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**Lecture - 46**
**DNA Transformation**

So, welcome back. Now what we are going to do is, so if you remember we have initially we have amplified a particular gene of interest from our mouse spleen c DNA and after the PCR amplification, we digested it and along with that we digested the vector in which we want to ligate them following which we ligated our gene of interest into the digested vector and till that step you are already familiar.

Now what we are going to do is, we have a ligated product and the overarching goal of all these experiments is to finally get a construct which contains only the gene of our interest and from which we can isolate DNA and use that DNA for certain experiments and also later on from that DNA we can get the protein which that DNA codes for and use that protein purify that protein and use it for certain other experiments.

In order to achieve that the next step that we must do is, we need to transform this DNA into chemically competent bacterial cells. So, what do I mean by chemically competent bacterial cells? So, the goal of our experiment, the next goal of our experiment is to in simple terms insert our ligation product into the bacteria and how do we do this.

So, normally bacteria will not take up DNA foreign, DNA foreign plasmids or even if they do that, the efficiency is extremely low less than 1 percent, but we cannot do our experiments with such odds. So, we need to turn the odds in our favour and in order to do that what we do is, we make our bacterial cells chemically competent to take up foreign DNA.

So, I will give you a brief explanation not too detailed, a brief explanation of how we make chemically competent cells. So, the exact mechanism is not known, but we all know the DNA is negatively charged and the basic idea is if you can grow your is your if you have your bacterial cells and you incubate them in ice cold conditions with positively charged metal ions such as calcium or magnesium or rubidium, then the bacterial cell wall gets coated with these positively charged ions. And one you have these

competent cells and you add your DNA. DNA being negatively charged now gets attracted to the positively charged divalent cations which are present on the bacterial cell wall.

Keep in mind that I am giving a very simple simplified explanation of how to make competent cells. You will learn these in detail elsewhere. So, we have our chemically treated bacterial cells which are treated with normally what we use is calcium chloride and these treated bacterial cells to this we add our DNA, our ligation product, we add say 1 micro litre of our ligation product and since DNA is negatively charged, it will get coated on the bacterial cell surface.

Since that surface is coated with divalent cations positively charged divalent ions. Once we have done that we keep that mixture in ice. One point that you need to always remember is when you are working with competent cells, you need to keep them cold always. The reason being that the mechanism by which DNA is taken up involves a process called heat shock.

So, only at that stage will the bacterial cells be exposed to heat. Before that they are always to be kept when under storage conditions they have to be kept in minus 80 degree centigrade and when you are working with them when you are taking them out from the minus 80 degree centigrade fridge, you immediately keep them in ice. When you are adding DNA to the bacterial competent cells, you do that while keeping the tube in ice.

When you let them incubate for half an hour or 1 hour, you maintain that condition in ice in the fridge and only when you are ready to proceed further when you are ready to actually transform your DNA that is insert your DNA into the bacterial cells, only then what we do is? We take the bacterial cells, the vial in which they are kept along with the DNA and we put them in a 42 centigrade water bath for 45 seconds.

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What happens is because of the sudden heat shock, if you remember they were stored for their entire life time in minus 80 degree centigrade. Once we began the experiment in the initial phase, they were kept at 4 degree centigrade. After DNA was added, they were still incubated at on ice. They were kept in ice not at 4 degree centigrade, they were always kept in ice and only during process of transformation they are suddenly kept in 42 degree centigrade for a very short duration of 45 seconds.

Because of this heat shock what happens is the cell wall of the bacteria cracks open. There are certain fitches and cracks and the DNA that is coated on the surface of the bacteria gets internalized. Immediately after the heat shock we put them in ice again we put the vial containing the DNA and bacterial cells competent cells in ice we keep them around for 5 minutes. You can keep them for 2 to 10 minutes. I usually prefer to keep them on ice for 5 minutes to sort of help the bacterial cells recover. And after that what we do is? We add some nutritive media. So, I will use LB media, we add some LB media and allow these cells to grow for one and half hours at 37 degree centigrade.

So, the cells will proliferate and keep in mind that our ligation product is present in a vector in a plasmid which has antibiotic resistance marker, but immediately after heat shock we keep them in ice and when we add LB, we do not add any antibiotic because till now they are not expressing the gene product to work against the antibiotics. So, we just add simple media and allow the bacteria to proliferate for one and half hours. During

this time they will start expressing the antibiotic resistant gene and after one and half hours what we will do is? We will take LB agar plates containing the appropriate antibiotic and on to that we will take say 20 micro litres of that LB broth.

And we will plate them on to the plate keep them in 37 degree centigrade incubator overnight and it is expected that the next day you will get colonies and those colonies will contain bacterial cells which contain the plasmid which you have prepared.

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So, the instrument that I have here behind me is a minus 80 degree centigrade freezer as you can see it is now maintained at minus 81 degree centigrade. So, this is a very important instrument in any molecular biology or experimental bio chemistry laboratory because many of our bacterial cells or constructs are stored here. So, what I am going to do is, I will just open the fridge and I will show you how it is arranged and how all the metal racks are kept and how we all have different boxes containing out different bacterial plasmid constructs.

