Experimental Biochemistry
Prof. Snigdha Maiti
School of Bioscience
Indian Institute of Technology, Kharagpur

Lecture - 40
Bacterial Culture for Plasmid DNA Isolation

Hello, today we will learn how to isolate plasmid. So, it is a molecular biology technique which is very useful in normal reactions or setting up different kind of experiments in a molecular biology lab. So, before we start doing this method or of isolating plasmid, we need to know what are the plasmid. So, the plasmid is an extra chromosomal DNA it usually comes from bacteria and most of the u bacteria and r k have plasmids and this plasmids are double stranded and small in size. So, basically if we look into the size difference of plasmid and the genomic DNA of the bacteria, it differs in the range of few mega base pair. Like if a plasmid is usually 1 kilo base pair to 1000 kilo base pair where the genomic DNA of equally is around 4 to 5 mea base pair.

So, there is a huge difference in size also the plasmid is circular in nature and it is it can independently replicate itself. So, why we need to isolate plasmid in molecular biology lab? So, there are different experiments you can design regarding the plasmid isolation and when you isolate the plasmid, you can design any kind of PCR reaction, you can digest the plasmid you can clone the clone a particular gene inside the plasmid also you can do transfection and different other procedures. Now the isolating a plasmid is useful technique from microbiology or molecular biology lab and that is what we going to do today.

So, as the bacteria possess plasmid they are not essential for their survival, but often this plasmid gives the super power to the bacteria. So, suppose a bacteria is living in a stress condition, the plasmid can help the bacteria to survive in that particular stress condition like different antibiotic are present in the media. So, bacteria can survive that kind of condition. Because most of the cases plasmid express different kind of antibiotic resistant gene and this product of this genes are often helpful for the bacterial survival. And we can use this kind of plasmid to easily manipulate or regulate or whatever gene or other product we want from the bacteria, and we can incorporate those gene product gene inside that plasmid and can express that plasmid.

Now, the copy number of the plasmid also differs in different strain, there are bacterias which have large copy number of plasmid or there are bacterias which have less copy number of the plasmid. During plasmid extraction you should be careful there which kind of bacterias strain you are using and how many copy numbers are present in that particular plasmid. And also another important aspect of plasmid extraction is that when you are growing the bacteria, there the media should contain some kind of selectable marker like some kind of antibiotic and that particular antibiotic resistant jean should be present in the plasmid. If you do not put the stress condition if you do not put the bacteria stress condition, the bacteria can eliminate the plasmid it called plasmid curing. So, you need the bacteria is to grow in a stress condition so, that the bacteria will keep the plasmid and when it will replicate, the plasmid will also replicate itself and make higher copy number.

So, before we start getting into the plasmid extraction there are few things we need first one is the bacterial culture. So, usually we give overnight culture. Now overnight culture means around 12 to 15 hours growth of the bacteria and this kind of bacteria strain usually there are excel one strain or b l 21 or d h 5 alpha strain of E.coli, this have high copy number of plasmid and they maintain the plasmid in cell.

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So, here we are using a l b medium where we have given the bacterial culture. So, l b is an enrich medium. So, you should give an enrich medium where the bacteria can happily

grow and the culture is around 15 hours time it has taken around 15 hours. So, the o d is usually 3 to 5 at this o d 600.

So, you need to have a large number of cells before you start extracting the plasmid from those bacteria. Now after you have grown overnight particular culture, then another things which you will need is a centrifuge because the steps required during the plasmid extraction requires the centrifuge, also the solutions which we will need for extracting the plasmid. Now here today, we will use a kit which is provided by a particular company, there are different companies provide this kind of kit also you can make this solutions in the lab following a molecular biology book.

So, I will be talking about the solutions and what the solutions contain and how the solution help us to extract the plasmid. So, three things we need one is the bacterial culture and overnight culture and that bacterial culture should have the anti biotic which is and the anti biotant registered gene should be present in that particular plasmid and another thing is that centrifuge machine ah. So, there we can centrifuge the cells and also later steps, we will need the centrifuge and the solutions which will use.

So, now I have mentioned that we need an overnight culture before we go for a plasmid extraction. So, here I have given this overnight culture in a cultrate tube and if you can see that the culture tube is not fully filled. So, it is around one third or less than one third filled. So, here it is around 7 m l of culture I have given and I have taken l b and the small in a (Refer Time: 06:07) from a cells tog which is kept at minus 80 degree centigrade. So, otherwise if you have colony you can pick colony and put it inside the cultured tube. And also the l b media has a particular selectable marker or the antibiotic register antibiotic in my case the bacteria contains the 28 vector. So, the pay 28 vector has kanamycin resistant jean which express when it grows and ah. So, that I have put kanamycin in my media.

