Experimental Biochemistry
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Lecture - 45
Cloning: Polymerase Chain Reaction, Restriction Enzyme Digestion and Ligation

Welcome back, I am Aditya Joshi Basak and today I am going to begin Cloning Experiment. So, I have already isolated cDNA from the spleen of mouse and today's experiment my objective is to amplify a particular gene of interest. So, as you can understand the spleen of mouse or any organism contains 100 of 100's of different genes. My research work dictates the requirement of a particular gene and my objective is to amplify that gene, extract it and then later on use it for my own experimental purposes.

So, for doing that the technique that one uses is polymerase chain reaction or PCR reaction and that is what we are going to demonstrate today. So, in order to set up a PCR reaction; a polymerase chain reaction, there are few components that you need to have at hand. So, you will require nucleus free water, you will require your template DNA for today's experiment as I had mentioned, I will be using cDNA isolated from the spleen of mouse so that will act as my template DNA. I will also use 2 primers a forward primer and a reverse primer with which I will specifically amplify a single particular gene of interest from my template DNA and most importantly I will need a PCR master mix.

So, basically the PCR master mix will contain a DNA polymerase for today's experiment, the master means that I am using contains Taq polymerase. Taq polymerase is a polymer is that has been isolated from a thermophile bacteria; thermus aquaticus.

So, the special property of this particular polymerase is that, it is stable even at high temperatures. Because as you will be knowing from your theory classes during the polymerase chain reaction, the enzyme periodically has to cycle through very high temperatures greater than 90 or 95 degree centigrade. So, normal polymerase might lose their activity at such high temperature, but Taq polymerase is remain functional during the PCR reaction.

Apart from Taq polymer is the PCR master mix also contains four types of DNTP's, it contains magnesium chloride and all this is suspended in a suitable buffer, usually Tri's

buffer. So, to summarize in order to set up a PCR reaction, you need to have a template DNA, you need to have your forward primer and reverse primer, you need to have your PCR super mix and to make up the volume to the right amount, you need to add nucleus free water to your reaction mixture. Be careful while you are using water do not just use any source of water, make sure that your water does not contain DNAs or RNAs. So, normally what we use is type 1 de-ionized water which has been specially treated; so, that the DNAs is have been already remove.

So, the first step when we are setting up a PCR reaction, at the very beginning what you must do is where fresh clean pair of gloves because, PCR is a very sensitive reaction and you do not want to contaminate your sample with nucleases are the do not use your bare hands while setting up this reaction because even the sweat in our hands they contain nucleases, they could damage and negatively impact your experiment and.

So, to summarize do not use used gloves, suppose in the lab you used a pair of gloves to clean, you have bench top and then wearing those you came to set up a PCR reaction try to avoid that, gloves are not expensive, each time you set up a new reaction where a fresh pair of glass. After you have done the PCR, you can use these gloves for some less sensitive purposes, but to emphasize for PCR new gloves always.

(Refer Slide Time: 04:58)

Ones you have prepared yourself in this manner, the next thing that you need to do is you need to thaw all the reaction components in ices. So, keep in mind that your PCR master

mix, your template DNA all your primers they are all stored in minus 20 degree centigrade. And, most importantly the PCR master mix that you have purchased or prepared in your lab. The main vial or the main stock is stored in minus 20 and a good practice is to make small aliquots of say 150 microliters, in different tubes that way you do not freeze thaw your entire amount of master mix at one go.

Each time you set up a reaction you take one tube and put it in ice you do not roam around with it in your hand, you put it in ice and by keeping it ice you thaw it gently and then you set up the reaction.

So, one thing that I recommend before setting a pure PCR reaction, what you should always do; is write down the components that you will be mixing together in your laboratory notebook. And each time you add a component just put a tick because keep in mind that in the molecular biology lab, we mostly deal with colorless liquids and today I am going to be demonstrating the setting up of a single PCR reaction. But while working in the lab you will see that, many times you need to set up simultaneously 5 or 6 different PCR reactions, at that time it is very difficult to lose track of how amount of which component has been added in which tube.

