, bactofection as name suggest in this particular method you are using the bacteria so this bactofection method is more popular in the case of plant. So if you are trying to deliver a gene to the plant you can use the bacteria to facilitate that process. In that case (we) people are very

oftenly using a bacteria which is known as the Agrobacterium tumefaciens and agrobacterium tumefaciens mediated gene transfer is very-very popular and successful in the case of plant.

Whereas in the animal cell the bacteria is actively been taken up by the host cell through phagocytosis and then it is entrapped in a membraneous vesicle known as the phagosome. Then the bacteria get escaped from the phagosome and the, it get lysed so once the bacteria is getting lysed it is getting to be release the DNA into the cytosol and the bacteria get; whereas in some cases what happen is that the bacteria get lysed inside the phagosome and the DNA is released into the cytosol. So both are these mechanism could be possible that the bacteria get escape from the phagosome and then it get lysed one it reaches into the cytosol and that is how it is going to deliver the DNA into the cytosol and ultimately the particular DNA will reach to the nucleus to express the recombinant gene.

The bacterial species what you use for the mammalian cells are salmonella, shigella and some more other species. So most of the bacterial strain what you use to deliver the DNA are mostly being infectious bacteria, for example the salmonella or the shigella but when you use them for the mammalian expressions or when you use them to do transfect the mammalian cells what the first thing what you do is you attenuate them. So that they should not cause any kind of disease because if that will happen then (the) it will going to make the mammalian cells susceptible for that particular disease and then the mammalian cells may not be useful for downstream experiments or suppose you are asking some crucial basic questions and you would like to use the transfection of some of the genes inside the mammalian system then it also going to get the disease of salmonella or shigella then these particular then your purpose of doing an experiment may not be fulfilled. So that it should not harm the host cells.

Whereas in the case of agrobacterium tumefaciens the (this) the agrobacterium tumefaciens is actually having its own plasmid which is called as the TI plasmid and the people are using the TI plasmid to clone the recombinant DNA or recombinant gene which they would like to transfect into the plant so what they do is, they first they clone the gene into the TI plasmid and then they transform the agrobacterium tumefaciens with this recombinant TI plasmids. So as result what will happen is you are going to have the bacteria which contains the TI plasmid which is actually

not the natural TI plasmid but the recombinant TI plasmids containing your recombinant gene and then this bacteria is going to be used for infection to the plant.

So agrobacterium tumefaciens has the ability to infect the plant, once it infects the plant it causes the wound to the plant and in this process the bacteria is actively transferring its genetic material and once it is transferring its genetic material it also transfer the TI plasmid along with that and because of this natural transformation performed by the agrobacterium tumefaciens, the efficiency of the transformation or transfection by the agrobacterium tumefaciens is very high and you can use the agrobacterium mediated transfection to most of the plant parts which means you can use this to express the protein into the leaf you can use that for other plant parts as well. So that is why the agrobacterium tumefaciens is very-very popular for doing the transfection studies into the plant.

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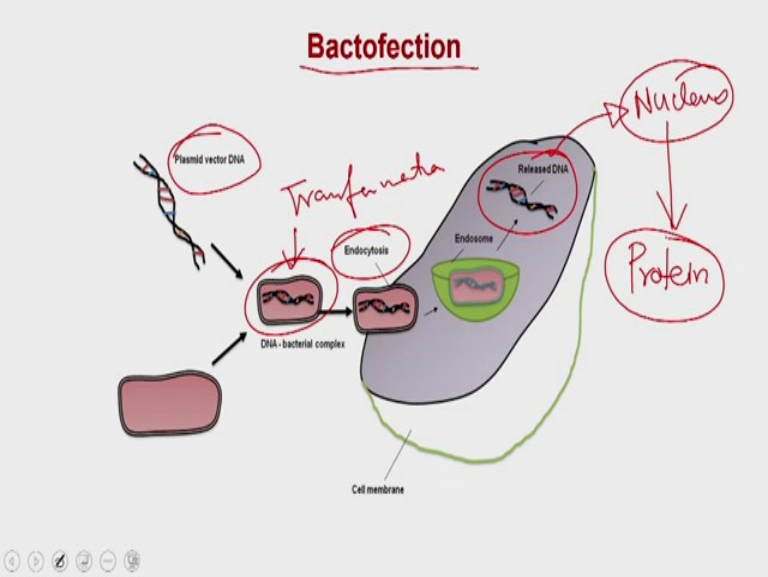

Now let us move on to the next method, so in the typical bactotransfection what you have to do is you take the plasmid DNA or the vector DNA you somehow then first, in the first step you insert that into the bacteria which is actually the transformation so with the help of transformation you first insert your recombinant DNA into the bacteria and once this bacteria will reach to the cell it will be taken up by the process known as the phagocytosis and once it will be going to be get into the inside the cell it will form the phagosome and then inside the phagosome the bacteria will either release the DNA or the bacteria will come out and get lysed

and release the DNA, either of these approach, either of these scenarios this release DNA then reach to the nucleus and it will going to express the protein or it is going to express the gene and that will produce the protein.

