Experimental Biochemistry
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Lecture - 39
Isolation and Characterization of DNA Part – II

Welcome back. So, in the previous lecture I talked about isolation and characterization of DNA and I just went over some basic concepts.

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So, this is the second lecture and in to this lecture, I will go through the same topic and I will focus mostly on the isolation of plasmid DNA from a bacterial source, because this is something that we will use in the next topic when we talk about recombinant DNA technology.

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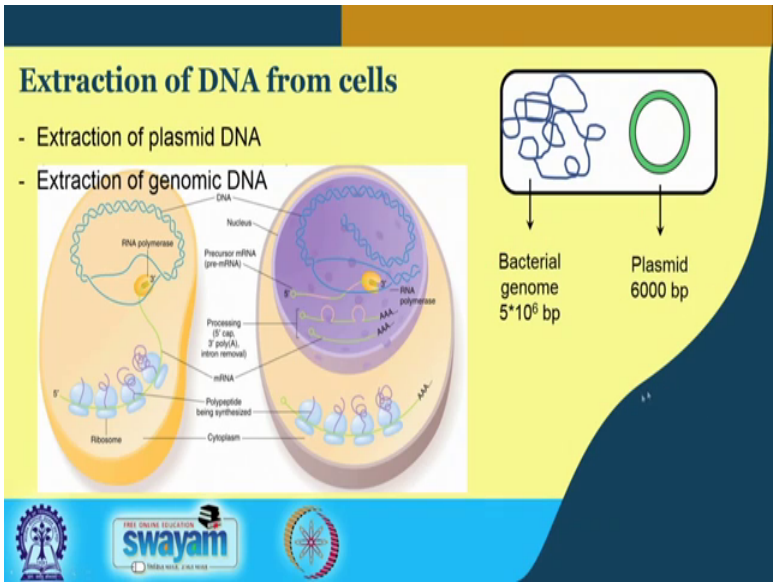

So, just a brief recapitulation of what we learnt in the previous lecture. This is let say a bacterial cell which has both the genomic DNA and the plasmid DNA. The genomic DNA of the bacteria let say it is a equalized cell will be in the range of 5 million base pair law and the plasmid DNA is again a circular DNA, but it is much smaller it is only 6000 base pairs law both are double stranded DNA.

So, if you look at the cellular organization, this is how a bacterial cell look's where everything is in the cytoplasm and so it means that when we break up in the cell all of these cellular components the DNA, the RNA that is synthesized, all the proteins that are there all of these will come out into the (Refer Time: 01:47). Similarly if we crack open eukaryotic cell where we have even more compartmentalized components in the cell what we can when we crack, crack open this cell all of these will come out along with a lot of membranes cellular membrane or different organal membranes.

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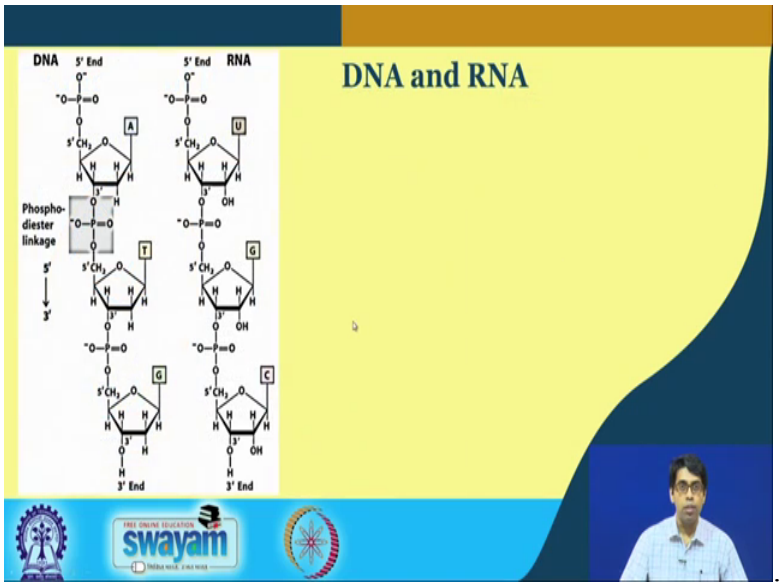

So, we talked about DNA and RNA because this is one of the challenges that we face when we are purifying DNA because DNA and RNA, they are chemically very similar. Eve is what we saw is that the sugar moiety is very similar except the H and OH at the 2 prime position the nitrogenous basis are very similar except one nitrogenous base which is T or Thymidine in case of DNA and Uracil in case of RNA and the third component phosphate group is exactly the same.

