Essentials of Biomolecules : Nucleic Acids, Peptides and Carbohydrates

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Lecture - 12
Major components and steps involved in Polymers chain reaction

Hello everybody and welcome back to biomolecules. So, we have been discussing about the technique of PCR Polymers Chain Reaction. And we have seen in the last lecture that how the process of DNA replication that happens in living cells can be modified in vitro in the laboratory and can be simplified by using only a handful of the ingredients rather than the whole lot of the enzymes.

So, we have seen that instead of using the many of those kinds of enzymes you can simply use heat and can generate 2 single stranded DNA out of your sample double stranded DNA and then the process can be further can be amplified or you can do the extension using the DNA primers instead of the RNA primers.

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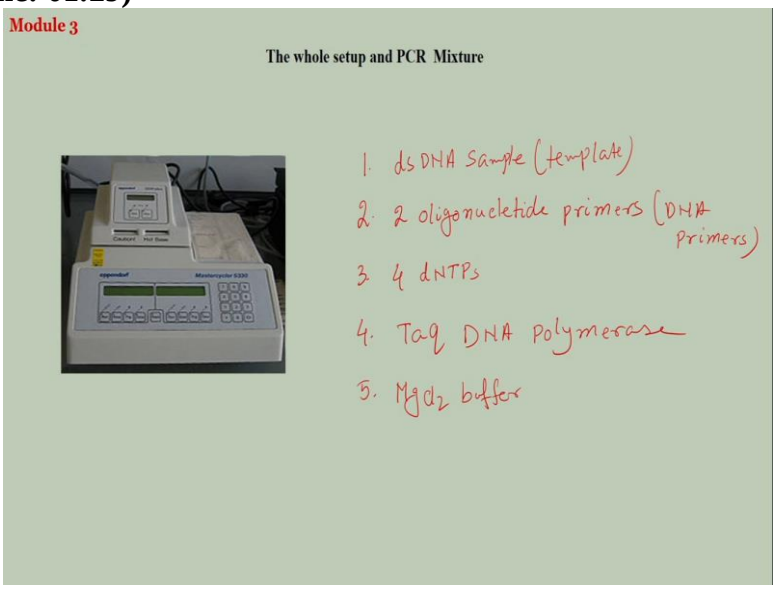

So I will write one second, write down 1 second, what are the ingredients that will be required for the PCR and then we will move we will show the individual steps how it works. So, first, what you need is ofcourse, your DNA sample, double stranded DNA sample that will add as your template. That is your template DNA. And next what you need is, you need the oligonucleotide primers, and we need 2 DNA primers, each for 1 strand, 1 for each strand, so 2 oligonucleotide primers or DNA primers.

Number 3 unit is all the 4 individual dNTPs, 4 dNTPs dATPs, dCTPs, dDTPs and dGTPs individually. And number 4, ofcourse, you need a single enzyme that is the DNA polymerase. And we need specifically we use taq polymerase or called taq DNA polymerase. And you as a fifth ingredient that is you need a medium across medium we use the buffer and in that buffer you have to use some salt because as I mentioned, when you have double stranded DNA, it is because of the negative charge for spread groups, they repel each other.

So, in order to nullify the negative charge repulsion, we usually use some metal salts in it. You can use sodium hydrochloride, you can use magnesium hydrochloride for this specific purpose we have we usually use magnesium chloride, because you will see that the DNA polymerase taq polymerase is also request or also uses magnesium 2+ as its cofactor or for its function buffer. So that is what all these ingredients you need.

And all these steps are they are put in a small vial, which is called the PCR vial and that is kept in this machine. This is the machine and then the machine is you can program the machine to do the individual steps.

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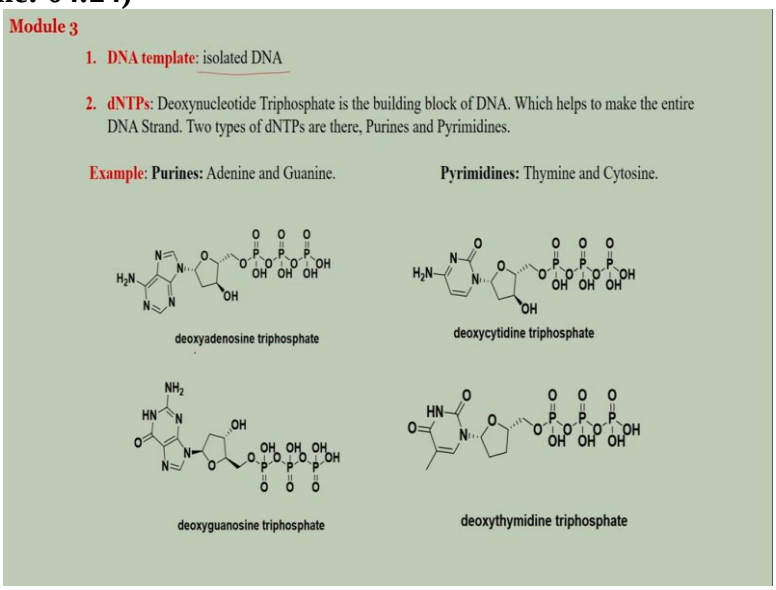

So, here it is, if you look at the structures, so you need the first one is the DNA template, which is the isolated DNA or your target DNA. Then you need all the individual dNTPs deoxynucleotide triphosphates that is the building block of DNA, which helps to make the entire DNA strand. 2 types of dNTPs are there, ofcourse you know purines and pyrimidines these are of purine class. So, this is the triphosphate three phosphate midines it can stay as protonated or in buffer solution depending upon the pH it will stay as deprotonated also.

So, this is adenine and this is deoxyguanosine triphosphate. Pyrimidines ones are the cited in triphosphates, this is cytosine. So this makes us cytidine and this is the thymidine triphosphates. So, first, two and second.