But the whole objective for our next step is that I will take out a vial of chemically competent DH5 alpha bacterial cells and then I will keep them in ice. So, the last process or the actual process required for the experiment I have to do it very fast because I ideally should not take out the whole box and ideally once I take out the vial from minus 80, I cannot roam around holding it in my hand. I have to immediately put in ice. So, during that portion I will not be able to talk much, but before that I am giving you an

overview. So, this is our one of our minus 80 degree centigrade freezers in our laboratory and so, I will just open it once and see it is covered in ice right.

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And we have different see you see we have DH5 alpha boxes here, we have different plates containing different types of cells cell stocks and all these are metals. So, be very careful I am wearing normal gloves, but ideally you should wear cryoprotective gloves while working with minus 80 degree centigrade, but if you are experienced like me and if you are a quick worker, normal gloves are fine.

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So, what I am going to do is? I will just open my cell stock notice that I am not taking out the box I will just look for my cells and I have here an ice bucket. So, what I am going to do is, I am just going to take a vial immediately put in ice and I will close the minus 80 degree centigrade freezer.

So, keep in mind that for normal conditions you should not keep the minus 80 degree centigrade for so long. I just kept it open, so that you could get a view, but normally this is a very important instrument which contains a lot of very priceless reagents which are not to be handled frivolously.

So, normally when you are working in the lab you immediately open the minus 80 degree centigrade, take out what you want and you proceed. You do not keep the door open. Today just for an educational purpose I kept it open for some time. So, the bottom line is when you are working with minus 80 degree centigrade, you have to be fast and if possible wear cryoprotectant gloves because it is actually very cold. The metal surfaces do not touch them for too long or you might get some cold burn; that is it.

So, what I have here in my ice bucket is a vial containing chemical competent DH5 alpha cells. So, it is labelled as that DH5 alpha CC is for competent. We made this couple of weeks ago and if you prepared them correctly, they can be fine for several months. And also in this vial what I have is our ligation product, what we have already done in our previous videos we prepared a ligation product. So, this is that and what I am going to do in our next step is take a small volume maybe 1 micro litre of DNA from the ligation product vial and add it to the competent DH5 alpha cells.

So, this vial I am not taking out of ice because that will defeat the whole purpose of the experiment. So, this vial contains 50 micro litres of competent DH5 alpha cells. So, what we are going to do next is add the DNA to the competent cell which you will be seeing subsequently. So, we have taken out our chemically competent DH5 alpha e coli cells and we have kept them in ice and along with that we also have our ligation product also in ice.

So, what I am going to do now is, I will take 1 micro litres of our ligation product and add it to our competent DH5 alpha cells. This vial contains 50 micro litres of competent cells. So, it depends on how you have prepared how many aliquots you have prepared? Couple of weeks earlier when I prepared these competent cells, I had made maybe a 100

aliquots of 50 micro litres each. You can make smaller aliquots and increase the number of vials you have or vice versa.

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So, I am now going to set my pipette at 1 micro litre because that is the amount of DNA that I am going to be adding to competent cells. So, you can add slightly more DNA as well you can add may be 2 micro litres or 3 micro litres if required, but one should be fine. We tested our competent cells earlier with different constructs, so, they are very nicely competents. So, even 1 micro litre of DNA is sufficient for all purposes. So, the DNA I can take out; I can take out and it was stored in minus 20 earlier, but it has already thought. So, what I am going to do is, I am going to take 1 micro litres of the digation product and sincere working with small volumes always check visually whether you have actually taken out 1 micro litre or whether you are just taking up air, but now that is done I am not going to take out the vial of competent cells. I will keep it embedded in ice because I do not want it to warm up.

So, if you are a very fast worker, you could just take out the vial, immediately add the DNA, mix it and put it back. Some people do it that way, even I do it that way, but now for this demonstration purpose what I am going to do is I will not really take it out too much, I will just wait just a second. So, I will just open this vial and keeping it embedded in ice I will just add the DNA and I will thoroughly mix it, give to it a gentle swirl, close it and leave it in ice make sure it is cold.

So, now what is happening is, I will just cover this. So, what is happening is that the DNA is being incubated with the bacterial cells under cold conditions and we will keep it in this manner for at least 1 hour. If you are in a hurry you might reduce it by maybe 10 or 15 minutes at max, but my advice is keep it for at least 1 hour or if you are in too much of a hurry maybe 45 or 50 minutes. Do not reduce the incubation time below that because that will reduce the efficiency of your transformation and ultimately you will get lesser number of colonies or may be no colonies at all the next day. So, what we are going to do is since we have time, today we will leave it in ice for 1 hour. Let me just release the pipette.

Another point is you have to keep an eye on the ice, if you leave it like this. So, since ours is a tropical country it gets very hot during summers if I just leave it like this after one hour what I will see is that maybe the ice has melted and the tubes are floating around, that is bad. That will defeat the entire purpose of the experiment. So, always keep an eye and make sure that you keep refilling the ices or packing it tightly, so that most of it does not melt or what you can do is, you can take the centre ice bucket and keep it in the 4th degree fridge. So, these modifications are applicable only when the ice can melt quickly like in our case during summer.

It gets very hot, but if you live in a if your lab is in a very cold place, all the ACs are always on and you do not have any issue, then you do not need to carry out this modification. What I am going to do is? I will just make sure that the DNA and bacterial cells are incubated for an hour after which we are going to proceed to our next step.