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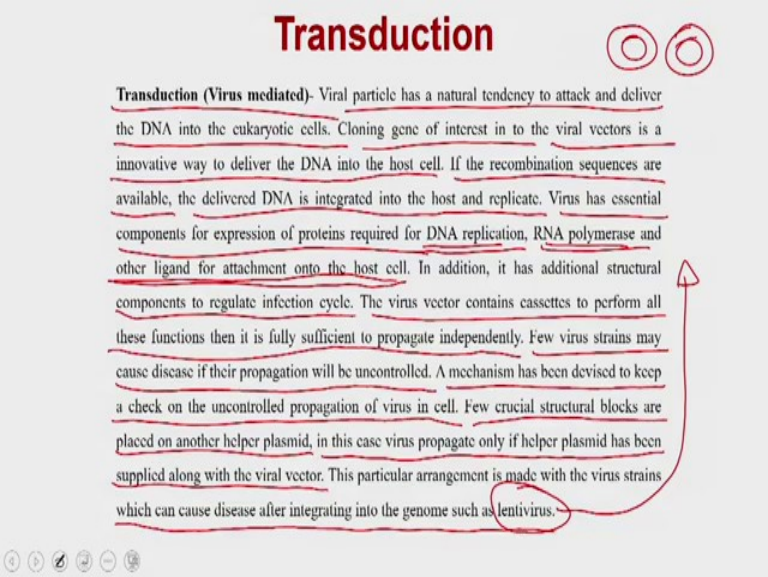

Now let us move on to the next approach, the next approach is called as the Transduction. So in the bacteriofection we have used the bacteria as a source to deliver the DNA whereas in the case of transduction you are going to use the virus as a vector to deliver the recombinant DNA. I think if you remember when we were discussing about the viral plasmids or the bateriopharge lambda based plasmids we have discussed that the those bacteriophage plasmids are going to be packed and formed the virus particle and then you can take these virus particle and add it to the mammalian cells.

These virus are going to insect the mammalian cells and in this process they will inject the, your recombinant DNA as well as their genome. So the virus particles have the natural tendency to attack and deliver the DNA into the eukaryotic cell. Cloning gene of interest into a viral vector is a innovative way to deliver the DNA into the host cells. If the gene or the adjoining recombination sequences are also been provided, then the delivered DNA is going to be integrate into the host and that is how it is going to replicate along with the mammalian cells.

The virus has the essential components for expression of a protein required for DNA replications, RNA polymerase and other ligands for attachment onto the cell. So in this process what you do is, when you preparing the virus particles you have to ensure that the vector what you are going to use should have the components for DNA replications, should have the component for RNA polymerase and it should also have the other protein which are essential for making the virus particles and so that it should continue its infections cycle.

The virus vectors contains cassettes to perform all this functions and then it is fully sufficient to propagate independently okay. Few virus strains may cause disease if their propagation will be uncontrolled. In this case a mechanism has been devised to keep a check on uncontrolled propagation of a virus in a cell. Few structural blocks are being placed on another helper plasmid, in this case the virus propagation only, virus propagates only if the helper plasmid has been supplied along with the viral vector.

What it mean is that, you have the multiple you what you do is you take the multiple plasmids and in some plasmid you keep the structural components, in some plasmid you keep the DNA polymerase, RNA polymerase and all that. So until all these (particles) all these plasmids are not available the virus will still be able to replicate but it will not be able to cause the active infection into the cell (may) active disease into the cell and because of that you can actually cut down the pathogenic site of the virus but you can still continue with the infection mode and that is how these viruses can keep delivering the recombinant DNA.

The virus which actually caused the disease after integrating into the genome is called as the lentivirus and that is why the lentiviruses are much more dangerous and much more risky to use compared to the adenovirus which you very often use for expressing the foreign gene into the mammalian cell or when you use the adenovirus for transfecting the mammalian cells but the lentivirus infectivity as well as the lentivirus propagation to cause a disease can be controlled simply by devising the strategies where you keep the different components on the different plasmids so that whenever you require to develop the disease then only you transfect the mammalian cells with the multiple viruses components and that is how when they will come together they will form the virus particle which will be infectious.

If you keep some of the components away it will still form the virus particles but those virus particles may not be infectious or may not be self-propagating. They may require that those additional components. So in those cases they will still able to serve the purpose of transfecting the other mammalian cells but they will not be able to spread the disease on their own. So with this I would like to conclude our lecture here, thank you.