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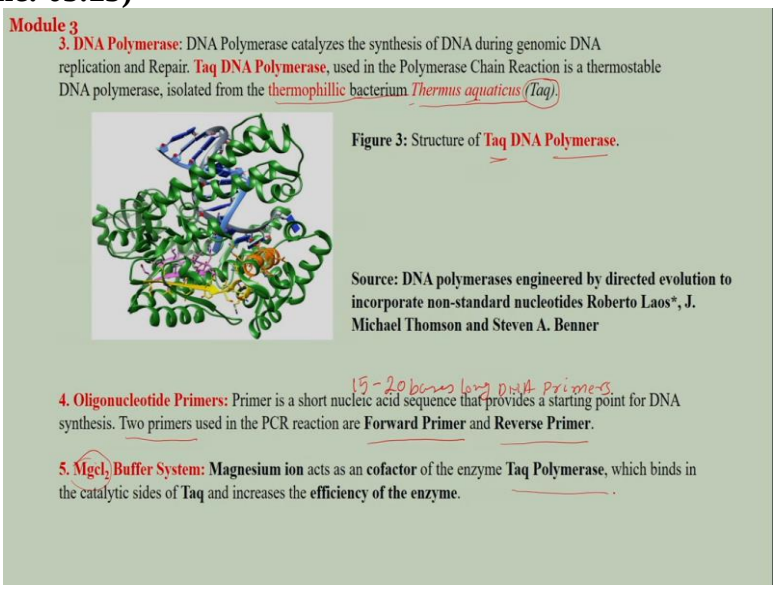

Third unit is the DNA polymerase. This is the structure of a typical DNA taq polymerase, typical taq polymerase. So, DNA polymerase that catalyzes the synthesis of DNA during genomic DNA replication and repair. So, that is for the normal cells cell replication. Now, taq DNA polymerase that is used in the polymerase chain reaction, that is a thermo stable DNA polymerase as I mentioned. Because since we use the high temperature to break open the double stranded DNA, you need your DNA polymerase to function at higher temperature as well.

So, that is where this particular polymerase was isolated from a thermostable bacterium which is known as the thermophilic bacterium. Thermophilic bacterium are stable at higher temperatures are they actually preferred to function at higher temperature. And this particular type of bacteria have been isolated from hot springs, because that has very high temperature actually. And so, this is the species Thermos aquaticus. Aquaticus means water, Thermos is temperature.

So, if you look at the name Taq, came from actually there. So, T for the thermos and aq for the aquaticus. So, that is why we call it this specific type of polymerase are known as taq polymerase, taq polymerase or taq DNA polymerase. This is typical structure and you can see

this is the double stranded DNA helix. The blue color is the double helical DNA and it shows how the target DNA is bound to the polymerase to the enzyme.

So, this is the enzyme substrate co-crysto structure that demonstrates how the DNA is bound to the active side of the DNA polymerase. So, the fourth is oligonucleotide primer as I have talked about, we no longer use RNA primers. Since we can synthesize oligonucleotides we use the DNA primers. So, and this primers are actually short nucleic acid sequences. Typically, for PCR, those primers are usually 15 to 20 bases long DNA primers. So, primers are about 15 to 20 nucleic bases long.

That we use for PCR, because that provides a starting point for DNA synthesis. Now, we need 2 primers, one for each strand, one is called the forward primer, other is called the reverse primer. And fifth the last one is you need magnesium chloride buffer solution, because magnesium iron acts as a cofactor of the enzyme that DNA polymerase, which binds in the catalytic sides of taq and increases the efficiency of the enzyme.

You will see later on also, or maybe you already know, that most of the enzymes they require either a coenzyme or a cofactor for its proper function. Because all these cofactors are all those coenzymes they actually participate in the catalytic processes. Sometimes electron transfer sometimes redox reaction, sometimes other things. So, most of them will do require cofactor or a coenzyme.

So, in this case, taq polymerase requires magnesium 2+ as a cofactor. Now, I will come back to the steps. So, mainly the whole of the PCR goes in 3 different stages. I will call them stages, 3 different stages.

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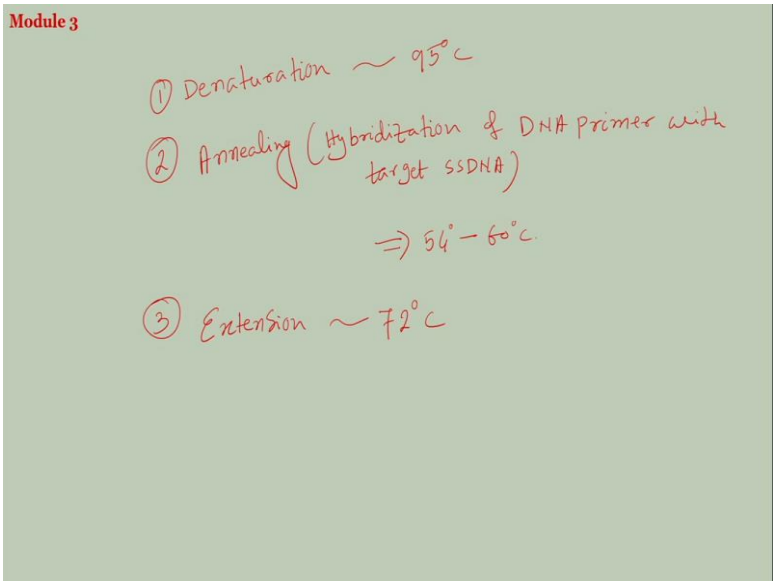

The first stage is known as Denaturation that is where your target double stranded DNA will be separated into 2 individuals single stranded DNA. And obviously, this requires a higher temperature, round about 95 degrees Celsius temperature we use for that. Second step is what you have used the DNA primers, so, they have to hybridize to the target DNA. So, that is the term is known as annealing or hybridization Hybridization of DNA primer with target now it is a single stranded DNA SSDNA. And this here the temperature can vary.

