Experimental Biochemistry
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Lecture – 41
Isolation of Plasmid DNA

So, after the centrifugation in the solution part we will have our plasmid DNA whereas, the genomic DNA and other protein and contaminants will be precipitated down.

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So, we will take that supernatant where the plasmid DNA is present, and then we will load that supernatant into a spin column.

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So, a spin column basically have a silica column. So, this is the tube which is the washed tube. So, whatever solution you put here, we need to centrifuge it and the solution will come down to this wash tube. And this column is basically a silica column when we will load our sample, the DNA will get attached to the silica membrane and later part we will isolate that DNA using a low ionic buffer. So, now that we have understand the basic steps are the theory of plasmid extraction will go and start isolating the plasmid.

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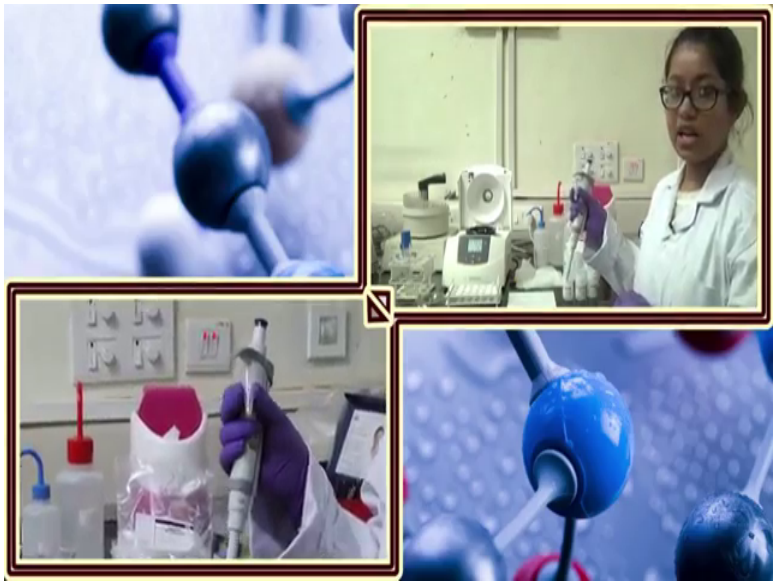

So, till now I have the cell pellet here. Now I will a resuspend the cell pellet into the resuspension buffer which has stress EDTA and RNAse.

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And that particular buffer is kept in an ice box because it is written that it will it has to be stored at 2 to 8 degree centigrade.

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Now, all I have is the cell pellet from a 5 ml to 6 ml culture. So, this culture or the cell pellet has to be re-suspended into the re-suspension buffer. The re-suspension buffer contains as I mentioned earlier stress EDTA and RNAse.

So, for that reason the bottle itself says that it has to be stored at 2 to 8 degree centigrade temperature. So, we usually keep it at 4 degree fridge, here I have taken it out and kept it a ice box. So, has this as RNAse and enzyme which is a very happy at a low temperature that is why we need to keep it at a low temperature or in a fridge.

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So, now the protocol mentioned here is that way I have to take 250 ml of resuspension buffer to dissolve that 5 ml pellet or pellet from a 5 ml culture. So, I will take a tip, I will adjust the micro pipette. So, as the protocol mentioned here is to I have to add 250 micro litre of resuspension buffer.

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So, first I will take a micro tip.

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Then adjust the pipetman 2 to 250 micro litre. So, now it is 250 microlitre then I will take the p 1 buffer.

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Carefully I will take 250 microlitre of this buffer which is basically the re-suspension buffer.

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Then I will add this resuspension buffer into my pellet. So, whatever I pellet I have got from this 5 ml of culture, I will re-suspend it. So, re-suspension means you can vortex it or you can pipette it up and down, do not vigorously pipette it up and down otherwise you might lose cell pellets and also damage the cell pellet. So, here what I am doing is, I

am continuously taking some solution and again putting that solution back and by that I am actually resuspending the pellet into this buffer.

So, you should not be able to see any kind of pellet or any kind of clump after you are done with the resuspension. So, gently pipette it up and down multiple times. So, that there is no clump of cell pellet and everything comes into a same buffer. Now that I have resuspended my pellet I will put this pellet into a lysis buffer. So, again the protocol says I have to add 250 microlitre of lysis buffer. Now after adding the lysis buffer it is tricky because I need to have a after resuspending the pellet into this resuspension buffer I will add the lysis buffer. Now, I have to add the lysis buffer and again 250 microlitre.