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So, we have been incubating our ligation product with the chemically competent DH5 alpha cells for an hour now. Next what we are going to do is, we are going to take out our vial containing the DNA mixed with the competent cells and what I am going to do is I will go I am going to move on to the step of heat shock.

So, this instrument that we have here is a water bath which is being maintained at 42 degree centigrade. What I do is? I will take the vial, keep it here for 45 seconds and once it is done, I will again take it out and put it back in ice to let the cells recover for 2 to 5 minutes. So, before that I am going to I have a timer with me I am going to set up the timer to 45 seconds because, transformation you need to be sort of careful of the numbers. If you have the temperature at 42 degree centigrade, you give a heat shock for 45 seconds. Now suppose instead of 42 degree centigrade, you have a bath which is at maybe 40 degrees, does that mean heat shock will not work or you cannot carry out heat shock?

What you do is? You increase the time duration for which you are keeping yourselves under heated condition. So, suppose instead of 42 degree it is at 40 degree, then instead of 45 seconds you can keep it for maybe 65 seconds. However keep in mind that do not go below temperatures of 38 and also more importantly do not go beyond temperatures of 42 degree centigrade because then the transformation efficiency will be really low and if it is too hot, you might actually harm the bacterial cells. So, my take on this point is 42

degree centigrade heat shock for 45 seconds. If it is at 40 degree, then just go a bit longer, increase it by 15 seconds and at 42 at 40 degree give a heat shock for maybe 1 minute.

Do not go below 40 or 39 degree centigrade and do not go beyond 42 degree centigrade. So, my timer is set up. I will just remove the lid for the water bath and the valve which contain the mixture of DNA and competent cells I will just leave it in water and I started my timer. So, once 45 seconds is over since this is at 42 degree I will just take it out and put it back in ice to let the bacterial cells recover. So, to recapitulate what is happening during the heat shock step is that because throughout its entire lifetime, it has been either at minus 80 degree centigrade or at present at 0 degree.

It has actually never encountered heat. So, because of this sudden change in temperature, it undergoes a heat shock which results in the bacterial cell wall slightly cracking open and it is through these cracks that the plasmid DNA which we have incubated with the cells gets internalized. So, my 45 seconds is over. What I will do is I will put it back in ice and we will leave it in ice and allow the bacterial cells to recover from the heat shock for 2 to 5 minutes.

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So, the instrument that I have behind me is a laminar air flow cabinet and what we are going to use it for is when we work with bacterial cultures and we need to maintain a high amount of stability, this instrument is necessary. So, what it does is it maintains a

positive pressure air flow which prevents air from the outside into entering this region. Once I remove this lid and switch it on there will be a positive pressure air flow which will be blowing preventing the normal air from entering and apart from that right now we have a UV lamp which is switched on. You will see that there is some bluish reflections from a metal surface. So, basically we have an ultraviolet lamp on top.

So, what it is doing is? It is ensuring that the inside is sterile. Once it finishes its cycle, only then can we switch on the laminar air flow and take out the lid and start our work.

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So, we have switched on the laminar air flow and now we will begin our work ah. Remember during normal experimental procedure you should not talk too much when working in the laminar air flow because you do not want you to forcefully spit into this and contaminate the entire place, however for this demonstration I have to talk. So, I am talking, but do not learn this portion. What you should notice is when I begin this I take some ethanol, spray it all over the surface and give a nice wipe down to further sterilize the surface.

I know that there is a UV lamp and it is going to keep the internal area sterile, but even beyond that it is a good practice to always wipe down the laminar air flow hood with 70 percent ethanol before you begin your work and after your work you should again wipe it, so that the next person gets a very sterile place to work on.

So, now that I wiped it up, I am going to take our stuff that we need for the experiment switch on the like not switch on, just turn on the burner just to create the sterile zone here, excuse me. So, what I am going to do is I have 50 micro litres of bacterial competent cell to which DNA has been added and it has undergone heat shock and it is now being incubated in ice for roughly 5 minutes; 5 minutes is not over. It is going to be over soon I can see it in my timer.

So, next what I am going to do is I am going to add 450 micro litres of LB to those bacterial cells. So, first so this is autoclaved LB it is sterile right. So, I am going to just get things ready. Make sure that your LB stock does not get contaminated. In case it gets contaminated overnight, you will see that it has turned cloudy and then you have to discard you media. So, yes 5 minutes is over and I will just stop the timer and now what I will do is add take 450 micro litres of this media. So, now you can take out your bacterial cells because so I added 450 micro litres to the bacterial cells and I am mixing them thoroughly.

And next what we are going to do is we are going to let them grow at 37 degrees for one and half hours. So, now, it is no longer mandatory to keep it in ice, you can keep it in a normal centrifuge holder as well let me just take care of my media, so that we can use it again in the evening and yes I will also release my pipette.

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So, now you can actually take a couple of minutes to make sure that your pipette is released and you have turned off the spirit lamp and you have taken care of your media, but during the earlier step of heat shock timing was more crucial, but now it is ok.