So, for such cases just write down what your adding and each time you add something put a tick this will help you, while working in the lab. So, while mixing the different components of the PCR reaction, keep in mind that the order in which you add the components is also quite important.

(Refer Slide Time: 07:05)

So, usually for so, today I will be setting up of 50 microlitre PCR reaction and the first component that I am going to add is 20 microlitres of nucleus free water; I have written down the recipe and you will be shown the recipe in a subsequent slide.

(Refer Slide Time: 07:20)

So, I am taking the nucleus free water from the ice bucket and now I am going to pipe it out 20 microlitres.

(Refer Slide Time: 07:43)

And add it to my PCR tube. So, PCR reactions are small volume reactions we only usually set them up in either 25 microlitre volume or 50 microlitre volume or 10 microlitre volumes.

So, you will notice that PCR tubes are much smaller in size than a regular centrifuge tubes. And while pipetting into PCR tubes, keep in mind that is very easy to form bubbles and it is not good practice to form bubble. So, pipette things very carefully, take your time, be slow, but make sure that you have as minimum number of bubble are possible with practice, you can end up mixing everything without forming any bubbles.

So, first we added 20 microlitres of our nucleus free water; now I am going to add my template DNA. So, I am going to take 3 microlitres of template DNA, in this case for today's experiment its cDNA isolated from the spleen of a mouse; so, I am going to measure 3 microlitres of template DNA and added to my PCR tube.

(Refer Slide Time: 08:53)

So, after adding the nucleus free water, next what I am going to add is the template DNA for today's experiment, I am using cDNA isolated from the spleen of mouse as the template DNA.

(Refer Slide Time: 09:09)

And I am going to add 3 microlitres of that to my PCR cube keep them back an ice take a chap.

(Refer Slide Time: 09:23)

Be careful so that you mix them properly, but at the same time you do not introduce bubbles into your PCR tube ok. I have added the template DNA; so, I am just going to put a tick here and after this I am going to add by primers, I will begin by adding 1 microlitre of my forward primer. And ones I have added that I am going to add 1 microlitre of my reverse primer.

So, first I am going to add my forward primer just you do not need much of it, 1 microlitre is sufficient if you have a 20 micromolar working solution of your primers. So, yes I have added the forward primer I will just put a tick because see, while working in the lab you will notice that I am adding very small volumes. So, even by seeing the tube it will not be possible to understand whether I have added 1 microlitre or not. If I was adding say, 500 microlitres to a particular tube I could look at it and say, I have or have not added, but since we are working with very small volumes of colorless liquids add them and put a tick in your laboratory notebook that will help you about many mistakes.

So, I have added the reverse primer as well just discard the tube, release my pipette and yes once it is released just put a tick. So till now, I have added nucleus free water, the template DNA, my forward primer and reverse primer.

The last component that I am going to add to my PCR tube is my 2x PCR master mix. Since I set up 50 microlitre reaction; I want to set up a 50 microlitre reaction I am going to add 25 microlitres of my 2x master mix.

(Refer Slide Time: 12:07)

So, just set up my pipette.

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And now, we are going to take the PCA master mix, when we had taken it out from the minus 20 refrigerator it was (Refer Time: 12:23) on ice, I am going to put it back on ice again.

(Refer Slide Time: 12:32)

And now I am going to add this to my PCR tube ok. Now that I have added it before proceeding further I need to make sure that all the components have been properly mixed together. So, I am just going to pipette the whole mixture up and down a couple of times. Be very gentle it is very easy to introduce bubbles at this stage and it is not good lab practice to have bubbles in your PCR tube careful.

So, I have thoroughly mix together all the different components of the PCR reaction in the tube, you can pipette it sometimes some people prefer centrifuging the tube once just to mix together everything thoroughly, you can use whatever technique is comfortable with you. And once that you have added everything just put your PCR tube in ice, until and unless you are ready to set it up in the thermo cycler machine.