Other vectors like pay 3 a have ampicillin marker gene. So, you have to put ampicillin. So, depending on the vector or the plasmid you are using and you have to check what kind of antibiotic resistance jean it contains, and accordingly you have to get that particular antibiotic inside your medium. So, it has been grown in the incubator at 37 degree centigrade because equalize grows best at 37 degree centigrade in an incubator

the RPM is almost around 200 RPM. So, the incubator has a parameter where it has to rotate. So, that the proper aeration can happen and the bacteria can grow perfectly.

So, as I am mentioning I was mentioning that, the culture tube should not be filled to much. So, whenever you are growing bacteria suppose you are taking any kind of conical or a tube like this, should not fill that particular container more than one third. So, suppose you want to make 250 m l of culture, you should not take a 250 m l or 500 m l conical flask. You should take at least one liter of conical flask where it can grow properly otherwise the aeration will be hampered and the bacterial growth will be less. So, now, that I have prepared my culture and it has been grown for 15 hours at 37 degree centigrade, I will isolate first to isolate the plasmid I have to take the cell out of this media. So, that for that I need to centrifuge this culture in a small append of tube in multiple go. So, because the appender tube is almost 1.5 m l and I have almost 7 m l of culture here.

Now, today we will use a mini prip kit. So, mini prip means basically you can isolate plasmid from 5 to 7 m l of culture media. If you want to isolate more plasmid you can isolate up to 50 m l of from 50 m l culture media you have to use a midi prip kit. And if you want to culture isolate more like 200 m l or 300 m l of culture media then you have to use a maxi prip. So, all this different types of kits are available from different kind brands and you can take them or otherwise you can also follow any other kind of molecular biology book and you can make those solutions and accordingly you can step up in your culture volume.

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So, another thing I would like to mention about here is the another important instrument which we need during plasmid extraction is a centrifuge. So, centrifuge this is simple centrifuge machine where we will isolate the pixels from the midi culture media. So, basically when we will start the centrifuge, it will rotate and it will put a centrifugal force into the different components present inside the culture. And depending on their size and their shape of the molecule it will precipitate because it has a centrifugal force. So, in every centrifuge there is a you have to plug it into a any kind of electric channel and then it has a body switch and you have to switch it on.

Now, here the centrifuge has two options one is the RPM another one is the RCF. So, RPM means Rotational Per Minute. So, the it depends on like how fast it is rotating this is called the rotor of a centrifuge. So, suppose it is rotating 5000 RPM s so; that means, per minute it is rotating 5000 times it does not depend on the size or the radius of the rotor. So, RPM can small centrifuge like this can rotate 5000 RPM a bigger centrifuge like different kind can also rotate 5000 RPM, but this RPM does not mean that all the force will be equal to the all the particles that are present inside the solution. Because the then we come to another term which is RCF or the rotational centrifugal force which is applied to the solutes or the different kind of constituents present in a solution.

Now, in that RCF what g is dependent on the size of the rotor because then it depends on the radius and also the RPM. So when you go for any kind of centrifugation you should

know what kind of g force or RCF force you need because that is kind of universal in terms of centrifugation because if someone says that you have to rotate at 5000 RPM depending on different centrifuge machine and depending on the rotor size that 5000 RPM can generate different kinds of g force, but if someone says 5000 g; that means, in this centrifuge machine that 5000 g will be similar to another centrifuge machine which has a bigger rotor or smaller rotor than this.

So, you should be careful what kind of g force you are applying for a particular centrifugation procedure. So, here if you see there is an written as RPM also you can select for the r c f. So, this is shown as 16162 into g. So, this is the g and if you go down this is 4800 RPM. So, this two different value differs from each other because RPM basically RCF depends on the rotor size also the RPM and the gravitational force that is applied into the solution.

So, now I will take this culture I a small centrifuge tube and start isolating the cells from this culture. Before that another important aspect of centrifuge is that you always have to balance the sample. So, suppose you are putting one sample here, at the opposite side you have to put another sample which is of equal weight otherwise this machine will show error and you will damage the rotor. Another important aspect of centrifuge is that you always have to balance the sample.

So, as I am removing the lid of the centrifuge, if I am putting something here one of my solution I have to put another sample at the equal at the opposite side of the particular sample which is of same weight otherwise it will damage the rotor and it will not give a proper value and the centrifuge will show error.

So, you always have to balance your sample, if you have one sample then you have to balance with some other solution like water or something else otherwise it will damage the centrifugation rotor. So, now, I will take my culture in this small centrifuge tube before that I will label it properly.