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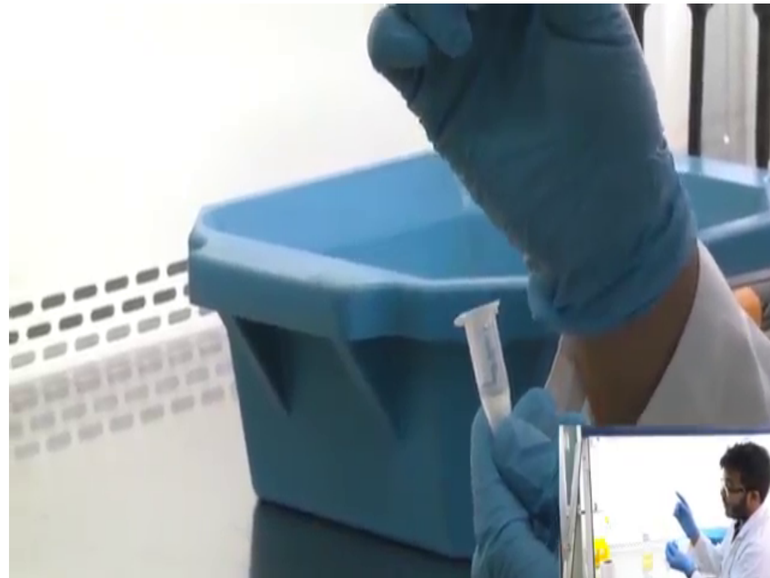

So, if you see it is slightly turbid because there is already 50 micro litre of bacterial cells to which we added media and what you will see is after one and half hours of growth at 37 degree centigrade much of the bacteria, there is much more population of bacteria.

So, there is some growth and proliferation. I will again repeat that although the construct that we have has some antibiotic resistance, but now we added plain media we did not add any antibiotic to this because till now the bacteria has not had time to after internalising the plasmid. It has not had time to start expressing the antibiotic resistant gene products. So, if we add the antibiotic right now the bacteria will die.

It is only after growing it in LB for one and half hours at 37 degree centigrade after that when we plate on an antibiotic LB agar plate, only then we will be able to survive in that antibiotic condition in case it has internalized our plasmids. So, that is the way in which we know which of the bacterial cells have been successfully transformed and which have not.

So, those bacteria which have taken up the plasmid will now be resistant to the antibiotic in the plate while those which did not will die on the plate and will die on the plate and

will not grow overnight in the plate; so, that is that. So, now what I am going to do is, we are going to put this in the shaker incubator at 37 degree centigrade and let it grow for one and half hours.

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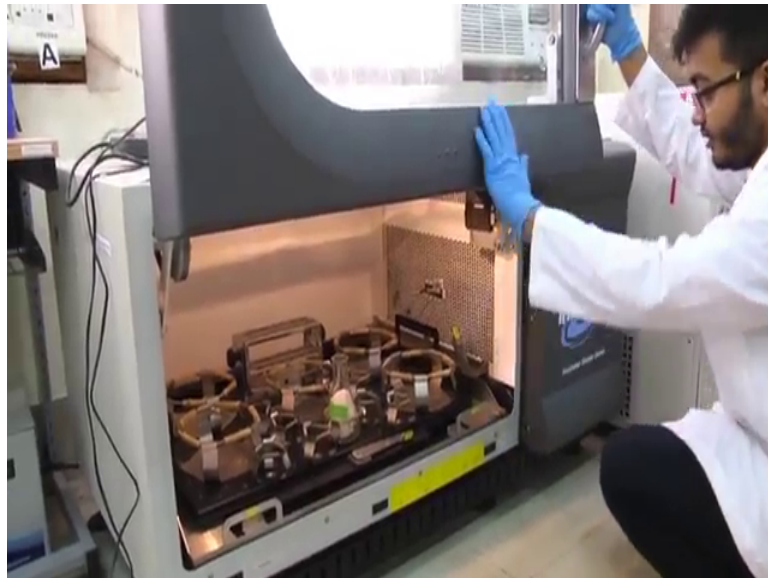

So, this is the vial to which we added the LB without antibiotic and I am just putting it here and now what I am going to do is its. So, this is our 37 degree shaker incubator it is going to maintain the temperature at 37 degree centigrade and once we put it in here.

So, at present it is 36 because we opened it and it was just switched on so, but it is said at 37 and it is going to reach that in a couple of minutes and also the RPM that we have set we set it at around 200 RPM. So, that is the condition under which this is going to be kept for the next one and half hours to allow it to grow. Because of the tissue you probably cannot see the vial, but the vial is inside and it is under constant shaking condition.

I order to ensure I will switch on the light in order to ensure that it is properly aerated and the bacteria can grow in a optimum temperature condition after one and half hours what we are going to do is, we are going to take a certain amount of this bacterial culture and plate it on an LB agar plate which contains the appropriate antibiotic for this construct. And so, now we are going to leave it for one and half hours, let it grow and meanwhile what we are going to do is? We are going to pour a few LB agar plates which I am going to show in the next part of the video.

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First what we are going to do is for plates is give the surface a nice wipe down using 70 percent ethanol. Normally you should not talk while working in the lab, but this is an exceptional case. So, what I have here is LB agar and it was autoclaved at 121 degree centigrade for 15 minutes and then maintained at 65 degrees. Once it reaches around 55 50 to 55, we are going to add the antibiotic to this ok.