In case of both DNA and RNA the linear chain is formed by this Phosphodiester linkage between the 3 prime and the 5 prime positions in both DNA and RNA. So, that we have a 5 prime end and a 3 prime end the only difference that we saw in terms of structure was that DNA forms a double helical structure whereas, RNA is single stranded and no such double helical structures are seen, but it can form other complicated structures.

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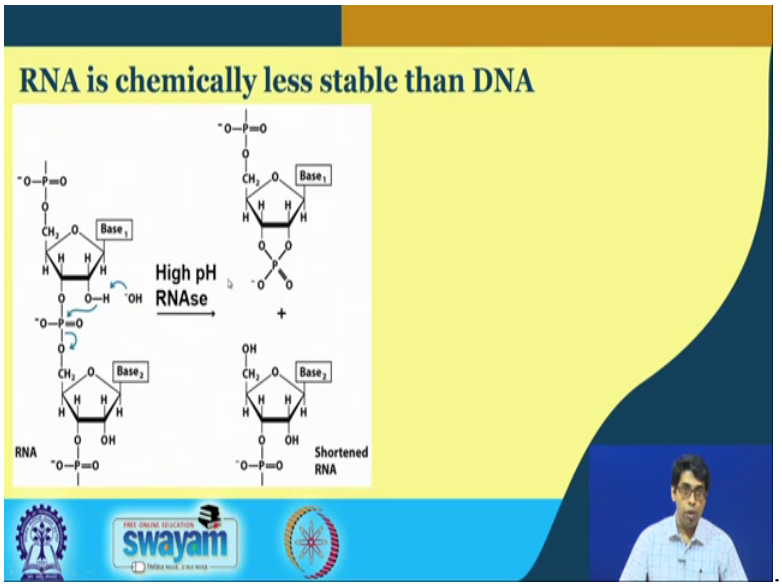

So, we also learnt that RNA is chemically less stable compared to DNA because of the presence of this OH group at the 2 prime position. So, if the ph of the solution is high then this hydroxyl group can attack this OH resulting in a disruption of the Phosphodiester linkage. So that this Phosphodiester linkage is broken and the RNA chain is also broken.

The same reaction can also be catalyzed by enzymes like RNA's. So, there are different types of RNA's and we can use some of these enzymes to selectively degrade the RNA molecule present in our solution without degrading the DNA. So, this is something that we are going to exploit when we purify our plasmid DNA.

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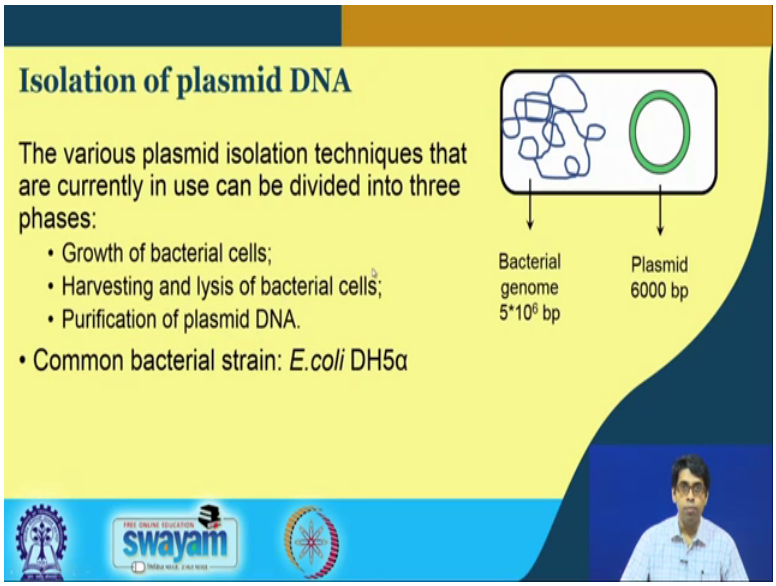

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So, there are various techniques of isolation of plasmid DNA and they vary in different terms, but if you look at all these techniques you can divide them into these 3 major phases and we are going to use something that is very common in all this different techniques that are available.