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So, here also I have labeled what kind of plasmid or what kind of culture I have given. Its a good practice always label your culture tubes or the any kind of a culture or any kind of solution you have label it properly in the lab. So, you give the name of that particular culture which you have given then you give the date like when you have put the culture. So, today. So, I have given the culture the yesterday night and also you should write you own name. Because if you are working in a lab there are other people and they should also know that you have given the culture if you do not give your name of your culture or your name then the problem will be the other people might take your culture or there might be some kind of mismatch and you might not get the result.

So, for easier recognition of your culture you should give the name of your and the name also of the sample. Also when you are taking out the culture in a centrifuge tube you should label that tube as well. So, here I am I have written the name of my sample in one tube in another tube since I am isolating plasmid from only one culture another good practice in lab is to always label your sample. So, here in the culture tube I have written the sample name which I have provided or given and also the date, like when I have put the culture. So, yesterday night I have put the culture. So, I have given the sample name also the date and.

Also I have written my name because when you are working in a lab there will be other people and if you do not write label your sample properly or write your name then it

might create any problem like people might take your sample or there might be some kind of mishandling of the sample.

So, for that purpose you should always properly label your sample put down the date and also your name. So, always it is a good practice to label the sample properly it is not only for the culture in any kind of solution or any kind of buffer you are using, any kind of bottles you have always label them properly. Otherwise it might create problem, because today you might be remembering what it is, but after two days or few days back you will forget what it is and then it might create confusion on of your own. So, for that always label your sample properly. Now I will take culture from that sampled to another small centrifuge tube. Now those small centrifuge tube also should be labeled properly.

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So, her I have labeled the one centrifuge tube, where I will take my culture and pellet down the cells. Since I am isolating plasmid some only one sample I need balance as I have mentioned that in a centrifugation procedure, you should always balance your sample. So, in another tube I have to take water or any kind of sample of equal volume or weight. So, I am writing in this tube as balance. So, in the balance I will not have any kind of culture only the water to balance my sample. So, now, that I have prepared the two tubes beforehand, I will start taking culture from the tube. Now as I have mentioned this small centrifuge tube is around 1.5 m l.

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So, I have to take multiple times from the tube because in a mini prip kit, you can isolate plasmid from 5 to 7 m l of culture

So, as I want to take 5 m l of culture, I will take using a micro pipette I will take 1 m l first and small volume discard the tip, close the culture tube. Now as I have mentioned you always need a balance in the centrifuge.

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So, I am taking equal volume of water in the other tube which is written as balance. And now I will keep the balance and then at the just directly opposite of the balance, I will put

the culture tube. Now always remember to put the lid on of the rotor otherwise it will again show error, close the centrifuge tube in the centrifuge there is a mention of RPM as well as the time.

So, in the centrifuge tube there is no parameter called temperature, other centrifuge also have temperature probe, but as during plasmid isolation we are not really need the temperature. So, it happens in the normal room temperature which is around 25 degree centigrade. So, we do not need the temperature sensitive centrifugation centrifuge machine. So, it only requires the time and the RPM or RCF. Here we will reduce the time to around 1 minute or one and half minute until there is a pellet formation and then we will start. Now during this centrifugation what will happen is that, there will be a centrifugal force applied to my solution and as the bacterial cells are quite heavy they will precipitate down from the culture tube and they will create a pellet at the bottom of the culture tube.

So, till that time we have to wait till the centrifugation process is over. Now another few things about centrifugation is that if it makes to much noise or if it is showing error or other things, which is not usual in any case then you should immediately stop this centrifuge machine. Because the rotor is rotating at a very high speed it might call if there is any kind of imbalance or any kind of proper labeling was not there. So, it might cause some kind of accident in the lab. So, you should always be very careful while doing a centrifugation process. So, now, the timing is going down and it has gone up to 16000 g. So, how we will know that the pellet down of the cells has been properly done or not.

So, when you take out the sample as I will be showing it to you. The supernatant will be very clear and it will look like the normal media when you have previously given the media l b media the it will look like a clear media. And there will be a small (Refer Time: 20:04) formation at the bottom part of the centrifuge tube; now it is almost over. So, you told me to stop the centrifuge machine when you give a time like 1 minute or one and half minute after that timing is over the centrifuge will itself go down and then it will show me that the centrifugation is over.

Now, it is over and the lid has been open.

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So, now I have taken out the culture tube. As you can see that the supernatant highly clear and there is a small pellet formation at the bottom part of the centrifuge tube. So, this pellet means these are the pellets from the equalized bacteria and the media has been cleared.