(Refer Slide Time: 13:47)

So, we have mixed together the different components for setting up the polymerase chain reaction and we kept the PCR tube in ice. So now, I have switched on this machine; so, this machine is a thermal cycler. And now I am going to set up a particular reaction condition under which the PCR is going to take place. So, as you can see here there are 3 stages in this particular PCR reaction that I have chosen today, in stage 1 for 3 minutes the temperature will be made at 95 degrees at the very beginning of the reaction.

After that, the reaction will move on to stage 2; so, stage two is where the actual amplification of our gene of interest will occur. So, what will happen is initially for 45 seconds the temperature will be kept at 95 degrees, after which the temperature is going to be brought down to 56 degree centigrade for and be maintained there for another 45 seconds and then finally, the temperature will be raised to 72 degree centigrade and maintained for 1 minute. This entire pattern or triplet of temperature cyclings constitutes one entire PCR cycle, this stage 2 will be repeated 35 times; so, I have 35 such cycles.

So, to summarize initially for 45 seconds we maintain the tube at 95 degrees, then we lower the tube 256 degrees and maintain it for another 45 seconds then the temperature is slightly raised to 72 degrees and maintain for 1 minute. After that again the temperature is raised from 95, 295 degree centigrade for maintain for 45 seconds, brought down to 56 degree centigrade, maintain for 45 seconds, raised to 72 degree centigrade, maintain for

1 minute and again the third cycle begins as I have described. So, 35 such cycles is what I have chosen for today's PCR reaction.

So, at 95 degree centigrade what happens is? Basically your double stranded template DNA become single stranded, the double strand opens up. At 56 degrees what will happen is that the primers will (Refer Time: 16:16) to their complementary sites and at 72 degrees what is expected to happen is that, primer elongation will take place. Which is why we need 3 different temperatures and this is why this machine is commonly called as a thermal cycler. It can quickly change temperatures in very short span of time from 95 to 56 to 72 and it keeps on doing it repeatedly.

After 35 such cycles have occurred, we will move on to stage 3 of our PCR reaction which is the final stage where we are going to carry out elongation at 72 degree centigrade for one last time, for a prolong time period I have set up for 7 minutes. After that, the machine will bring down the temperature to 4 degree centigrade and it is written that it will be maintained for infinite. So, basically what means is that after the reaction is over, the tube will be maintained at 4 degrees until and unless you take out your sample and switch off the machine. So, we have a 50 microlitre PCR reaction and this is the template that I am going to be using.

(Refer Slide Time: 17:37)

So, now I am just going to choose the block.

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So, we have 3 different blocks, which means we can run 3 independent PCR reaction simultaneously.

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Today I am going to run only one, so it is in block 2 I will just raise it, I will take my PCR tube from ice and put it here.

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So, its very simple just put you tube back here and carefully bring down the lid you need to be careful. So, I have set up the reaction and now I am going to take my PCR tube in this block, I will put my PCR tube here and you need to be very careful the inner surface of the lid of this block is actually very hot do not touch it, you might end of burning your fingers, then you bring down the lid.

So, basically in this thermal cycler we can set up three different PCR reaction simultaneously, today we are going to do only one. Now that I have put my PCR tube in the thermal cycler block 2, what I am going to do is I will start the PCR reaction. So, it is beginning excuse me.

(Refer Slide Time: 18:42)

So, as you can see now it will take around 2 hours for the reaction to complete and the temperature is raising I just show you what is happening.

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So, at this state right now the temperature is being raised to 95 degrees and subsequently we will see that for the next 3 minutes, it will be maintained at 95 to cover stage 1, stage 2 is the stage at which actual amplification of your gene of interest will occur. So, for 35 times it will keep on varying the temperature from 95 to 56 to 72 repeatedly.

So, these temperatures have been decided upon depending upon the experiment that one wants to do. And the machine is showing that after close to 2 hours, the reaction will be done and they will store the PCR tube at 4 degrees until and unless I come and retrieve the tube and switch off the machine.