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Now, after adding the lysis buffer we have to invert the tube to mix the lysis buffer properly. Now, at this stage do not vortex or agitate to much the cell pellet otherwise the lysis will be hampered and the DNA you might cause the genomic DNA to break into small parts. If the genomic DNA breaks into small part then you will get genomic DNA contamination into your plasmid DNA, because the plasmid DNA is also small and if the genomic DNA becomes smaller, then it might get stuck to the membrane and you will get genomic DNA contamination.

So, be very gentle and to mix the tube the lysis buffer into the solution you just have to invert it like this. So, I have not added the lysis buffer, because after adding the lysis buffer there is a incubation time and it should not go beyond 5 minute. So, we will here

in our lab we stick to 4 minute time because we do not want to overdo the lysis process. So, we will stick for 2 minute 4 minute and at 2 minute interval we will mix it by inverting the tube.

So, inverting the tube is this. So, you just invert it like this do not vertex it, do not centrifuge it do not agitate it too much otherwise you might cause shearing of the genomic DNA which is undesirable. So, now I will add the lysis buffer. So, lysis buffer contains high alkaline solution which is sodium hydroxide and also SDA or sodium dodecyl sulfate. So, this 2 will cause lysis of the cell.

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So, I have taken 250 microlitre of lysis buffer, then I will add directly to the tube and immediately I will invert this 2 to 3 times, now I will wait another 2 minute. So, keep clock beside you. So, that you can monitor the time and after 2 minute, I will again mix it for another 2 times by inverting the tube and then when 4 minute is over, I will immediately add the neutralization buffer.

So, at this time keep an eye and it should not be agitated to much otherwise it might hamper the lysis process. So, you have to wait another 2 minute. So, as I have mentioned the lysis buffer contains SDA it will break down all the cell membrane because its an ionic detergent and it also has alkaline or alkali basically. So, high amount of sodium hydroxide, it will denature all the proteins and the DNA r n a whatever is present and when we will give the neutralization buffer the plasmid DNA because of the size

difference and the shape of the plasmid it will readily anneal and coming to the solution where others will precipitate out.

So, here the timing is really important do not over do this, and even in the kit itself the protocol. It is mentioned that you should not keep the solution in lysis buffer more than 5 minute. So, it s 2 minute is over I will again invert it another 3 times for a proper mixing of the lysis buffer and again keep it for another 2 minute. So, at twelve 4 minute incubation will be given. So, you can make all the solution in your lab using molecular biology book protocol book. So, basically the components if you know then you can make it and if you depending on the nature or how many how much plasmid you want to make, you can scale it up. Here I am taking 5 ml culture to isolate the plasmid you can take around amount 50 ml to even 250 ml of culture almost 3 minutes is over.

Now, as the next step requires me to add 250 ml micro litre of neutralization buffer. I will set my pipetman to 350 micro litre and I will be prepared whenever the 4 minute is over I will add the 350 microlitre of neutralization buffer. So, now, neutralization buffer will actually neutralize the solution since it was alkaline, it has acidic solution which is potassium acetate. And just after I give the potassium acetate I will show you there will be some precipitation and the whole solution will curdle up. So, always keep an eye at the timer do not over do this lysis process because it is critical at this stage.

Since my 4 minute is done, now I will add 350 microlitre of neutralization buffer to the solution. And immediately after adding I will vigorously shake it the centrifuge tube and you can see the whole solution becomes turbid. And small small white curdle curd like precipitation will occur. As I have mentioned that the neutralization buffer has sodium acetate sorry potassium acetate, this will form the potassium dodecyl sulfate which is a salt and it is insoluble in the solution.

So, it will precipitate out of the solution and also the genomic DNA will precipitate out because it is of huge size and it cannot readily anneal with another strand of its own. So, it will mix it will clump together and precipitate out of the solution and all the proteins will come out of the solution only the plasmid DNA will remain in the solution.