So, as I was saying this is the LB agar and once it reaches around 50-55 degree centigrade, we can add the antibiotic to this. If we have the antibiotic at an earlier stage what will happen is the antibiotic might get degraded because it is too hot. So, it will take maybe another few minutes to cool down sufficiently to add antibiotic. So, what I am going to do is we have so these are sterile petri plates. So, we purchase them in sterile condition. So, I am just going to open them and make them ready for use while the LB agar cools sometimes the packets are too good.

It is hard to open them. So, I am going to add 200 litres of the antibiotic to the LB agar. Yes it is now cool enough for adding the particular antibiotic. So, different plates have different antibiotics based upon what construct you want to grow on them. So, some might be kanamycin, some might be chloramphenicol, some might be ampisilin. So, it depends. So, this is my antibiotic I am just taking it and what I am going to do is? I am going do is I am going to add it to the LB agar, give it a nice ensure that it is firmly mixed and the issue here is we cannot let this cool down too much.

Because if it cools down below a certain temperature, it is going to start solidifying. That is why around 55 we add the antibiotic and then what we do is, we start pouring the plates. So, I poured the plate, keep it semi covered. So, we poured 4 plates and what we are going to do is? We are going to let these solidify. So, it is going to take some time for these plates to solidify. So, what we did was we have taken this plate? So, this was a sterile plate I am not pouring media, but I will just open it to show you what it is like. So, there is an inner dish and an outer lid the diameter of the outer lid is greater than the dish.

So, that it can cover it and what we did was? So, you see the dish we tried to ensure that maybe two-thirds of it is covered with the LB agar media and the rest of it is kept empty and after we pour it and it solidifies, we are going to close the lid and leave it like this until we are ready to plate our bacteria also. Keep in mind that during storage plates are always stored upside down and not like this because otherwise the condensation that might develop will fall of the condensation that develops on the lid might fall on to the plate surface, the LB agar media surface and make it watery and also hamper the bacterial growth and so on so forth.

So, normally when you store plates, so now it is what you see is the dish contains the media and you cover it like this, but when we are going to store this in the fridge or elsewhere, you have to keep it upside down because otherwise condensation which develops on the top of the lid you can already see some because it is hot there is some condensation, right. So, if you leave it like this condensation the water droplets will drip off and fall on to the LB agar bed and that is not good lab practise to avoid that. You have to keep it like this when you are storing it or even when after plating you are keeping it changes and it becomes more whitish in nature. .

I do not know if you can see it or if you can appreciate the colour change, but while working in the lab with experience you will be able to notice these differences and this is a good point to look forward to look towards when judging whether the plate has solidified. However, if you leave it for around 20 minutes, it will solidify along with the colour change and then what you will do is since these are solidified I am just going to put the lid on for all of these and then a very important fact that you must keep in mind is you need to label these plates.

You need to label whether they are LB agar and what antibiotic is present and on which date you made it and you should also keep your initials because in case you are in a big lab, people should be able to know which plate was made by whom and so that when you are storing them in the 4 degree centigrade fridge, they do not get mixed up and you can keep a track of things.

Also when labelling mandatorily, you must label the bottom dish because the lids might get interchanged. You can additionally label them on the lid, but whatever you do the lower dish must be labelled because that is where the LB agar media is and that is where the bacterial colonies will grow because by mistake you inter change the lids and the lower dish is not labelled and only the lids are labelled, then you are going to draw completely wrong conclusions if a switching happens.

So, what I am going to do is now I am going to take a marker and I am going to label these as LB agar with the antibiotic and also today's date and my initials. So, it will be something like this. I do no know if you can see it. So, LB agar the antibiotic, the date, my initials they should be there and once it is ready you can keep the in the inverted position, as I was mentioning earlier the plates. So, when pouring they were in this position. Now that they are solidified and they have been labelled we have to keep them in the inverted position because you will notice already there is some condensation because of the heat that came out of the warm media.

So, if we keep on storing it in this condition after sometime the water droplets will fall on the bed and that will create a mess. It is not good lab practice. It makes the media soggy, the surface soggy and it should be avoided. I will just label all four of them and meanwhile of you remember we have been growing our transformed bacteria with LB in the 37 degree shaker incubator.

So, it is almost one and half hours, there are there is some time left. Once one and half hours is completed what I am going to do is I am going to plate a small amount of culture from that on to these plates and then we are going to leave these plates in the 37 degree incubator and overnight and by next day we are going to get bacterial colonies which contain our gene of interest.

This is because the gene of interest is in a particular vector and that vector has an antibiotic resistance marker. So, the bacteria which have successfully taken up the DNA,

they will be able to survive in the corresponding antibiotic and hence they will be able to grow in LB agar plates containing that antibiotic, but those cells which did not take up the DNA. They are not going to survive the plate containing that and particular antibiotic and hence if we get colonies, when we get colonies you can be more or less more or less certain that those are the DNA those are the bacteria which took up the plasmid DNA.

Of course, subsequently what we need to do is from those bacteria also we need to make cell stocks and isolate plasmid DNA again and send them for sequencing to finally confirm if our gene of interest is present or not. But getting colonies is a preliminary indication that transformation has occurred and from there we will move on to the next experiments. Yes let me just finish labeling the last plate then we are going to move on.