The first one is the growth of the bacterial cell because we are going to isolate the plasmid from some bacterial cell. So, you have to grow the bacterial cell and then isolate the plasmid from them. So, once the bacterial cell is grown you harvest it and we typically do it by centrifugation where the bacterial cells are precipitated out with through the media and then we work with that precipitated cells then we break open the cells to bring everything out into the solution that is the lysis of the bacterial cell and once everything is out into the solution then comes the purification step where we selectively purify we are going to selectively purify the plasmid DNA. So, for this exercise we are going to use a common E.coli strain called DH5alpha we choose this strain for the extraction of plasmid DNA because this strain can maintain a high copy number of plasmids ok.

So, the genomic DNA will have only one copy, but the plasmid DNA can have fifty or even hundred different copies in the same bacterial cell which results in a very high yield of the plasmid DNA that you will recover from your purification step. In the next lecture

when we talk about recombinant DNA technology we are going to again mostly talk about the DH5alpha strain and the extraction of plasmid and we will talk about what downstream experiments you can do from this purified DNA. When we talked about protein purification we talked about a different type of E.coli strain and that was bl21 de3 strain. So, in that case we used bl21 de3 strain because we wanted to make protein and there are certain a characteristic of that particular strain which are conducible for over expression of the protein of interest that we want to use. So, that we want to make more of our protein and then purified from that particular strain.

So, for proteins we use bl21 de3 and for plasmid DNA we use DH5 alpha E.coli strain.

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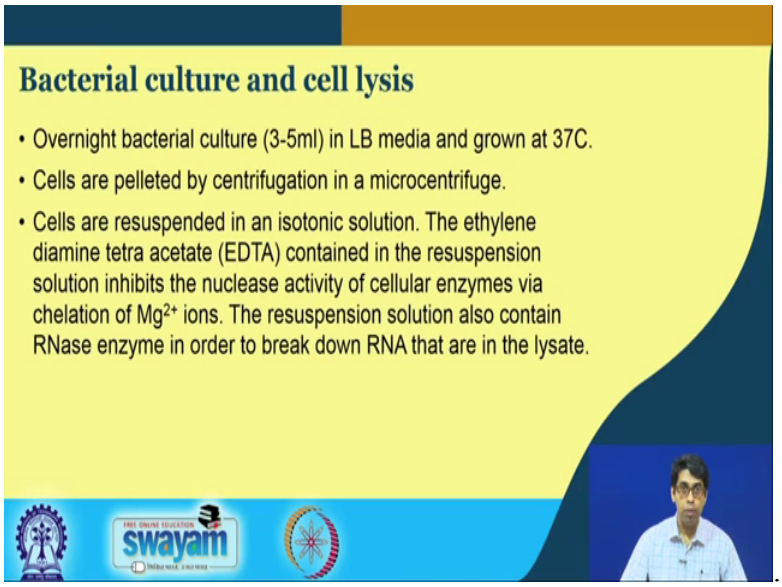

So, once you have your bacterial strain which is the DH5alpha and you have your plasmid which is inserted into that bacteria you growth at overnight culture this is something very similar to what we have done before to during protein purification the only difference is that in this case our cell culture is very small it is only 3 to 5 milliliter in the (Refer Time: 07:25) media in case of protein expression we used at least thousand ml culture. In this case we are using 5 ml culture and it is again grown at thirty seven degree centigrade and it can be grown overnight. So, that you let it grow for several hours 12 hours or 16 hours throughout the night and in come back in the next morning and do your plasmid purification.

So, you start your bacterial culture let say in the evening at 5 pm, you let it grow come back in the next morning at 9 in the lab now you have your dense bacterial culture in this 5 ml media you spin down the media in a in a centrifuge where it will result in the precipitation of all the cells. So, that is the cells are pelleted out and you have a clump of cells at the bottom of your centrifuge tube you throw the media from the top and now you work on the cell that is precipitated out. So, what we have to do is we have to re suspend these cells. So, we do that using a buffer which is called a isotonic solution. So, what is an isotonic solution isotonic solution means that if you have 2 different solutions and they will be called isotonic if the ionic strength of these 2 solutions are exactly the same. So, that if these two solutions are across a semi permeable membrane then you will not get much osmosis from one side to the other side.