Typically, you can use 54 degrees Celsius to maybe 60 degrees Celsius temperature. Number 3 is the actual thing that is where the polymerase is bound and doing the extension. So this is known as extension. So, that is where your actual replication is happening using the target single stranded DNA as the template and using the DNA primer as the base. So extension typically happens at 72 degrees Celsius. And ofcourse, you need the polymerase for it.

So, if you look at these, these are the main stages that pressure undergoes. Now, you do not have to do anything else, it is just only controlling the temperature. So, the whole of the PCR technique is actually once you add all the ingredients to it, all you need to do is to change the temperature and you can program the machine to change the temperature over time. And that is all you have to do, then it will go on by itself and it will, the cycle will keep on going. So, now if we go to the individual step. So you have that double stranded DNA at the beginning that you need to separate into 2 single stranded DNA.

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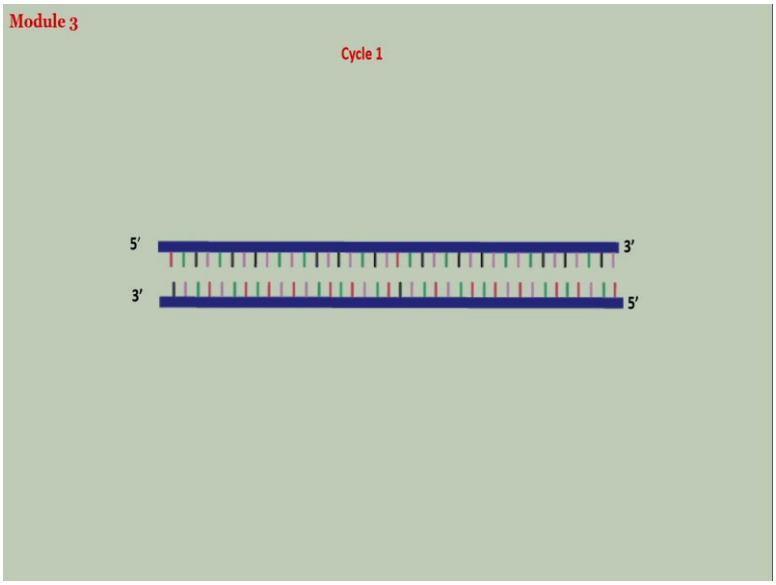

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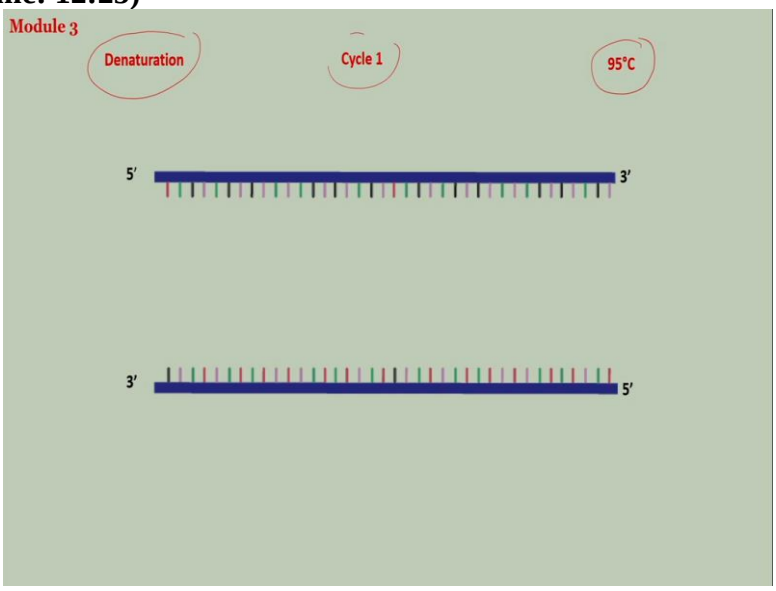

So, the cycle 1 and the first is your denaturation and that is typically done at 95 degree Celsius temperature. So, that will make open the double helix into 2 individual strands. So, ofcourse you need the higher temperature, because otherwise what will happen, you may have the non specific hydrogen bondings or non specific interaction is still left over.

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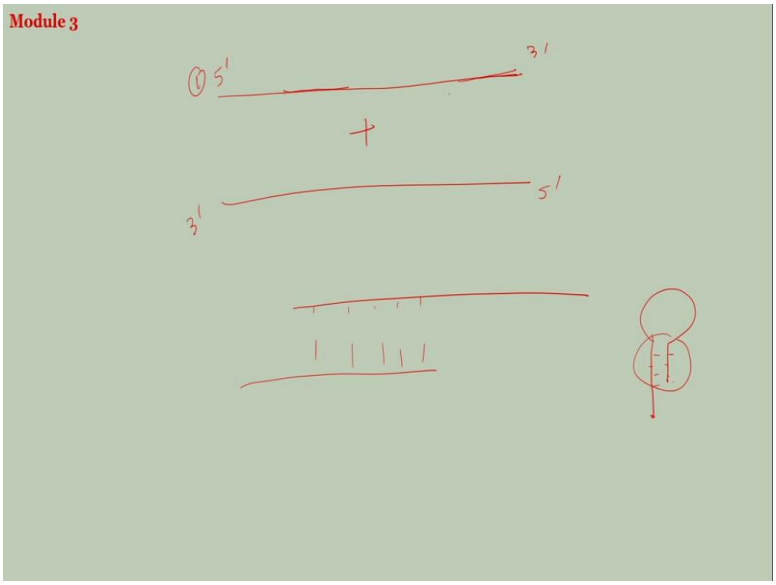

For example, if this is your single stranded DNA, DNA 1 and so 5 prime to 3 prime and 3 prime to 5 prime. You have opened them, let us say that they might have opened and what can happen if you do it at lower temperature, you can have the non-specific interactions or maybe the non-specific interaction to already exist, such as the full length of DNA will not be complimentary to exactly this sequence maybe half of this.