Yes now that we are done we can move on to the next part of our experiment. So, now what I am going to do is, we prepared 4 different plates right. Two of them I am going to store for subsequent use in the future. So, what I am taking is? I am taking para films. So, it is basically a type of sealant. So, what I am going to do is these two plates I am going to store. So, we take the para film and it is very stretchable. So, as we keep stretching it creates an air tight seal and in this manner once the entire petri dish has been sealed, so it does not suddenly open up. We are going to store it in 4 degree centigrade. For now I am setting it aside.

I will take another strip of para film, remove the protective cover and I will store this plate as well. So, it is basically like a sealant a stretchable sealant with which are we sealing the prepared petri plates and what we are going to do is later on once this experiment is over I am going to just take them and store them in the 4 degree centigrade fridge. So, these two plates we are going to use for today's next step of the experiment. First I am going to switch on the burner or turn on the burner the spirit lamp .

And so, this is the media containing our hopefully transformed cells after it has been grown at 37 degree centigrade for one and half hours. We will notice it has become turbid because of the growth of bacteria and so this contains 440 micro litre of LB and we originally had 50 micro litre of bacterial cells, competent cells and we have added 1 micro litre of DNA. So, out this 450 micro litre what I am going to do is, so I have 2 plates.

In one of them I am going to take 200 micro litre of the culture, put it at the center of the plate and then spread it across the entire plate using a spreader. I am going to show you how it is done in the next couple of minutes and in another plate what I am going to do is, I am going to take a smaller amount 10 times less since I took 200 micro litre of this.

What I am going to do is I am going to take 20 micro litres of culture and spread it on the other plate. So, in case transformation efficiency is very good may be in 200 micro litre, we might not able to get single colonies. We will have a lawn of colonies in which case in 20 micro litres we will be able to get well dispersed and spread out colonies which will help us to discretely identify separate colonies. For that reason we are going to make 2 plates having a 10 fold difference in the amount of culture that is being plated before we can begin plating.

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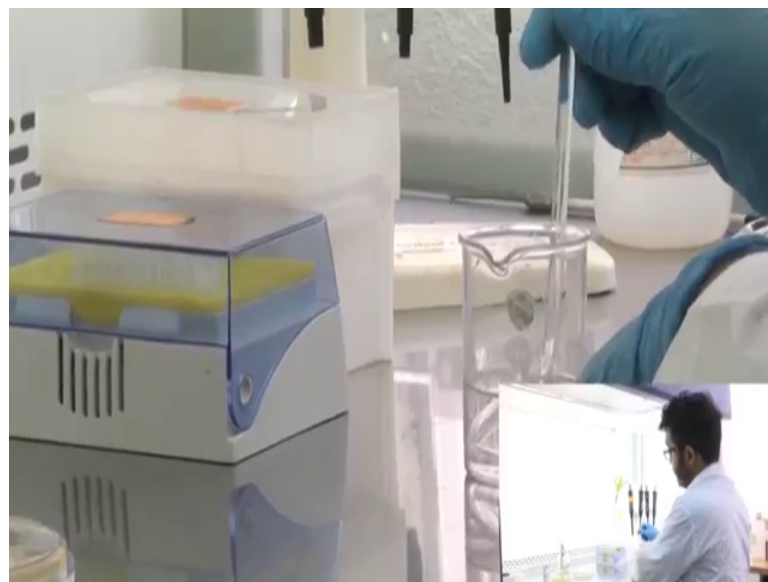

So, I have a glass spreader here and it is dipped in 70 percent ethanol to make it sterile. So, what we do is once you take it out, you add the bacterial culture to the surface of the plate after removing the lid and then you just spread it around using this. So, you need make sure that this surface does not contain any other bacteria other than the bacteria which we have been using for the transformation. So, for that purpose you have to sterilize it in 70 percent ethanol. So, that is what I am doing, I am making sure that the entire surface is dipped in 70 percent ethanol and after that what I am doing is I am holding it to the flame.

So, holding it to the flame ensures that the ethanol is removed because as you know 70 percent ethanol kills bacteria, right. It is for sterilization purpose. So, in case your spreader after you have sterilized it with 70 percent ethanol contains a residual amount of ethanol and with that if you spread your transformed bacteria there is always a very high that you are going to end up killing your bacteria then and there.

So, to remove the ethanol is volatile right. So, to remove the ethanol we are just heating it up in the burner I am going to repeat the process again. So, that you can see it one more time and dipping it in ethanol and I am going to heat it in the burner to evaporate the ethanol and dry it out completely.

Cool it for some time and then I am going to use it to plate to spread the culture. So, first I am going to spread 200 micro litres of culture. So, keep in mind that all these information should be labelled on your petri plate because, unless its properly labelled you are going to be making mistakes in the subsequent steps. So, I will set no I will just use this pipette they both the same its just that i prefer using my own pipette. So, what I am going to do is? I am going to set the volume at 200 micro litres and once that is done I am going to take 200 micro litres of this. So, now, when we spread the plate we need to invert the plate again.

So, now its kept in the normal conventional not conventional normal position where you take out the lid mix thoroughly. So, that you take sufficient amount of cells and I am going to add this to the center of the plate keep it in the center of the plate release the pipette quickly. So, you need to be a bit fast in this sort of work its nothing to worry about with practice you are going to get that eventually and my spreader it was dipped in 70 percent ethanol its sterilized and now I am getting rid of the ethanol. So, you will see that the ethanol gets burnt off, it gets burnt off is to be thorough. So, that there is no residual ethanol left and then you have to cool the spreader.

