Molecular Biology

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Module - 10

Molecular Cloning

Lecture-43 DNA Delivery (Part 1)

Hello everyone, this is Dr. Vishal Tewedi from Department of Biosciences and Biogeny IIT Guwahati. In the previous module, we have discussed about how you can be able to isolate a gene from the genome, whether you are aware of the genomic sequences or whether you are unaware of the genomic sequences, you can be able to use multiple approaches to isolate the gene of your interest, which is going to code for the enzyme of your interest. And then once you have got the fragment, we have also discussed in the previous lecture, how you can be able to clone that fragment into the vector of your choice. Now, once you have cloned the fragment of your choice into the vector, the vector has to be delivered into the suitable host. So, in the today's lecture, we are going to discuss about the various strategies what you can actually be able to use for delivering the DNA into the suitable host.

So, what you can see is this is a scheme for enzyme production, right. And in this scheme, we have already discussed many aspects of this scheme where we have said that the you are going to isolate the gene fragment from the from the from genome, right, either you are aware of the genomic sequences or you are unaware of the genomic sequences. This means either you will use the genomic library approach or the cDNA approach, or you are going to use the PCR. Once you have these genomic sequences, then you are going to perform the decision digestion of this particular sequence.

And that's how you're going to get the sticky ends. Same you are going to do for the vectors, right. In this case, we have taken an example of the plasmid, which is a bacterial vector. And then you're going to put the decision digestion and you're going to have the sticky ends of the plasmid, then you're going to put them for the ligation reaction and that's how you're going to get the recombinant clone. This recombinant clone is has to be delivered into the suitable host so that you can be able to you can be able to use that for protein production.

So, when we talk about the DNA delivery in host, we have to, you know, we have to understand the many aspects of this particular process. So, DNA delivery in host, so you can imagine that how the people have discovered or how the people have get to know about the DNA delivery in host. So, you can imagine that you have a donor cell from which you are actually going to have the, you know, the DNA molecules. And these DNA molecules are, you

know, that are negatively charged. So, these DNA, the charge on the DNA is negative charge on the DNA molecule interacts with the host cell, especially with the cell surface, which means if I change the cell surface chemistry of this donor cell in such a way that it is actually going to take up these DNA and they will go and you know, stick to this particular, you know, cell wall or the plasma membrane, they will be going to taken up by the cell.

So, the surface chemistry of the host cells, host cell surface chemistry either will attract or the ripple DNA as a result of the opposite or the similar charges, which means the cell surface of the host is either going to be positively charged or the negatively charged. If it is positively charged, it is actually going to attract the DNA, if it is negatively charged, it is going to ripple the DNA. So, presence of the cell wall in case of bacteria or fungus or the plant causes the additional physical barrier to the cellular uptake and the cellular entry. So, actually the entry of the DNA is not a facilitated process, it has, it has multiple barriers and multiple hurdles. And all these hurdles has to be overcome by preparing our competent cells.

So, what is mean by the competent cell is the cell which is actually going to be readily be taken up the, so which is readily going to take up the recombinant DNA. And you are supposed to prepare their competent cell, which means you are not, wild type cells are not competent enough, they are not going to take up the DNA. But you can actually be able to, you know, treat them in such a way that it is going to change the surface chemistry of the cell surface and that is how it is actually going to take up the cells. Naturally, the, this process is called as the transformation, which means the process in which the DNA fragment from the one cell is going to be taken up by the other cell. So, you can imagine that if the donor cell is resistance for the antibiotic, for example, you have taken an antibiotic and that is been, you know, some, some bacteria cells are actually acquired the resistance for that antibiotic cells by many means because of some presence of some DNA.

So, if you have a presence of DNA, which is responsible for providing the antibiotic resistance, this particular donor cell is actually going to be overcome or it is actually going to be overcome from the antibiotic mediated killing. So, in that case, what it will do is it will actually going to throw this particular DNA and this DNA is going to be very, very valuable for the other bacterial members. So, what they will do is they will going to accept by the other cells and as soon as the other cell is going to take up this DNA, it is actually going to also acquire this cell and this is actually the way in which the transformation is actually going to be you know, going to help the single bacteria to acquire the resistance and also to spread the resistance throughout the colony. How it happens? It happens that you are actually going to have the donor cell from the donor cell, the donor DNA or the DNA fragment, which is actually going to have the antibiotic resistance genes or any kind of phenotypic gene is actually going to put into the extracellular media and then this extracellular media is actually going to interact with the acceptor cells. So, you are going to have the competent recipient cells.