So, this is very important because the bacterial cell membrane acts like a semi permeable membrane. We have intra cellular components so there are solids. So, it is basically a solution inside the bacteria and the bacteria is suspended in some buffer which has some ionic strength, if the ionic strength of these two of the interior of the bacteria and the buffer that we are using do not match then there will be osmosis which will result in either water flowing into the cells or flowing out of the cells and both are detrimental because water flowing into the cells will disrupt the bacterial cell. And it will result in lysis when we do not want lysis we want to lysis the cell eventually, but we want to do it in a controlled manner ok. So, that is why we use an isotonic solution. So, that we do not get lysis of the bacterial cell. This isotonic solution contains several things one of them is EDTA-Ethylene diethylene diamine tetra acetate.

So, this is something that binds specifically binds different divalent ions for example, it binds the magnesium ions and it reduces the, or it inactivates various nucleases which are present inside the cell. So, this nucleases can be de ionizes which can break down a DNA molecule and we do not want that. So, to protect our DNA molecule we add EDTA that will inactivate these nucleus enzymes in the bacterial cell. We also add from outside RNA's enzyme. So, this enzyme is something that very specifically breaks down RNA molecule as we saw that RNA is less stable and it this enzyme exploits that chemical reaction that mechanism to break down RNA specifically. So, that will remove the RNA contamination from our sample.

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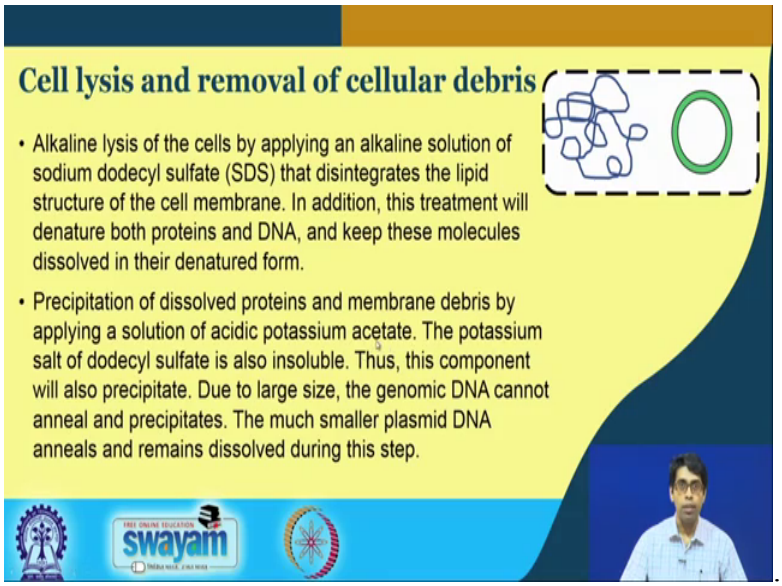

So, once we have re suspended our cell in the re suspension buffer we add the lysis buffer the lysis buffer has high salt concern concentration and it is also highly alkaline in nature, alkaline means the ph is very high. So, when we do that it results in the disruption of the cell membrane and this lysis buffer also contains sodium dodecyl sulfate or SDS. This is the same SDS that we use during SDS ph electrophoresis and we know the structure of SDS it has a charged head group and a very long hydrophobic tail. So, this is something that is very similar to the lipids in the cell membrane. So, it can easily disrupt the cell membrane structure and once the cell membrane is disrupted. So, now, the cell membrane is disrupted. So, everything that was inside now comes out into the solution. So, all the genomic DNA the plasmid DNA, RNA different proteins they are all out in the solution SDS will denature both proteins and DNA through hydrophobic interactions the high ph of the lysis buffer disrupts the formation of any hydrogen bond.

So, the double stranded DNA will also get denatured because of the disruption of the hydrogen bond the presence of RNA's a will chew up all the RNA that is present in the solution the there by converting it into very small fragments or monomeric nucleotides. So, at this point we have a mixture of all this things in the solution we have denatured genomic DNA we have denatured plasmid DNA and we have denatured protein. Now what we do is we add another solution which is called the precipitation solution, this solution is a solution of potassium acetate. So, this salt potassium salt of the acetic acid and it brings the ph again to the acetic range. So, it brings up the ph to the acetic range.

Now, since we are in the acetic range ph you can again form the hydrogen bonds the presence of dodecyl sulfate has already denatured the protein. So, the protein will not be able to refold and it precipitates the potassium salt of SDS is not very soluble. So, it also precipitates now the major difference comes between the genomic DNA and the plasmid DNA because chemically these two are very similar. So, this is where a proper experimental technique comes in. So, when we add this precipitation solution what you will see is this solution turns white because all this denatured protein and denatured genomic DNA they will start precipitating out and you will have to mix these two solutions because initially we had suspension solution then we had the alkaline lysis solution and now we have the precipitation solution we have to gently mix them so, that the genomic DNA if you mix it very vigorously then the mechanical agitation can actually start breaking the genomic DNA. So, we do not want that.