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So, our PCR reaction is over, what I am going to do now is remove my sample from the machine stick out the tube which is back so, keep it here. So now, what I need to do is I need to check if at all my gene of interest has been amplified or not and the way we do that is I am going to take 5 microlitres of this sample and I am going to run it on a agoras gel. And if I see any bands corresponding to the length of the jean or PCR product that I am expecting; it means, that the PCR reaction has occurred successfully.

After PCR amplification of the gene of interest, we will next move on to the double digestion step; however, prior to that what we need to do is, we need to remove the excess or unused, primers that were used during the PCR or we need to remove the different enzymes that are present. And the way we do that is we used commercially available kits known as PCR clean up kits which help us to isolate or extract only the DNA and get rid of the small primer fragments as well as enzymes and other impurities. After that what I am going to do is. Now I am going to setup or double digestion reaction.

(Refer Slide Time: 21:47)

So, in order to do that I am going to take 40 microlitres of my PCR my product that I have, I will just set a pipette at 40 so basically I am setting of a 50 micrometer double digestion reaction depending on the concentration of DNA that I have.

(Refer Slide Time: 22:14)

So, I will going to need for today's reaction 40 microliters of DNA, if you remember I had set up a 50 microlitre PCR reaction.

(Refer Slide Time: 22:33)

I had used 5 microlitres for analyzing it on a agoras gel and I still have sufficient amount for digesting. So, I have now taken 40 microliters of the DNA. Next, I am going to add water I will add 3 microlitres of nucleus free water, added the water, I had already added the template the amplifying DNA and I have added water.

Next I am going to add 3 microlitres of the buffer in which my restriction enzymes are most active. Be careful to note that different restriction enzymes are efficient or active in different buffers. So, always check the product information of your restriction enzymes and find out which buffer is ideal for showing 100 percent activity of the enzyme. So, I am going to take that buffer and I am going to use 5 micrometres of that buffer (Refer Time: 24:30) from buffer and add it into my digestion mixture, add added DNA, I have added water, I have added buffer.

So now I am going to add my two different restriction enzymes 1 microliter of each and yes. So, restriction enzyme one take 1 microlitre of that. So, when your pipetting small values always check that you actually have collected some liquid in your pipette tube. Often it might be that specially when your tube is almost empty might be that, you think you have taken out something, but you do not and then if you miss adding that your reaction will not work. So, when your pipetting such small volumes 1 micrometre or 2 microlitre, the best way is to look closely and see that you have some liquid in your tip and then proceed.

So, I have added my second restriction in the nuclease gives. So, just release my pipette is for proceeding, always release pipettes do not store them under stressed condition because then you will damage your instrument. So, I have added both my restriction enzymes and thus I have set up my 50 microlitre double digestion reaction.

(Refer Slide Time: 26:31)

So, what I am going to do is? Now, I am going to incubate this at 37 degree centigrade for 1 hour. So, how long you need to carry out digestion also depends on the type of enzyme that you are using some enzymes have been modified or enhanced; so that they can work in a quick manner and some enzymes need a bit more time depending on the enzymes that you are using check the information. For today's experiment I am going to set up a digestion reaction which is going to continue for 1 hour and it is going to take place of 37 degrees. So, I am going to basically keep the digestion reaction in a water bath.

So I have now set up the digestion reaction for the insert, keep in mind that you also need to digest your vector into which you want to clone your gene of interest. So, I have also done that I am not showing that to you, but similar to this I have also set up a digestion; double digestion reaction for digesting the vector which I want to clone my gene of interest. And I am sure you know from your theory classes that for cloning to occur, you need to make sure that both your gene of interest as well as your vector have been

digested with the same pair of restriction enzymes. Now, we are just going to set up those tubes in the 37 degree water bath.

So now we are going to digest both are vector as well as insert we have already added the restriction enzymes and set up the digestion reaction, our enzyme works based at 37 degrees when kept for 1 hour.