So, that donor DNA fragment will bind to the competent recipient cells and that is how it is actually going to be taken up. So, mechanism of transformation is the process by which the cell free DNA is taken up by the another bacteria. The DNA from the donor bacteria binds to the competent recipient cells and DNA enters into the cell. The DNA enters into the recipient cell through an uncharacterized mechanism. The DNA is integrated into the chromosomal DNA through homologous recombinations.

Naturally, transformation is common between the closely related species, which means there are you know, through natural transformation, we have taken an example where you can actually be able to use that for transferring the resistance from the resistance or the phenotype from the one cells, one bacterial population to another bacterial populations. But the in the lab, what you are going to do is you are going to add the extracellular DNA or you are going to add the recombinant clone recombinant DNA and in presence when you add this recombinant DNA to the competent recipient cells, the DNA is actually going to interact with the competent recipient cells and that is how it is actually going to taken up by the cell. And once it is going to taken up by the cell, it is actually going to go for cell division and that is how you are going to have the transform cells. So, where the copies all on the both side of the DNA. Some time you actually going to have the integration sites on these DNA.

So, they will integrate into the genome, otherwise they will remain as the extra chromosomal DNA in the form of the plasmids. Now, how you are going to prepare the competent cells for your laboratory experiments. So, you can use the depending on the bacterial species, you can be able to use the different types of chemical reagents which are actually going to change the surface chemistry and will make the cell competent so that they will take up the new cells, they will take up the the extra cellular DNA. So, for example, in the case of tetra focus new in new many, you are going to you are going to treat the cells with the mitomycin C or fluoroquinol. So, when you treat the cells with mitomycin C or fluoroquinol, they are going to be competent and they will actually going to take up the extra cellular DNA.

Similarly, you have the basillus subtilis, you are going to treat the cells with the UV light and that actually is going to induce or that is going to change the surface chemistry and that is how it is actually going to be competent cells. In the case of helicobacter pylori, the bacteria which is responsible for the ulcer or is going to be treat with the ciprofloxacin, ciprofloxacin is an antibiotic. So, the treatments are different because your surface chemistry is going to be different, the physiology of that particular bacteria is going to be different. So, that is why you are actually going to treat it with the different competent agents. Then we have the dibunopila pneumophila, then that is actually going to be make the competent by treating you with the mitomycin C, norfoxacin, ophloxacin and all these kind of antibiotics and hydroxyurea and as well as UV light.

For example, E. coli which is called as laboratory strain. So, this is the lab strain and that

can be competent by treating with the covalent, you know, the covalent, covalent, you know, chemicals like calcium chloride and rubidium chloride and the cells are how you are going to make the competent cells. So, you are going to treat the cells with the different agents, so all of these agents. So, what we are going, so just for sake of how we can be able to make the competent cells, I am giving you an example of the E.

coli cells. So, we in the E. coli cells, we are going to take an example of how you can be able to use the calcium chloride to prepare the E. coli competent cells. So, preparation of the competent cells, so bacteria is incubated with the divalent cation like the calcium chloride, manganese chloride or the rubidium chloride for 30 minutes at 4 degrees Celsius. And what, so bacteria you are going to, first you are going to grow the bacteria and then your bacteria is going to incubate it with a divalent cation such as calcium chloride, manganese chloride or rubidium chloride for 30 minutes at 4 degrees Celsius.

What will happen in this period? During this process, the cell wall of the treated bacteria is going to swell and it gathers the factor required for the intake of the DNA docked onto the plasma membrane. So, you can imagine that when you are doing all this preparation, it is actually going to cell swell, it is actually going to take up the calcium chloride or the divalent cations. And these divalent cations, what they are going to do is they are actually going to take, they are going to make the cells a little fragile. So, in that case, you might have to take care of these cells very nicely because if you, they also get susceptible for any kind of shear stress. So, shear stress is a, it means that you are actually going to, suppose when you are swimming into the water, right, the water, whatever the stress you will feel when the water is actually hitting you, that is called as a shear stress.

Shear stress means you have two layers and these two layers are actually rubbing to each other and that is actually being responsible for causing a friction and that is actually called as shear stress. So, they are very susceptible for shear stress. For example, if you spin them a very high speed, you probably will actually go into live cells. So, that is why these computational cells are very fragile, you cannot run that very high speeds, okay, and you also cannot do the pipetting at a very high pipetting. Like for example, if you do a pipetting a lot of, you know, with the thin bore tips, it is actually going to destroy the cell because it is going to live the cells.