So, this is not a proper hybridization, partial hybridization can happen and that is a problem or sometimes even what happens, the same strand can have self-complementarity in it. In other words, maybe this part, this part is complimentary to this segment. Then they will fold together the DNA sometimes exist in this fashion, they will fold together like this and make a self-complimentary. So, in this fashion, you have a double stranded DNA and that is the driving force to form the 4 lead structures.

So, in order to avoid all these non-specific hydrogen warnings or non-specific based interactions at the beginning, the temperature is kept at high shootup. So, that is what your denaturation and you get you to single strands.

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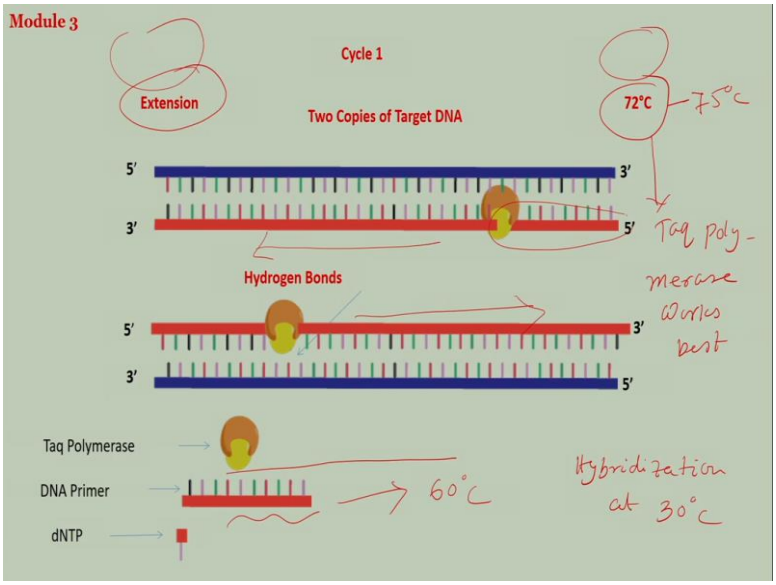

After that your DNA primer has to come. And now, since you have to hybridize your DNA primer with the target DNA for a partial hydrogen bonding of the whole primer was this the part of the now single stranded DNA or now the template DNA. So, therefore, now you cannot keep the temperature still high, because at higher temperature, these all these hybridization cannot take place.

The melting temperature of these double stranded DNA with the primer and the target with the primary and the target, the melting temperature are maybe in the range of, typically it can go for 55 to 75 degree Celsius. So, therefore, above this temperature, they will be hybridized or they will be stay as single stranded DNA. So, 95 degree Celsius temperature you cannot keep for the hybridization of the DNA primer with the target, which we call annealing or annealing hybridization, annealing means hybridization.

So basically, you have to bring down the temperature. And here is something that you have to play a little bit around. It, of course, depends upon the sequence of the DNA primer you have, or sequence of the DNA primer that you have to use. Because depending upon the GC content, depending upon the number of AT content, the melting temperature of this DNA primer that will vary.

And therefore, you have to find a optimized temperature where you can have the maximum or the full hybridization, the desired hybridization. Now, let us say that if you are melting temperature of this DNA primer, is around 60 degrees and that means, above 60 degree, this will open up, below 60 degree, it will have the peering, full hybridization. Now, if you do this

reaction, or if you allow this hybridization to happen at too low temperature, let us say if you do the hybridization at 30 degrees Celsius, then what will happen?

You will get your hybridization ofcourse, in this temperature it should happen. But at the same time, there could be other hybridizations which are partial hybridizations. For example, as I have just shown in the earlier case, the full with the full target DNA, your full of the primer may not hybridize, half of this primer may hybridized and that melting temperature, maybe above 30. So at 30 degrees Celsius temperature, this hybridization is fully stable, and it will stay in solution.

But that will create problem for your extension. What your desire is, to get the whole of the primer, perfectly hybridized. So, to avoid all this non-specific interactions again, you have to play around with what should be your optimized annealing temperature. In this case, we are keeping it at 60 degree, just for a generalized thing. It can vary as I mentioned, it can vary from maybe 54 degree to 62 degrees, 63 degrees, something like that, depending upon your sequence of the DNA primer.

So, 95 degree back to lower temperature to get a good hybridization. And now comes your taq polymerase this. They will come, they will bind here and here. So, your second step was done. Now, we are on the third stage that is the taq polymerase is coming there. So, we have to again change the temperature that I will show again. So, the dNTPs that are floating around in the solution. So, once the taq polymer is bound, the dNTPs would be picked up switch heavily, seeing what is there in the opposition.

And then you will have the extension in this direction, here the extension in this direction and this stage is called the extension states. And that is done at 72 degrees Celsius temperature. So annealing at 60 degrees for the extension or for the synthesis of the new strand temperature has to be 72 degrees elevated again from 60 degree to 72 degree. The reason is taq polymerase you know, they are thermostable polymerase.

And in fact, they function at higher temperature. So, 72 degree temperature is the optimum temperature where the taq polymerase activity of taq polymerase is based. Taq polymerase is works best at 72 degrees Celsius temperature. That is why the extension always done at nearly around 72 degrees Celsius temperature.