We gently mix this now the plasmid DNA which is much smaller can form the hydrogen bond. So, it can go back to it is double stranded form and it remains soluble, but in case of genomic DNA since it is. So, big we saw that it is several million base pairs long it is almost impossible to re anneal because it will not be able to find the necessary sequences to form the hydrogen bonds. So, the genomic DNA will not re anneal and it will remain in soluble and it will precipitate out. If we use very vigorous mixing at this step then what happens is the genomic DNA will start breaking down and it will form small fragments now if the fragments are small then they can easily find each other and become soluble. So, it is important to do this step very gently where we have to mix the solutions without breaking the genomic DNA's. So, that it can precipitate out and the plasmid DNA which is because of it is small size can very efficiently re anneal and remains soluble in the in the solution.

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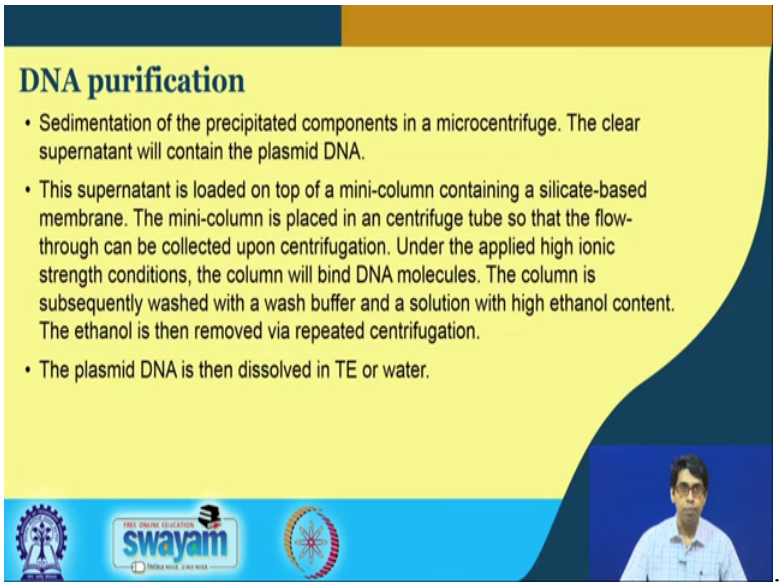

So, at this step what we get is insoluble protein, insoluble SDS, insoluble genomic DNA and soluble plasmid DNA. So, then again the microfuge micro centrifuge is put in a centrifuge and we spin it at a very high speed for a long time. So, we typically spin it for almost 10 minutes. So, that all this insoluble components are precipitated out and the soluble plasmid DNA remains in the solution. So, the solution becomes clear and in this clear supernatant we have the plasmid DNA so, that we can easily pipe it out from the top.

This clear supernatant which is taken out from the top is then loaded on to a small silica based column because this solution will contain digested nucleotides, it will contain several ions and all this different ions that we have added it might contain some of the small fragments of DNA and RNA. So, we have to remove all of them from the plasmid. So, that we get a very pure DNA plasmid DNA at the end of our purification step. So, to do that we add this supernatant on to a silica based membrane. Now silica based membrane has negative charge and this negative charge can interact with the positive ions that are on the surface of the double stranded DNA. This is done by using certain carotropic ions which can selectively facilitate this interaction between the plasmid DNA and the silica gel or silica membrane so, that the plasmid DNA sticks to this membrane everything else is washed out.

So, we do some washing step just like we used washing step during protein purification using various columns. So, here we also wash out anything that does not bind the membrane, our plasmid DNA binds to the membrane and it sticks there. Now to we have to elute out the plasmid DNA. So, we can do that by using a very non ionic solutions. So, we can use either water or we can use a buffer to elute out the plasmid DNA from the column. So, we also do this washing steps to remove ethanol because ethanol is something that is used in some of the steps and it can be detrimental for the subsequent use of the plasmid DNA as we will see in the next lecture we are mostly going to use this DNA to do polymerase chain reaction and there the ethanol can interfere with the polymerase chain reaction.