How you are going to store the competent cells? You can, once your competent cell preparation is over, then you can actually be able to add 15 to 20% glycerol and you can store it at minus 80 degrees Celsius and whenever you require, so 15 to 20% glycerol is actually going to work as anti-freezing agent, so it is not going to allow the formation of the water crystal and that is how it is actually going to protect the bacteria from getting any kind of damages. So, you have to be very careful that the cells are very fragile, they are sensitive for the centrifugations and they are also sensitive for the pipetting. That is why you can, once you prepare the competent cells, you aliquot them in into a suitable volume and then you can just use that same, that volume completely. You cannot just pipette it out

and prepare the more aliquots. How you are going to prepare the competent cells? So, what you can do is, first you are going to do, grow the bacteria, okay.

So, if you see the bacterial growth curve, what you are going to see is, it has the lag phase, it is going to have lock phase, it has the stationary phase and it has the death phase or the decline phase. So, the cells which are in the lock phase, stationary phase or the cells which are in the decline phase are actually under the extreme stress, which means these three phase of the cell, right, whether you are under the lock phase or the stationary phase or the death phase are not suitable for preparing the competent cells. So, you have to grow the bacteria in such a way that it should be in a lock phase, in a logarithmic phase, so that they are very healthy, they are actually acquiring the different types of factors and there is a no dearth of nutrition. So, there is no loss of nutrition. So, they are actually going to have a lot of nutrition and they are actually having the ability to produce a lot of factors.

So, that is actually going to be the best bacterial culture what you are going to use. So, the growth stage of the bacteria has a significant impact for its ability to take up the foreign DNA. The bacteria at lock phase is more active and efficient to perform the DNA damage and repair than the stationary phase. As a result, it is preferred to use a bacteria of lock phase for making the competent cells for the transformation. So, once you grow the bacteria, bring it to the lock phase and then you, you know, collect the lock phase bacteria and then you are going to prepare that using, use that for the competent cell preparation.

So, how you are going to do the transformation? So, on the day of transformation, competent cells are incubated with the DNA or the circular plasmid containing appropriate resistance gene, such as MPC in resistant genes for the 30 minutes on ice. So, first you know, what you are going to do is, this is the step one number one. So, in the step one, what you are going to do is you are going to thaw the competent cells, right? Because remember that the competent cells are going to be stored at minus 80 degrees Celsius in the 15 to 20% glycerol, right? So, you are going to first what, what you are going to do is you are going to thaw the cells and you are going to use that for transformations. Then in the step one, you are going to incubate the DNA, either the linear DNA or the circular plasmid within the ice, right? Along with that for 30 minutes. So, and then you are going to add the pestle in the resistance, okay? And then once this step is over, so you are going to have the 30 minutes incubation and once the 30 minutes incubation is over, then you are going to give the heat shock.

So, in the step two, you are going to do a heat shock. So, competent cells are given a brief heat shock such as 42 degrees Celsius for 90 seconds to relax the cell wall and high temperatures thus causes upregulation of the factor responsible for DNA recombination and repair. So, then what you are going to do is you are going to give the heat shock. So, in the heat shock step, what will happen is that because you are giving the heat shock, the cell is actually going to swell and it is actually going to take up the DNA into that. And because you are providing the heat shock, it also going to induce the production of the cellular

machinery which is a sample for DNA recombination and repair because that is very, very important event.

Because if the even if it has taken up the DNA and it cannot actually be able to recover from the damages, then the cell is actually going to die. Then in the next step, what you are going to do is you are going to add once the heat shock is over, then you are going to add a chilled media and that is actually going to you know that is going to bring the rigidity of the cell wall or the plasma membrane. And that is how it is actually going to seal the pores, what is going to be prepared into the plasma membrane and that is how it is actually going to help in terms of the faster recovery of the transform cells. So, once you add the chilled media, you can actually allow them to recover for 30 to 45 minutes at 37 degrees Celsius. And after this recovery, you are actually going to plate the cells onto the agarose plates.

So, after this, it is plated onto the solid media with the appropriate antibiotics such as empicillin and allowed to grow for another 18 to 24 degrees Celsius at 37 degrees Celsius incubator. So, when you are done with the chilled media, you added the chilled media, you let them to remain into the 37 for some time and then you are actually going to plate them onto the agarose Lb empicillin plate. So, for example, in this case, we have taken the empicillin resistance gene. So, when you do the empicillin plating, which is actually and then you incubate into the incubator for 18 to 24 hours, then it is actually going to form the colonies. And now these colonies are the transform colonies which are actually going to give you the which has taken up the DNA.