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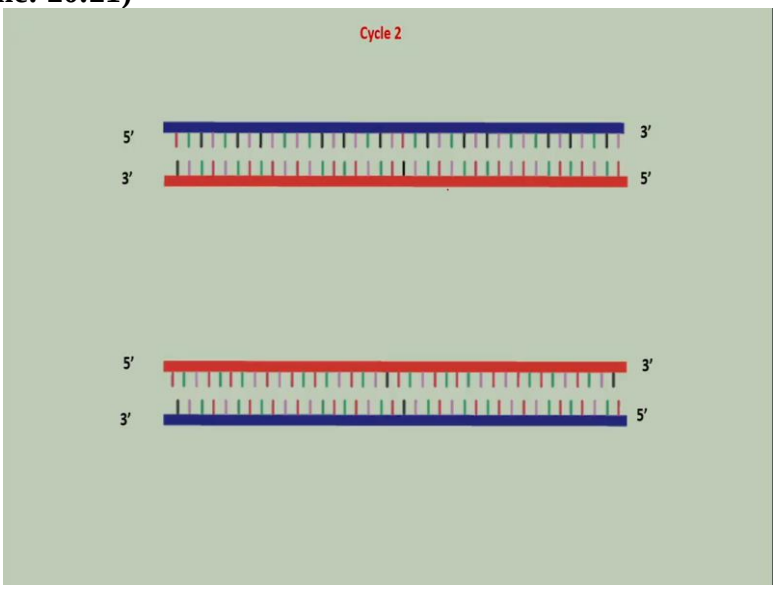

So, now you have your amplified DNA, first after the completion of the first cycle 2 DNA from one target DNA, you have now 2 DNA.

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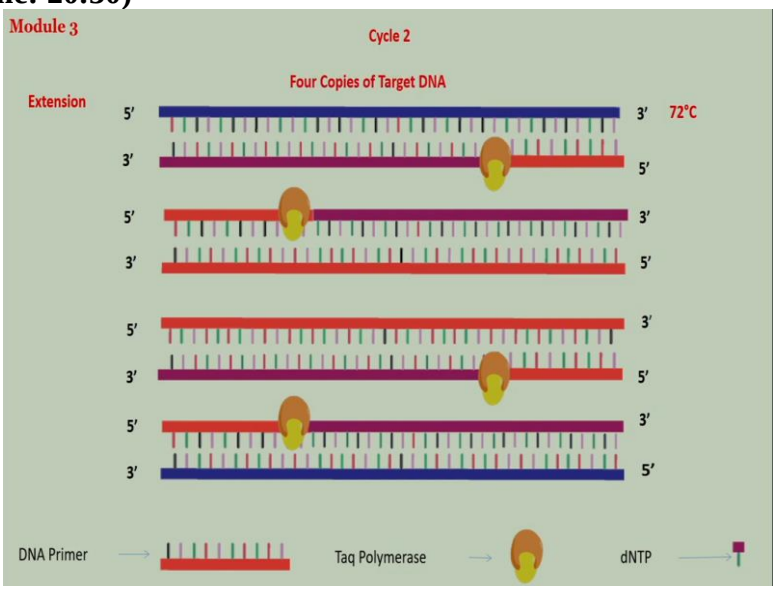

Now move on to cycle 2. Again you will have the individual strengths. Primer is the temperature bring in polyamerase all the dNTPs would be picked up and the new strengths would be synthesized. Again the temperature would be changed to 72. So, 4 copies, 4 copies of target DNA now.

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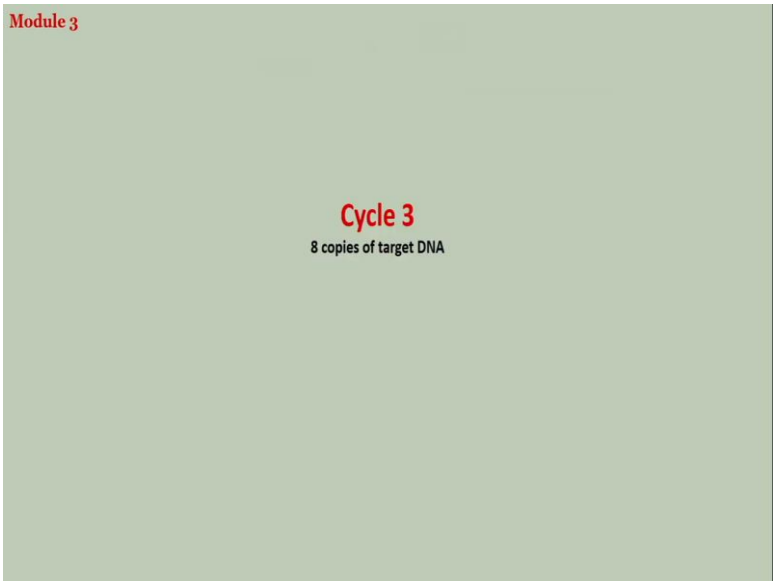

Cycle 3, you will have 8 copies, 8 copies of your DNA will be there.

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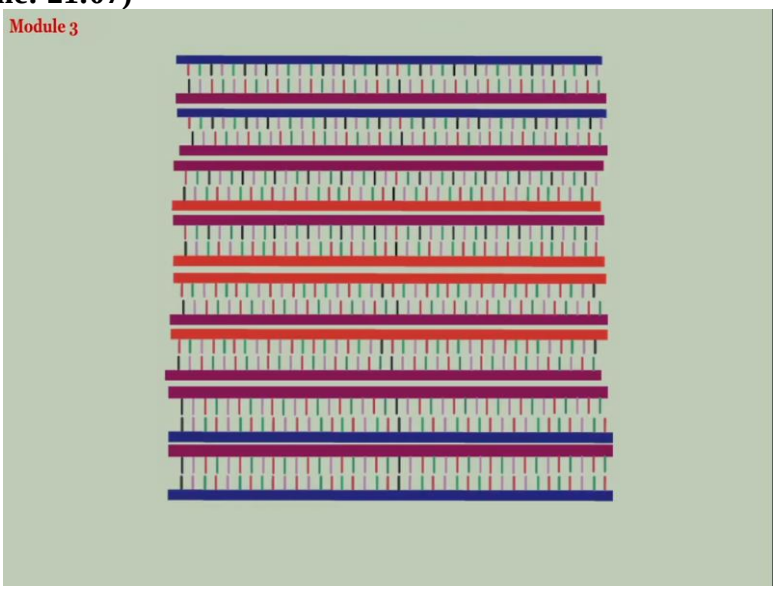

So, that is actually the steps or the stages involved in the PCR. Now if you look at the time frame, how long time it will take for the individual step.