So, it is important to remove any of these contaminants from the plasmid DNA and that is done very efficiently by washing the silica column using wash buffers and then eluting it out using water or TE buffer. So, TE buffer is nothing, but Trees EDTA buffer which is at ph 8.

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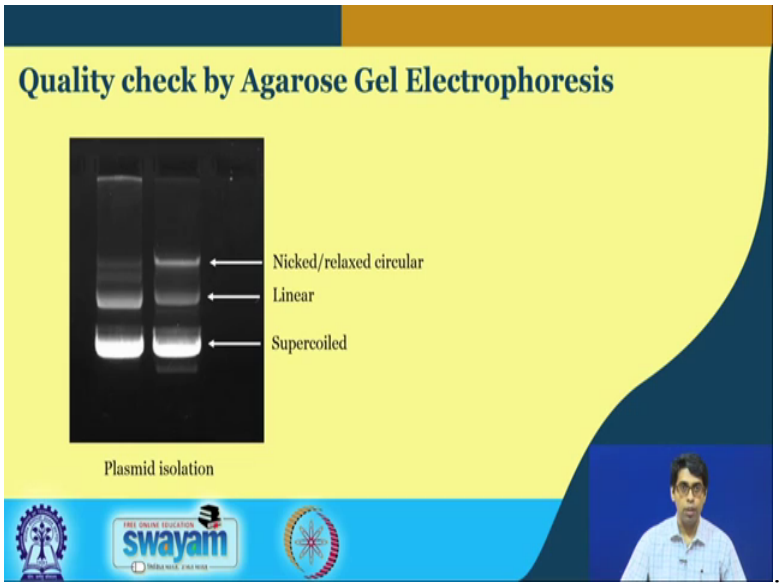

Now, once we have this eluted solution of our plasmid DNA we have to check it is purity and that is done using agarose gel electrophoresis, remember that DNA is a highly negatively charged molecule. So, we do not have to add anything like SDS to make it negatively charged. So, once you add your DNA sample to the wells and if this is the

positive end all the negatively charged DNA molecules will start moving towards the positive end.

So, in case of a pure plasmid sample we typically see several bands if you are not going to see just one band. The reason we see several bands is not because of contamination, but different states of the plasmid DNA. So, this one the one which is at the lowest at the bottom is the one which is running the fastest and this is the super coiled DNA. So, remember it is a circular DNA and that circular DNA can again form more coils on top of that resulting in a super coil which makes it is size highly condensed. So, since the size is small it will run very fast and that is what we see here some of the DNA molecules will get nicked it will not be remain circular. So, it will become linear. So, since the linear DNA has a bigger size it will run slowly compared to the super coiled DNA even though it is the same sequence same DNA it will run slowly and then the third one is where the circular DNA is highly relaxed and it runs even slower.

So, we typically see such 3 bands for any plasmid DNA that we have purified and this is quite acceptable. So, if you do not see any other bands here or here and all of this bands are in the right place that you expect. So, you have you should also run a molecular weight ladder and that will tell you that they are in the right molecular weight range then from that we can be very sure that our DNA is pure and we do not have any much we do not have much contamination from other genomic DNA. Once we have that we will also have to check the absorbance at two sixty and two eighty nanometers. Two sixty nanometer absorbance will tell us the quantity of the DNA that we have purified and the ratio of two sixty to two eighty will tell us whether we have protein or RNA contamination.

So, if the ratio is very close to 1.8, we know there is not much protein contamination and if it is not more than two so, it is between 1.8 to 2 then we know that there is not much RNA contamination.

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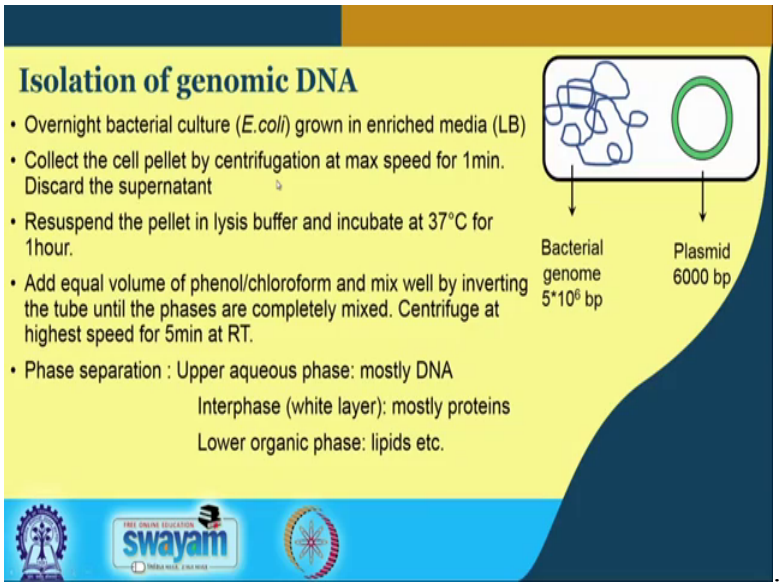