Because the untransformed colonies will actually not going to have the empicillin resistance and that is what they will actually going to die in this Lb empicillin plate. So, transform cells with appropriate resistance will grow and it will give you the colonies. So, once you are done with the transformations, how the how the bacterial plates will look like. So, this is actually the untransformed or the control plate.

And this is your transform plates. So, what you see here is, we have transformed a plasmid into the Lb empicillin. And if you count the number of colonies, it is 80 to 90 colonies what we got into this particular plate, whereas there is no colony in the control plate, which means the plate where we have not done any plating, we only contains the cells it does not contain that. So, this is without DNA and this actually has plus DNA. Now, how using this in use this number, how many colonies you got, you can be able to calculate the transformation efficiency. What is mean by transformation efficiency? The number of colony forming unit obtained by transforming 1 microgram of DNA into a given volume of competent cells.

So, number of colonies what you got from the 1 microgram of DNA is called as the transformation efficiency and it is a very, very important parameter to judge how good your competent cells are because if they are very bad, you cannot use them for the cloning reactions. For example, if you transformed the 1 microliter of 0.09 microgram per

microliter plasmid into 100 microliter of competent cells, you added 900 microliter of LB to your cell to get a total reaction volume of 1000 microliter and then plated 100 microliter of the transformation. The plate has 450 colony on its next day, which means you have actually added this much amount of DNA and this much amount was the reaction volume. So, what you are going to do is, you are first going to calculate the amount of DNA what is you have used.

So, if you calculate this, what you are going to know is that you have plated 0.001 nanogram of DNA. Now, if you want to calculate the efficiency 450 colonies from 0.005 nanograms into 1000 because this is what the dilution you have done and that will actually going to tell you that you have a transformation efficiency that is 4.

5 into 10 to the power 8. So, that is very good actually. So, 10 to the power 8 is a very good efficiency. Sometime you may get even more 10 to the power 11 and so on. So, that is going to be very, very good actually. So, any number which is above 10 to the power 4 or 5 is reasonably okay.

Apart from the transformation by the chemical method, you can also use you can also do the electroporation. So, electroporation is a method where you are going to use the electrical pulses into the cell. So, what will happen is that when you put the electrical pulses, you are actually going to make the hole into the plasma membrane and utilize the and since the DNA is already out, it is actually going to enter into the cell and that is all and after that you are going to put the chilled media and it is actually going to resell this pore and that is how it is actually going to be taken up the DNA. So, the plasma membrane is composed of the lipid and protein. These macromolecules give a partial conductance to the cell membrane.

So, when a high electrical pulse is given to the cell, the charge runs across the membrane and partially disturb the arrangement of the lipid molecule. As a result, it makes the formation of pore and allow easy passage of the macromolecule, especially the charged molecule like DNA into the cell. After the electroporation, the cell is allowed to recover from the damage and it forms a colony on the selective solid media. So, you can see that this is a plasma membrane.

So, plasma membrane is made up of the lipids. So, lipids are arranged like this and lipids are partially being charged. So, when the plasma when there is a very high pulse which goes on top of this, these lipid membranes are lipid molecules are getting rearranged and because of that, it actually allows the passage of the DNA into the cell. And this DNA is so this is actually very momentarily. So, once you add the media, this pulse is going to be over and that is how it is actually going to seal this particular thing. And that is how the DNA is going to be delivered into the host.

And then later on, you can actually allow the cells to recover and that is how you are the

transformation done. The advantage of electroporation is that it does not depend on the surface chemistry of the cell. It depends on the so that is why it is very robust compared to the chemical transformations. The disadvantage is that the electroporation is required a specialized instrument like the electroporator and it also requires the electrocubates. So, what are the factors affecting the transformation efficiency? The plasmid size, then the form of DNA, you can you know that when we were discussing about the plasmids, there are three different forms either the triple C forms, OC forms and super coiled form.

So, the transformation efficiency depends on the surface area of the molecule. So, surface area of the molecule the super coiled is smaller or smallest, whereas the other two forms are actually having the larger surface area. So, that is why if you use the super coiled DNA, the transformation is actually going to be very high compared to the closed circular DNA or the open circular DNA. Similarly, the plasmid size if you are working till 10 kB, the transformation efficiency is going to be very high. But if you go beyond that, if you go above to the 10 kB fragments, the transformation efficiency is going to be very, very low.