So, initially it had ethanol if you use that to spread it you are going to kill yourselves because 70 percent of ethanol is for sterilization. You do not want to kill yourselves that is why you are heating it in the burner. The ethanol being volatile it just goes away, it gets burnt off, but now the spreader is hot very hot. So, if you use this and dip it in to the culture that you put on this plate again you are going to kill your bacteria. So, just cool it,

keep it out for may be at least a minute around a minute and what I do is, I just sort of wave it around to cool it and then see I added the culture to the middle.

So, normally when you are work with petri plates, you do not touch it. I am just pointing at it from a distance I added the culture in the middle. So, the corners do not have culture. So, in case the spreader is still warm I am just touching the culture at the corners to ensure that the heat, residual heat gets dissipated and it comes as close to the ambient temperature as possible. Once that is done now what I am going to do is, I am going to use it to spread the culture uniformly across the entire surface.

So, what I am going to do is I use the spreader and then using my hands I keep turning it vial. So, there is a rotational motion with one hand and there is a translational motion with the other. I am just moving the spreader up and down and with my other hand I am just rotating the plate, so that the 200 micro litre culture gets evenly spread across the entire surface of the plate. So, when do you stop? So, 200 micro litres is quite a large amount of culture.

So, initially when you start spreading it will feel very slippery time. So, next we are going to plate 20 micro litres of the culture. My spreader is already in ethanol. Again I use this for spreading the 200 micro litre, but after that I put it ethanol and let me just get rid of the ethanol, burn it off in the burner. So, I do it twice, I repeat an ethanol once thoroughly get it sterilized with 70 percent ethanol, then I heat it in the burner flame to get rid of the ethanol and then I again repeat an ethanol and repeat that. So, I just do it twice. Now what I am going to do is I am going to take 20 micro litres of the culture and plate it on the different plate I have labeled it as 20 micro litre plate.

So, I will just take the culture 20 micro litres, add it to the center of the plate. Do not just scatter it all over the surface because, you need to keep the surfaces of the plate, divide of bacterial culture because initially you are going to help cool your spreader by touching those surfaces. So, you do not want your bacterial culture to be there and get killed by the heat. So, I am again reheating the surface sterilized spreader glass spreader and once the ethanol has been burnt off, I am satisfied that there is no residual ethanol left. What I am going to do is? I am going to again cool it. If it is too hot, it is going to kill my bacteria and if bacteria dead you cannot get any colonies, there is nothing left to grow right.

So, now I am just going to cool it quickly. So, rule of thumb is keep it like this for may be round 60 seconds close to a minute and I repeat once again that normally when you are working in the laminar air flow hood, you should not talk because as I am talking there is a possibility that I might spit into the lab or the breathing that I am doing it might overcome the positive pressure of the laminar flow and I have an open plate here, right. So, in case I am spitting then bacteria from my mouth might also be there and who knows may be it is resistant to this antibiotic in which case I will get a false colony.

So, when you are working and when you are carrying out your experiments or even when I am doing my experiments no talking in front of the hood. For this demonstration purpose it cannot be helped, but always keep in mind the lesser you speak the better. So, it is cool I believe. I am just going to touch the spreader to the edges of the plate, so that in case there is any residual heat it gets dissipated into the media and not on the culture which is at the center of the plate and once I am ready now I just do this to and flow motion with one hand for the spreader and with my other hand what I am doing is, I am rotating the plate. This will ensure uniform spreading of the culture.

So, you cannot see this, but since I added only 20 micro litres compared to the earlier plate where there was 200 micro litres it does not take too long. I can already feel that the slipperiness because of the liquid is going away since it is getting you properly spread. It is actually becoming sort of what to say stickier and drier already which means that the spitting actually complete.

So, spreading is complete. I am going to stop here, I am going to put on the lid and keep this for sterilization. Always keep in mind when you are spreading that the your hand should have a uniform pressure. If you put in too much pressure, there is always a chance that you are going to break apart clumps of the LB agar and so, you will ruin your plate.

So, your hand should be steady and there should be a steady uniform pressure and you should be rotating your plate uniformly. Too much pressure can actually break the LB agar plate into fragments and crimps and that is going to mess up your experiment. So, going back to our experiment that we are demonstrating today, these are the 2 plates which have 200 micro litre and 20 micro litre of the bacterial culture. I am not adding para films to these plates which I am going to store in the 4 degree centigrade fridge and use on a subsequently later day I have add a para film but these are already used.

So, what I am going to do is, now I am just going to put them in the 37 degree incubator and leave them over night for the bacteria to grow.

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So, what we have here the instrument next to me is the 37 degree centigrade incubator. There is no shaking component to this and what we use this for is to keep our LB plates containing bacteria that we have spread on it keep them inside and allow them to grow at 37 degree centigrade over night. You can also set it at other temperatures depending upon the temperature that your bacterial cultures requires, but mostly since we work with equally and the type of work what we do we need 37 degree usually. So, that is what this instrument is set at.

And so, I am just going to open this and so, these two plates that we plated the plates that we plated I am now putting them inside and so, we will keep them here. We will leave them over night and tomorrow we will see if at all we have any colonies. In case we do not have any colonies that is a problem. That means, our experiment failed and there could be many reasons for that. One reason is might be the bacterial cells that we used were not competent or they lost their competence because of storage.