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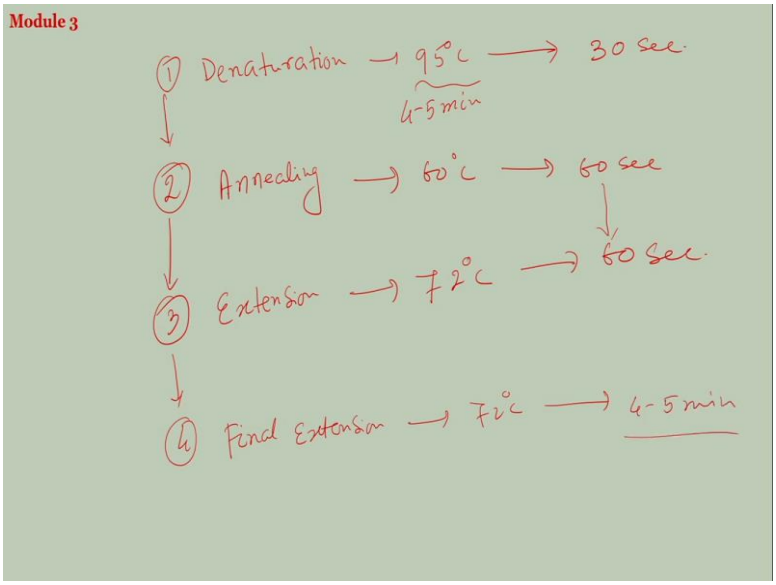

The first step or the denaturation step which is at 95 degrees Celsius, this is the starting point. So, before this, you keep your DNA at 95 degree temperature for around 4 to 5 minutes. This is for the first cycle to make sure that you have the full cleavage of the hybridization. And once you start the program, you keep in this temperature for just 30 seconds because you already have kept it for a long time. So 30 seconds here. From 30 seconds, you to come down to the annealing stage 60 degrees Celsius temperature and that is here for 60 seconds.

Roughly 1 minute 1 to 2, 2 to 3, that is the extension which is at 72. 60 degree temperature to 70 degrees temperature it has to come and stay at around. It comes around in 60 second and then call the final extension. So, here to here this jump takes around 60 seconds and you keep on the extension for around 5 minutes, 4 to 5 minutes again. Depending upon your requirement, it may not take that long also, minute most of the times is enough.

So, this is your time for him, for 1 complete cycle. So if you run it again for a couple of hours, you will have quite a good number of cycles. And you can have a concentrated DNA from the target. So this is all about the basics of PCR here. So, when you are doing the PCR, of course, it is happening inside the PCR machine and your cycles are continue. And after a certain number of cycle, you can stop it and you hope that your DNA sample has been amplified to a concentrated DNA or 2 more number of copies of DNA.

Now the question is, how do you know that when your PCR is going on, how do you know that whether your PCR is actually working or not. I mean, whether you are you getting your desired application or not. So if that is not happening, obviously you can see that there are

DNA primers or they are all these things are there, which may work non-specifically polymerase may not work properly all the time, they might not have been properly hybridized.

And most importantly which I did not tell is that when you have DNA isolated from organism and you do not know the sequence of the DNA at all. Then what kind of primer are you going to use. So, because you do not know the sequence, so you have to use all sorts of primers 15 to 20 let us say 15 nucleus base long DNA primer with every possible combination it was there in the solution. And hope that the suitable one will be hybridized. So, that makes sometimes a problem ofcourse because you have too many primers inside.

So, using this method there is no way of knowing until you complete your PCR technique, there is no way of knowing whether your PCR is actually working or not. So, there comes a little bit improvement that is the modified version, which we call a real time PCR or in short, this is known as RT - PCR.

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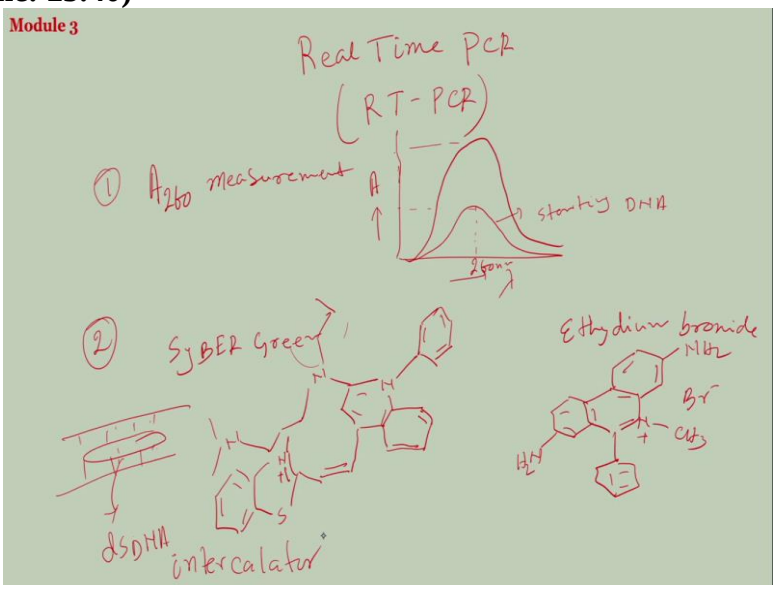

That is the modified version, which we call a real time PCR or in short, this is known as RT PCR. Real Time PCR will allow you to know whether your PCR is actually working, whether you are getting your amplification or not. In other words, it actually measures the concentration of DNA in your sample in real time so there are many ways of doing that. So I will show you 2 or 3 different ways that are used.