Now, as you can see the solution becomes too much of turbid if you feel like there is not turbidity is less or it is not properly mix you just do vigorous mixing and then we will

put it for centrifugation. Now at this stage the centrifugation is for longer time we have to do centrifugation for around 10 minutes at the highest speed.

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So, I am putting this into the centrifugation machine, I have also taken the balance, close down the lid close the whole thing. I will select the speed of the centrifuge at the highest also I will select the timer and make it at 10 minute because the protocol requires me to do the centrifugation for 10 minutes and then I will start.

So, when that particular centrifugation process is happening, we have to prepare this spin column basically the spin column as I have mentioned before as a silica membrane or silica column. After the centrifugation is over so, we need to take out the supernatant which will contain the plasmid DNA and put the content in this spin column. So, spin column as I have mentioned early silica membrane, where our DNA will get stuck and all other contaminants like if there any kind of protein contaminant or any salt it will elute it out.

So, after this step we need to have some subsequent centrifugations step and here our centrifugation is over. The volume it can hold in this pin column is around 800 microlitre. So, I will set up the pipette to 800 microlitre.

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I will take out my sample as you can see the supernatant has become clear now and all the precipitates are precipitate all the insoluble fragments have been precipitated out that contains our genomic DNA other proteins and salts of for same dodecyl sulfate.

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Now, I will take out the solution and put in the spin column. Now I will put for another round of centrifugation put on the lid, this centrifugations are shorts mean. So, basically I will reduce the time to 1 minute. After 1 minute we will see that all the flow through as gone down to the collector and we will discard that wash collector.

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After the centrifugation is done then we need to have to washing step one is we the high ironing strength buffer, that will provide us have a clean without any protein or soil contaminant DNA and after that we will wash it with 70 percent ethanol. Say ethanol will allow the DNA to be bound at the silica column, but other regions or other soils will wash with wash away from the DNA and then we will have a illusion step.

So, illusion will happen in the low ionic strength buffer or if you do not want to have it in buffer, you can do in a normal autoclator non deionized water as well; make sure the water is nucleus free otherwise it might degrade the DNA. The centrifugation is over, as you can see the flow through has come down into this wash tube I will discard this wash tube. Now according to the protocol I have to add around 500 microlitre of this p b buffer which is another binding buffers. So, basically it will ensure that the DNA becomes bound to the silicon column, while other contaminants will wash away.

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So, I am adding 500 microlitre of this p b buffer or binding buffer, then I will again go for another round of centrifugation for 1 minute. After this binding process we will wash the column using this washing buffer which contains alcohol which is ethanol 70 percent alcohol. So, I have to add 700 microlitre of this washing buffer, I have set up my micro pipette.

So, when if you buy a kit you will see that some of the reagents will be asked you to add some of the stuff like here before use it is mentioned that add 24 ml of ethanol to obtain 30 ml of buffer p e. So, you have to add those buffers when you get the kit and then you have to keep it. So, some of the terms you have to ensure that you add previously before you start using the kit and those will be mentioned at the bottle of those reagents or even in the protocol card which will be given by the manufacturer.

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Our binding has been done. Again the flow through as been collected in the wash tube, now we will wash the column using this wash buffer which contains around 70 percent of ethanol. Carefully pipette out this solution because alcoholic solution tend to drip while pipetting it out. I will again go for another round of centrifugation. Now here I am isolating plasmid from one particular culture and that is why I did not label the spin column. If you are isolating plasmid from different culture or different colonies you should always label all the spin columns otherwise it might happen that you might mix up with different cultures and that might cause any problem.

After this washing step is done we need to remove the alcohol. So, I will show you after I throw away the wash buffer, I will again spin it down without putting any kind of solution. So, that will ensure whatever residual alcohol is present in the column it will evaporate and it will go away. Otherwise if a solution contains alcohol the DNA will not solubilize, I mean whatever buffer you are actually trying to elate out your DNA. So, the column should be alcohol free before you try to innate out your DNA.

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So, one step of washing is done. So, I will throw away again the washing buffer and then again I will put this tube at the wash tube, and then the empty tube I will again centrifuge it to remove the residual alcohol that is present. So, all the steps will be written in that protocol kit protocol card or else you need to follow a book.