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So, we are going to use a 37 degree water bath as you can see the temperature of the water inside this water bath is maintained a 37 degree centigrade.

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And now what I am going to do is basically, I will take my two vials containing my vector and inserts set up for digestion and keep it here in 37 degree centigrade for 1 hour; so, the digestion will take place for 1 hour.

So, after digesting both the vector as well as our PCR product with the same set of digestion restriction digestion enzymes what we did was, we ran them on an agoras gel and after digestion, see no process is 100 percent efficient. So, there is always possibility that some amount of your vector is undigested, while usually a larger portion of a vector is digested by both enzymes.

So, the vector that I am using today, if it is undigested then it has a size of approximately 7 to 8 KB; Kilo Basis, but if it is digested by this particular set of enzymes then the portion which gets removed has a size of around 2 kilo base and the remaining vector is 5 kilo basis. So, what we did was after digestion we ran the vector on the gel and after analyzing the gel, the band which corresponded to the 5 kilo base VGL which is our digested vector, we just cut out that portion of the agoras gel and melted the gel and extract, the digested vector DNA from there.

So, after setting of digestion in a tube, you do not use the entire tube contains for ligation, you first purify and extract the digested portion of DNA by running it through an agoras gel and cutting out the region which correspondence to your; which correspondence to your digested product. So, similarly not only the vector, but also the PCR product of interest has been digested and then purify it by running it through a gel and cutting out only the band of interest.

So, I have already done that and now, what I am going to do is I am going to set up a ligation reaction. So, for that I am going to first take so the recipe we will provide you with the recipe elsewhere I have just written it down while I am doing the reaction so that I can keep tract what I have added.

(Refer Slide Time: 31:03)

So, first I am going to take 11 and half, 11.5 microlitres of nucleus free water yes.

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So, I will take 11.5 mictrolitres of nucleus free water, careful while pipetting.

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Next I am going to take, I will just release the pipette. Next I am going to add my buffer; so, keep in mind ligation, the ligase that I am going to use today T for DNA ligase, it comes with the special buffer which contains ATP. So, ligation is an ATP dependent process therefore, the buffer that you are going to use contains ATP and you should always store it in ice because if the buffer goes bad the ligation reaction will not take place.

So, I am going to just take 2 microlitres of that buffer now, let me just set up the pipette yes. So, a 10x buffer I will just take it out of ice pipette of 2 microlitres and put it back on ice and add the buffer here.

So, I have already added the water and I have added the buffer, now I am going to add 2 microlitres of my double digestion vector. So, I will take 2 microlitres of my double digested vector and add it to my reaction tube also as I keep adding, I just ensure the things are mixed properly by pipetting it couple of times, I have added excuse me, I have added my vector. Now I am going to add 4 microlitres of my PCR product which has also been digested by the same set of restriction enzymes.

Now, keep in mind that volumes here do not have much meaning basically, the way this reactions a set is that you or I personally referred that you should have a 1 is to 3 molar ratio where you have, 1 part if digested vector and you have 3 parts of double digested inserts. So, you have excess double digested insert compare to your vector, you can also

set up a 1 is to 5 or even sometimes 1 is to 1 ratio of vector and insert in your ligation reaction.

So till now, I have added the nucleus free water, I have added the 10x ligation buffer, I have added my double digested PCR product and my double digested empty vector. So now, I am going to add the last component basically, that is left for the ligation reaction to be initiated. I am going to add 0.5 microlitres of ligase; T for DNA ligase, I am using d for DNA ligase for today's experiment. So, I will just take a very small amount 0.5 microlitres add it to my ligation mixture. So, we can pipette it up down a couple of times, I will just increase the volume and I will mix it thoroughly.

And; so, the ligation reaction is going to take place at room temperature for half an hour. So, we will just leave the ligation mixture in a tube on our lab bench at room temperature for half an hour, after which we are going to proceed with our experiment.