And in those cases, you might have to use the electroporation or the other methods, you cannot rely on the chemical methods. Then the genotype of the cell, cloning strains for example, E.coli K12 strain have 4 to 5 times the transformation efficiency of the similar strain. For linear DNA, which is poorly transformed in E.coli, the RecB or RecD mutation can significantly improve the efficiency of its transformation.

Then the third is growth of the cell. So, log phase cells are best for preparing the competent cells. Then the method of transformation. So, we already have discussed that the chemical methods are actually going to give you the less transformation efficiency compared to the electroporations. And then we have to also have the damage. So, exposure the DNA to UV radiation is actually going to be another factor which is all to affect the transformation efficiency.

So, this is all about the transformation of the E.coli cells. And I am sure you could have got the better practical experience of how to perform the competent cells and how to you know, perform the transformations. Now, let us move on to the next step. And the next step is transformation in the yeast. So, yeast is also another host which you can use for the overexpression purpose.

And there are many methods which are available in the yeast. The worst method is lead acetate single standard DNA PEG method. So, in this method the yeast cells are incubated with a transformation mixture of the lithium acetate PEG 3500, PEG-1, polyethylene glycol PEG. So, PEG is polyethylene glycol, polyethylene glycol. Single standard carrier DNA and the foreign DNA or the foreign plasmid that is the recombinant DNA at 42 degrees Celsius for 40 minutes. The purpose of adding the carrier DNA is to block the non-specific site on the cell wall and made the plasmid available for the uptake.

Post transformation the cells are pelleted to remove the transformation mixture and re-suspended in 1 ml of water. It is plated onto a solid media with the appropriate selection pressure such as antibiotics. The second method is called as the isperioplast transformation method. An isperioplast transformation method has multiple steps. So, in this method the yeast cell wall is removed partially to produce the isperioplast and that you are going to do with the help of enzyme which is called as zymolase.

So, isperioplasts are very fragile for osmotic shock but are competent to take up the free DNA at a very high rate. In addition, the polyethylene glycol PEG is used to facilitate the deposition of the plasmid and the carrier DNA on the cell wall for the easier uptake. So, how you are going to perform the transformation of the yeast with the help of the isperioplast transformation method? So, in the step 1, in the isperioplast method the yeast cells are incubated with the zymolase the enzyme. So, first you are going to grow the cells at log phase and then the step 2 you are going to incubate the cells with the enzyme which is called as zymolase. So, what the zymolase is going to do is it is actually going to chew up the cell wall at a very discrete step.

So, if you are not going to do a complete hydrolysis or curve complete removal of the cell wall you are going to make the partial removal of the cell wall to produce the isperioplast. So, the species or the cell what is going to be generated once you have removed the partial cell wall then it is called as isperioplast. Now, what you are going to do is you are going the step step these isperioplast are going to be collected by the certification and incubated with the carrier DNA and the plasmid DNA for 10 minutes at room temperature. So, take the isperioplast you collect the isperioplast by centrifugation and then you are incubating the isperioplast with the PEG carrier DNA and as well as the recombinant plasmids. Then in the step 3 it is now treated with the PEG and the calcium for 10 minutes with the gentle shaking.

So, you are going to you know keep the cells like the isperioplast the carrier DNA and plasmid and then you are slowly slowly slowly you are going to add the PEG and as well as the calcium. The transformed isperioplast are plated onto a selective solid media and incubated onto the 30 degree Celsius for 10 4 days because it takes that much amount of time for getting the transformations. So, once you have got this you know transformed isperioplast you are going to plate them onto a selective media and then you incubate that into a 30 degree Celsius incubator remember that the yeast is growing optimally in 30 degree compared to the 37 degree Celsius and then you incubate that 4 days after 4 days you are going to get the plate with colonies and all these colonies are a distance for that particular antibiotic. So, this is all about these the methods the transformation methods what you can use to deliver the DNA into the bacteria or the yeast. In this particular lecture what we have discussed we have discussed about how you can be able to make the use make how you can be able to use the different chemical agents to prepare the you know chemically competent cells and how you can be able to use them for the transforming the with the transformations.

Apart from that we have also discussed about the electroporations and so with this I would like to conclude our lecture here in our subsequent lecture we are going to discuss about the DNA delivery into the mammalian cells. Thank you. Thank you.