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So, during this kind of experiment you should always have a small beaker where you can discard all this flow through. So, any kind of liquid discard you should not throw it in a normal dustbin, you should always take a clean beaker because this kind of solution have

media and if you throw it in a normal basin or wash basin, it can contaminate the environment.

So, you should take it in a clean beaker and then it needs some kind of treatment like we put detergent also some bleach so, that other or microorganism like other bacterias and fungus cannot grow further. So, now, I have taken around 1.2 m l, I will again take the same culture in the same tube as I have discarded the media. So, the tube is now free. Again in the same tube I will take more culture. Now as I have discarded the supernatant from this culture tube you can see only the pellet is there and the tube is free now. So, I will be taking another 1 m l of culture in the same tube. So, that around 5 m l of culture my all the cells are taken into a same tube.

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So, I am taking almost 1.2 m l you do not need to be very precise about this, because anyway we are going to take all the cultures present in this cells. So, I am not changing the balance because I took almost same amount as previously, always remember to put on the lid close the rotor and again start. And always keep an eye if the machine is making any kind of noise or showing errors immediately stop the centrifuge machine. So, again we have to wait for another 1 minute and in the same culture pellet there will be another 1 m l. So, another from another 1 m l all the cells will be into a pellet.

So, my second round of centrifugation is also over. So, it will stop on its own. So, now, you can see the RPM value is going down; that means, it has been stopped and it will

gradually stop and the lid will open on its own. Again you can see the upper part the supernatant is almost clear as a like a normal media and there is a big cell pellet formation at the bottom of the centrifuge tube. Again I will discard the supernatant into this beaker, do not through it here and there as the tube is free now or empty I will take another 1 m l of culture. Again put it in the at the opposite side of the balance run it for another time.

If you are changing the volume to much then you need to change the balances volume as well otherwise if you are taking every time the similar volume, then you do not need to change the balance volume every time. If it shows any kind of error further balancing is not proper then you should change the balance volume as well. So, here I am actually pelleting down the cells at the higher speed for 1 minute or more than 1 minute 15 second. If you feel that that the supernatant is not properly clear or the cell pellets are not properly formed then you can increase the RPM or RG RCA or you can increase the timing as well.

So, in most cases pelleting down from 50 m l or 15 m l of culture, we go for 10 to 15 minutes around 3000 or 5000 g. If you do not have that much at highest speed 1 minute is enough. So, you should always check this supernatant if you feel that supernatant is properly not properly clear, then you on go for higher speed and higher time. My third round is also done again the supernatant is clear. I will again discard the flow through and again I will take another 1 m l.

Put it at the opposite side, if you are feeling like you do not know exactly which one is the opposite side you should count the blank holes in the rotor. So, from here there is 1 2 3 4 4 5 and 5 again from this side it is 1 2 3 4 and 5 so; that means, is equally distant. So, it is at the exactly opposite side.

now as I was mentioning that the copy number varies in case of different strain of e coli. So, basically if you feel when you isolate the plasmid if you feel like the plasmid isolation the content of the plasmid is very poor, the yield is poor then it might happen that the bacterias strain the bacterias strain which you are using as low copy number. oOherwise if you feel like. So, in those cases it will have lower yield you can [FL].

Yeah.

[FL]. So, as I was mentioning the copy number varies in different bacterias strain. So, if you feel like that yield of the plasmid extraction is low, then you can go for higher volume suppose higher I have given 7 m l of culture then you can give 10 or 15 m l culture to get higher yield you should check the copy number of the bacteria as well at how many plasmids are present in one bacterial strain. So, in normal cases the p h 5 alpha have around 10 to 15 or hundreds even hundreds or more than 100 as well. If there are bacterial strain contains low copy number then the yield will be less. So, for the those cases you need to go for higher culture it the bacterial copy number is very high then 5 m l to 7 m l is fine. Also it will depend what kind of work subsequent work you will do with the plasmid if you want to do pcr very less number less amount of DNA s with your experiment. If you want to do restriction ligation any kind of ligation or transfection depending on your necessity it will change.

Now, my last round of centrifugation is also done. So, now, again I will throw away the culture or the supernatant.

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Here I have taken a piece of tissue paper I will just dap it. I will invert the tube and dap it so, that all the media has been soaked away and the pellet is as much as possible is without the medium. So, now, we will start the purification of the plasmid. So, there are different types of purification method in this case we will be doing alkaline lysis method.

So, as I have mentioned earlier that I will be using the mini prep kit, but you can make this kind of solution in your lab as well following any kind of molecular biology protocol book. So, the steps which we have to follow will be given by the prep kit manufacturer, also it is a similar protocol you can get it online or follow any kind of book.