The first kind is of course, 1 type is that you can stop your machine at some point take a small aliquot out from the PCR 2 and then measure the absorbance at 260 degree and see whether your observance is increased. So, A 260 measurement this is really not real time PCR. This is one way of knowing if you are having your amplification or not. So, if this is your UV graph.

This is your absorbance at 2 absorbance and this is your lambda. So at the beginning since the concentration of DNA is low, you will have low intense low observance of your DNA starting DNA and this is your 260 nanometer lambda max, this is your absorbance. Now if you are getting your amplification then your concentration of DNA. Or the number of molecules of DNA will be increased and therefore, the absorbance will also increase.

So, now after a certain time you should see like this, that you are getting higher absorbance. So, by simply saying or simply measuring the observance, you can at least have some idea that you are having your amplification or not. Second is now, the problem with the EV is, of course, you need a small amount of your sample which is actually kind of important quantity for us.

Because we do not have much spirit mode, this is a destructive process, you cannot stop most of the times you cannot put it back there, because you will dilute the samples. So, it is kind of you do not have the fancy to effort or to lose even as a small amount of sample from your PCR 2 that will be required to measure the UV much better than UV is of course fluorescents. Because fluorescent have they are highly sensitive compared to the 1000 times more sensitive than the UV.

So, if you measure the fluorescence, then your core amount of material that will be needed will be really, really small and you do not really lose much. So, that is what is one technique you use florescent technique to measure whether you are getting increase of signal. But DNA does not forests, DNA is non-fluorescent molecule. So you have to make it flawless. How there are molecules actually 1 is, for example, like SyBER green or a TDM bromide I will draw the structure.

This is SyBER green, 6 modules basically this and this another nitrogen 10 alkali chain basically here and then here you have a double bond and you have here thyridine N S 5

membrane. In methyl plus is you can find the structure of this double bond here, structure of SyBER green also and it will be this here or double bond there. So this is roughly a structure of the SyBER green or there is another one called ethydium bromide that also have the aromatics structure and this is a fused bromin.

So, this is plus or B r is the counter and then so this is your ethydium bromide. Here there is a MN here there is, I mean there are some other molecules of similar type. So, this molecule CPUC they are highly aromatic in nature, and they are flat structures hydrophobic to some extent. So, there are long groups are there. So, that their flat structures basically what they do is that they intercalate inside the double stranded DNA. So, these molecules are not fluorescent at the beginning.

Or some of them are fluorescent but shows fluorescence at one wavelength. But once you put it inside a DNA and that DNA is double standard DNA. So, these molecules get inside the double standard DNA, if this is your double standard DNA. So, these molecules will get into it and form by second interaction. So, they are intercalator we call them intercalated or double standard DNA intercalator.

So, they will invade into the double helix and once they invade into the double helix they are orientations. Orientations are changed and they will start flourishing or they will also shift the wavelength of fluorescence or emission spectra will be shifted. So, by seeing the fluorescence, if initially, if you do not see any fluorescence, and when you add that component into the doubles and the DNA, it will start swaying, seeing the signal is coming up.

So, therefore, a small amount of DNA double standard DNA present will generate a quite a good amount of or quite a good readable signal of the fluorescence. So, as you move on in your PC here, the number of double standard DNA is increased every cycle if it is working and therefore, if you add this compound they are or if you keep that compound inside it itself and measure the online florescence time to time.

It will show you the increment of florescence so, that is a good way to know whether you have more number of double standard DNA that is generated in the solution. So, that is another technique and that is what is most often used.

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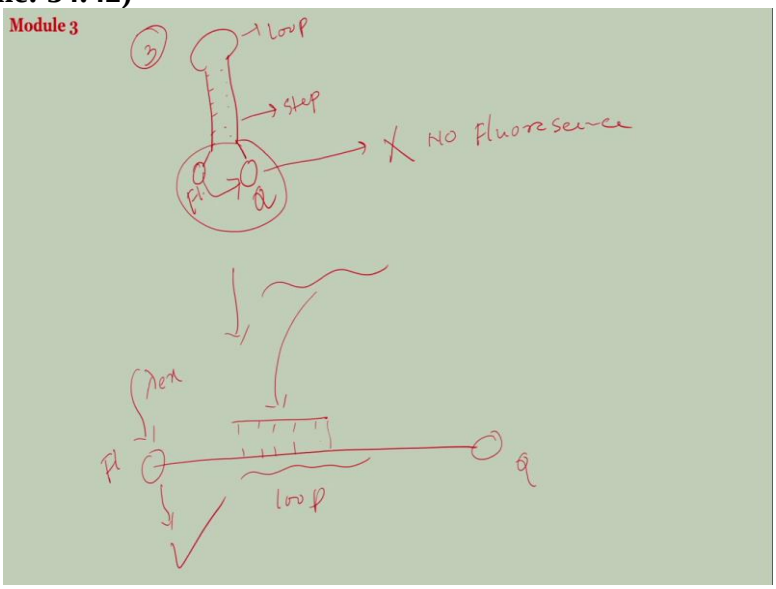

Other one is used a little bit advanced technique. That is, the idea is to find a complimentary sequence. This I will, we will talk later also. So, this kind of DNA hairpins safe DNA, they have self-complimentary at the tail towards the end that forces the molecule to form off hairpin structure or a loop and stem structure, this is the loop part, this is also the DNA sequence, but present as a single stand.