So, we talked about plasmid DNA isolation, but in many cases we have to do a genomic DNA isolation also. So, when we typically do a genomic DNA isolation we make sure that there is no plasmid present in that particular bacterial sample because it will be very difficult to eliminate the plasmid DNA from the genomic DNA. So, again you start with same bacterial culture in LB media then. So, you grow that overnight at thirty seven degree centigrade and in the next morning we pellet down the bacterial culture by centrifugation and discard the supernatant. So, the supernatant is the spent media and our bacteria will be at the bottom formula and then again we re-suspend the bacterial pellet in the lysis buffer just like the plasmid purification and break open the cell. So, that everything is now in the solution now in this case what we do is a different type of extraction.

So, we do instead of using the column that we used last time here we use phenol chloroform extraction. So, phenol chloroform extraction will result in different phase separation the top layer will be the aqueous layer which will mostly contain DNA because DNA is highly negative and it will be very soluble in the aqueous phase. There will be a interface a white layer which will contain mostly the denatured protein or even folded protein and the lower organic phase will contain all this cellular debris mostly the lipids which are highly hydrophobic.

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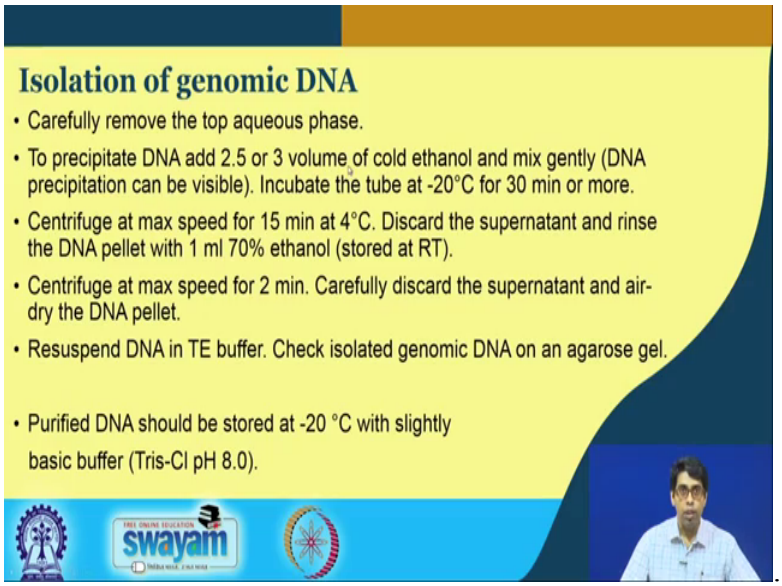

So, now we can carefully remove the top aqueous phase which contains the DNA and it can also contain some other contaminates. So, we have to now purify the DNA from this and it is done by selectively precipitating out DNA using cold ethanol. So, this aqueous solution is mixed with cold ethanol and this ethanol is something that is chilled at minus 20 degree centigrade and it is mixed. So, now, the DNA starts forming precipitates. So, what we see is a white precipitate we can centrifuge it at high speed. So, that all the DNA precipitated and then we remove the supernatant because that supernatant will contain the contaminants and our DNA is forming a white precipitate at the bottom. That white precipitate is re suspended in 70 percent ethanol. So, this is a washing step. So, we can do it once or we can do it several times. So, that we get highly pure white precipitate of DNA.

So, once we have done that we can re suspend the DNA in the same TE buffer as before we can check the purity of the DNA using an agarose gel and we can determine it is concentration using absorbance at a two sixty using the absorbance at two sixty nanometer and then we can store it at minus 20 degrees centigrade for downstream experiments.

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So, that is all for this week and in the next week we will see what we are going to use with this purified genomic or plasmid DNA how we use this DNA for recombinant DNA technology.

Thank you.