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After the washing is done then I will use a illusion buffer. So, here the illusion buffer is basically a low ionic strength trace buffer, which has around 5 molar milli molar to 10 milli molar trace and it might contain EDTA which will actually chillet down all the

divalent ions and so, that the nucleus if any kind of nucleus contamination is present your DNA will be not clewed because the EDTA will chill it all the divalent ion and nucleus need those divalent ions to work. So, it might contain EDTA or else it will have just 5 milli molar to 10 milli molar tris buffer. If you do not want to add this illusion buffer because you might not know the actual components of this buffer and if there is EDTA present.

So, you cannot work in subsequent experiment like you want to restrict restration digation of this plasmid. So, there you need to put some enzymes which will again need the divalent ions and if EDTA is present, then it will chill it all those ions and your restriction enzymes will not work properly. In those cases you can use just normal 5 milli molar or 10 milli molar trace page around 8.2 or 8.4 otherwise you can also elute out your sample or plasmid in nucleus free deionized water. So, now that I have remove the alcohol. Now I will take out this tube the spin column. Now that I have remove the alcohol that is present in the wash buffer I will remove this wash tube because I do not need it anymore.

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Then I will take this pin column and I will take a clean centrifuge tube or a collection tube, I will put the spin column in that particular collection tube. So, whenever I am putting the illusion buffer in this collection in this column and subsequently I will centrifuge this tube, the collection ellutant will contain my plasmid DNA and will be

collected in this centrifuge tube. So, now I will put 50 microlitre of this illusion buffer, take one pipette, this is my illusion buffer. And, just put the buffer at the right the middle of the tube do not touch the spin membrane or the silica membrane basically do not make any hole or damage the column otherwise it might get damaged. And then after putting the illusion buffer you need to wait around 1 minute or so.

This time the buffer should be at room temperature if it is a little bit warm it is great because the illusion will be perfect at that particular temperature do not cool it down and wait for another 1 minute. So, that the buffer will spread all over the silica bed and when you centrifuge it, it will come down to the collection tube and along with the plasmid DNA. Now, that we had waited for 1 minute I will put this whole thing for centrifugation. So, you cannot close the lid of the centrifuge tube, but always close the centrifuge tube the centrifuge machines lid, then centrifuge at the highest speed for 1 minute.

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After the centrifugation is done, you will see that the collection tube contains some solution which is basically the illusion buffer. So, basically it has been eluted the plasmid DNA which was attached to the silica bed, now you can throw away this spin column.

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And this is the plasmid you have extracted from the culture now to check the amount of DNA which it contains, you have to check at 260 nanometer of wavelength because DNA absorbs that 260 nanometer, then you can also check if other contaminants are present like other carbohydrates or salts or RNA present. So, you have to check 260 by 280 nanometer ratio, you have to check 230 by 280 nanometer ratios. So, those ratios you have to check for how much yield you have gained from this extraction process.

Now, all the DNA will absorb 260 nanometer. So, you might not know if all the DNA that are present in this tube are of your choice or plasmid DNA or genomic DNA contamination is also there or not. Another aspect of plasmid extraction is that the shape of the plasmid. So, the plasmid can be of circular double stranded shape and which can be super called or it might have some nick at one strand or it can be a nick of 2 strand.

So, depending on all this nick or your extraction procedure the plasmid might get distorted and the shape might change. To check if you have high quality plasmid which is basically the super coiled double stranded plasmid you need to run 1 person across gel, to check the plasmid how it looks and if there is any other contaminant present if you see sneer; that means, the DNA has been broken down in this smaller fragment. If you see different kind of fragments or at the very big chunk of DNA that might contains your genomic DNA, then you need to check the size and the shape of the plasmid by running in one percent agraose gel.

Now, this plasmid to store this plasmid for regular use you can store it at 4 degree, but for longer period of time if you want to store it, you have to keep it at minus 20 degree centigrade temperature. And you can use this plasmid in any kind of subsequent reactions like you can do PCR reaction, you can digest this plasmid using restriction enzyme you can use for transformation or transfection, you can do go for jean sequencing. If you have put any kind of gene in your vector and you have isolated that particular vector plasmid you can send it for sequencing to check if the particular jean is present or not.

So, based on your experiment you can isolate how much plasmid you need, how much good quality plasmid you can have. So, based on that you need to modify your protocol a little bit and try to get has good plasmid as possible.

Thank you.