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So, there are three types of solution, which we will need. The first solution is to. So, today we will be using a mini prep kit and the steps which we have to follow will be

given by the kit itself like the it will be there inside the kit or you can follow any kind of internet protocol or any kind of book the protocol will be similar in most of the cases.

So, there are few solutions which we will need, the first solution will be the resolubilizing buffer. So, as I have pelleted down my cells I need to again take this cells into a buffer. So, there are few things we need a few solutions the first solution which we will need is the presuspensionary solubilizing buffer. So, as I have pelleted down my cells I need to take this cells into a buffer solution where other solution will work into. So, the resuspension buffer is kept all mostly at the 2 to 8 degree centigrade. So, that is why I kept keep it in a ice box. So, this buffer content trace a buffering agent also it has EDTA which will chillet all the divalent ions like magnesium and manganese. So, this divalance ions are necessary for the action of the different kind of nucleus.

So, when subsequently we will break down the cells the nucleus will be ex will be out of the cells and it might damage the DNA which we want to extract. So, for that the EDT is there in the re suspension buffer it also contain RNA's. Since we are isolating DNA and we want to make take only the DNA part, there might be some contaminant from the RNA part. So, we degrade those RNA using an RNA (Refer Time: 32:33). So, this resuspension buffer contains a buffering agent which is stress EDTA, which will chillet down the divalent ions and RNA s a which will kill the RNA. After resuspending this buffer then we need to break down the cell walls or the cells. So, for that we need to have a buffer which is the lysis buffer. So, lysis buffer is mostly alkaline that is why the name comes alkaline lysis method. So, it has high amount of NAOH or sodium hydroxide.

Now, sodium hydroxide will actually denature all the proteins that are present, it will denature the membrane proteins the anything which are protein related, but will be degraded also it has ionic an ionic detergent which is sodium dodecyl sulfate. So, sodium dodecyl sulfate will actually break down the cell membrane and it will help to lyse the cell. So, this lysis buffer contains high amount of sodium hydroxide ah. So, it is an alkaline solution it will also denature the DNA or RNA whichever is present whatever is present inside the cell. So, everything will be denatured while we were giving the lysis buffer.

Now, after giving the lysis buffer the timing is important. So, in the kit it is written around 4 to 5 minute is enough more than 5 minute should be avoided. Because we want

the denaturing processes very time sensitive. So, do not keep the sample in denaturing buffer or the lysis buffer more than 5 minute, after this we need to have a neutralizing buffer. So, this buffer is all mostly three molar of potassium acetate. So, potassium acetate is acidic in nature when we will giving this buffer into the lysis buffer what it will do is it will readily the third buffer which we will need is the neutralization buffer. So, neutralization buffer has around three molar of potassium acetate. So, this is acidic in nature.

Now, immediately after giving the potassium acetate buffer or the neutralization buffer it will start the reannealing of the DNA. Since the previous buffer or the lysis buffer has a strong alkaline substances it will denature all the protein as well as the DNA and RNA. After you put the soluble neutralization buffer it will start reannealing the DNA. So, as I have mentioned earlier the size differs in case of plasmid DNA and the genomic DNA. For the plasmid DNA is from 1 k b to mostly 1000 kilo base pair, then genomic DNA is from 4 to 6 mega base pair. So, immediately after you give the neutralization buffer the plasmid DNA since it is very small and also it is very covalently close the two strands are very close to each other it will readily anneal together.

So, you will get the plasmid DNA in the solution whereas, the genomic DNA will require much more time to properly anneal the two strand will be oppositely annealed and it will form a clump and it will precipitate out of the solution. Also as I have mentioned the potassium acetate is there in the neutralization buffer, it will form potassium dodecyl sulfate. So, the potassium dodecyl sulfate is a salt which is again unsoluble in the solution it will precipitate out. And along with the precipitation all the proteins and other contaminants that are present in the solution will also precipitate out. So, when we will put the (Refer Time: 36:07) neutralization buffer we will see small curd like substances forming and those contains the proteins other contaminants are also the genomic DNA. And immediately after putting the neutralization buffer we will centrifuge the whole solution. So, that other substances will precipitate out and the plasmid DNA which is covalently linked with each other and they can readily anneal after we give the potassium acetate and it will be kept in the solution and we will isolate the solution.

Now, after taking out the solution, it which will contain the plasmid DNA.

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And in this kit we will use a column. So, column is basically a silica membrane. So, when we will give the solution inside this, at the bottom part of this column there is a silica membrane and our plasmid DNA will get stuck to the silica membrane. And then what we have to do is, we need to wash it watch the column to get read of all the other.