Another reason could be that they were competent, but while handling them vial we were taking them out before adding DNA may be while taking it out from the minus 80 degree centigrade fridge. May be we kept it in our hands for too long and therefore, we messed up the competence of the cells, that could be another reason if we do not get colonies.

Another reason could be that while plating we the spreader was too hot. We killed the bacteria. So, there could be many different reasons if we do not get colonies and we will have to troubleshoot them subsequently. However, if we get colonies that means that the bacteria which survived in this antibiotic plate have successfully taken up our ligation product which contains an anti biotic resistance gene in the plasmid which enable them to survive here.

And once we get colonies, we are going to subsequently make cell stocks and isolate plasmid from them and send them for sequencing to finally establish whether we have our gene of interest in them or not. So, yesterday we had put 2 plates containing our ligation product transformed into DH5 alpha competence cells.

And plated on to it that are containing that probate antibiotic. So, those 2 plates today morning I came and collected them and I filled them with para film and stored them in 4 degree centigrade until further need. So, I have taken them out and I am going to show them to you. So, these are the 2 plates we have plated 200 micro litres here and 20 micro litres.

Here you will notice that in the 200 micro litres the number of colonies is much more and also some of the many of the colonies are very small because of the space crimps. So, there was a lot of transformed bacteria as a result of which a 200 micro litres it is more cultured. So, more bacteria and in this limited space we could not grow as much as these in the 20 micro litre. See even this has a lot of bacteria, but you will see that the sizes of the colonies seem to get bit more also notice that there will be some colonies which are merged together.

So, may be 2 colonies side by side I do not move you can see it here, but while working you will be able to see you might see this that 2 colonies growing too close to each other when kept overnight, they might just merged and create a larger blog. So, when you are going to pick single colonies for your experiment make sure that you avoid picking such colonies which are merged cluster of colonies. It is very good practice.

In fact, it is almost mandatory to click perfectly single colonies when working with such bacteria. So, that is one point that you need to ensure. Also once you have kept them in the 37 degree centigrade incubator for sufficient time when you come in the morning and

see that they might be overload or there is enough growth you cannot keep them in definitely in 37 degree centigrade.

Because if you keep them instead of one day if you keep them for 2 or 3 days, these colonies will keep going larger larger and then they will all merge together to form a long and then you will not be able to collect single colonies. So, once in the morning you come, you plate in the evening, you leave the lab in the morning. First thing that you do is you check if there are any colonies.

In case there are colonies and they have grown to sufficient size and there is sufficient number by sufficient what I mean is you will learn that to experience when the colonies are round and well apart and not too small not too large. You should take off the plates, seal them with para film and store them in 4 degree centigrade to prevent further continuation of growth which might equal all formation.

To summarize this set of experiments that you have been observing in this week initially we had PCR amplified a particular gene of interest from particular DNA source and after PCR amplification what we had done is, we had digested them with a particular set of restriction enzymes and also the vector into which we wanted to clone the gene that was digested with the same set of restriction enzymes.

After digestion for both these components we had ligated them together and after ligation what we did was we transformed them into chemically competent DH5 alpha equalize cells. And after transformation we plated them on LB agar media containing the appropriate antibiotic which is dictated by the vector which you are using or the plasmid which you are using depending on the plasmid what anti biotic gene it encodes for you use an LB agar plate containing that exact same antibiotic.

We did that and as I showed you just now after growing them at 37 degree centigrade incubator overnight, we also have colonies. Next what are we going to do with these colonies what we are going to do is subsequently we will or you should since it is your experiment what you should do is, you should take one single colony, use a pipette tip scrip of one single colony ensuring that you do not touch any other colonies, put it in a small amount of LB media may be 5 ml of LB media with the same antibiotic, grow it overnight. So, now what you will have is a culture containing bacteria from that

particular single colony. Once you grow it overnight what you can do is the next day you can isolate plasmid from it and also make a small cell stock.

So, take 500 micro litres of that cell culture add 500 micro litres of glycerol to it in an append of tube, mix it thoroughly and store it in minus 80 degree centigrade. This cell culture you can subsequently use for regrowing further bacterial cultures because see from the plate you have already picked the colony you no longer have it on the plate, since you have grown 5 ml or 6 ml of it overnight.

You take 500 micro litre in a centrifuge tube and 500 micro litre glycerol and store it in minus 80 degrees and after proper mixing and this will now act as a source for this particular culture. So, each time you need to grow more of the culture, you go to the minus 80 degree centigrade, fridge that vial out immediately scrip of a small pic, it will be frozen solid. Just take a small spic, drop it into media containing antibiotic and leave it overnight bacteria will grow from there.

So, what you should be doing is making a cell stock and with the remaining four and a half five and half ml of culture that you have from the colony overnight growth of the colony, you should isolate plasmid. And so, we have shown plasmid isolation in other lectures and after you isolate the plasmid what you should do is send it for sequencing only.

After you get the DNA sequence, will you be able to confirm whether your gene of interest is exactly present or not. Once you see that your sequencing has been successful, you need to move on. In case you need to express the protein as well you need to retransform the plasmid into BL 21 the 3 equalize cells. So, those cells are cells which are meant for protein expression vial DH5 alpha cells that we used during the.