And this part is self-complimentary so this presents as a double strand and this is known as step now if you have a fluorescent stag here, so, the molecule so fluorescence and if you have a fluorescent squinter like a black hole that absorbs all fluorescence, there are molecules that absorbs all florescence then what will happen these two are very close together, when it is in loop and stem.

So, these two are very close together and therefore, all the florescence of this flora for will be absorbed by the quencher and there will be no ultimate florescence no florescence when the molecules exist in this form. Now, if you add our complimentary sequence, which is complimentary to the loop, then what will happen? This and this will hybridize together and that will force open this step.

So what we will get is something like the, so, this is your this DNA this is the loop sequence they will hybridize that will force open this step part. So, here is your fluorescence now, and here is your quencher now and they are now far apart fluorescence and quencher are far apart.

So, they cannot quencher the fluorescence. So, now, if you excite lambda excitation, you can see your fluorescence coming up.

Signal will come up. So, if this is your single standard DNA that has been amplified more and more if that quantity is high, so, the increase of fluorescence here will also tell you that you are having your amplification so, these are few ways to know, or to read and the progress of the PCR amplification one line, so that is more or less of the PCR. Now we can do some numerical.

How can you calculate or what are the ways if you can know the number of increments that are happening in the PCR, so question number 1.

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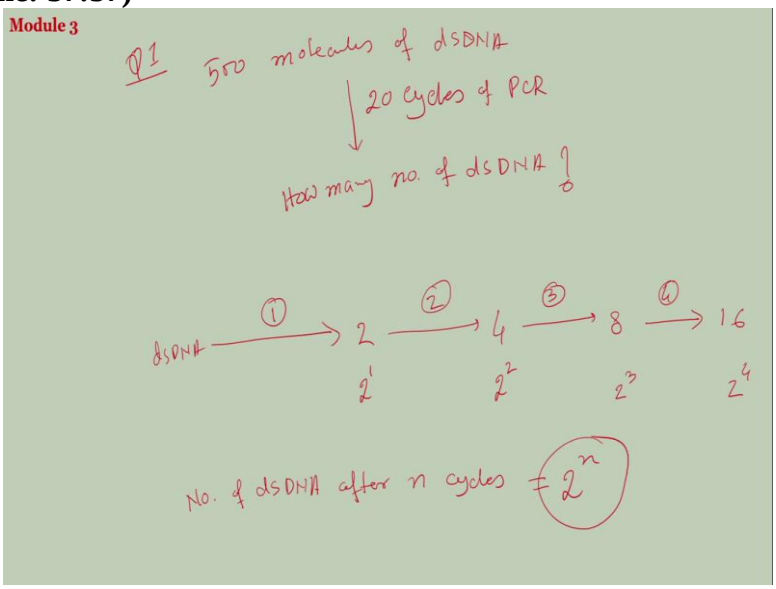

If you have initially if you started with 500 molecules of your target double standard DNA and if you do, let us say 20 cycles of PCR, then how many number of molecules now more of double standard DNA you are going to get can you calculate that, can you find out formula how to calculate, If there are a number of PCR cycles show there is a formula that you can find out actually, general formula.

So when you are doing let us say you started with 1 DNA, ds DNA. 1st cycle, how many number of DNA you are going to get 2 2nd cycle, number of copies would be 4. Cycle 3 number of copies 8 cycle 4 number of copies would be 16 can you find out or can you elucidated a general formula to find out what will be the number of molecules after the end cycle or n number of cycle.

So, if you look here, 4 cycle you have 2 this basically means 2 to the power 1 2nd cycle you have 4 that means 2 to the power 2 3rd cycle you have 8 is 2 to the power 3 number of cycle here 2 to the power 4 number of cycle. So, the number of double standard DNA after maybe n cycles would be equal to 2 to the power n that is the general formula. Now, using that, can you calculate how many number of double standard DNA will ultimately get.

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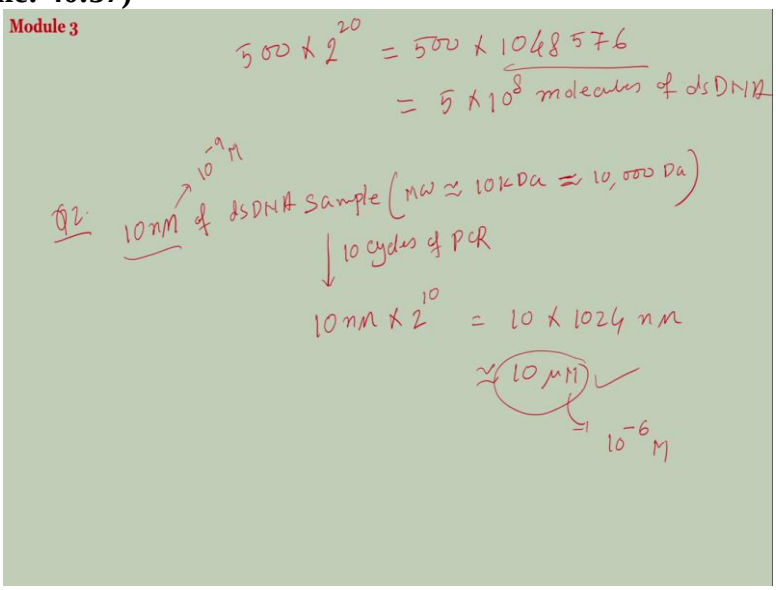

So, this will be of course, now it is very easy 500 multiplied by 2 to the power of 20 number of cycles 20 right. That is equal to look at the power of the machine; look at the power of this technique 2 to the power 20 is equal to 1048576. So, which means, if you have started with one DNA after the 20th cycle, you will have these many number of DNA 1234567 digit so, huge powerfully technique, brilliant technique.

So nevertheless, so this will be the number of records so it is roughly equal to 5 into10 to the power 8 molecules of double standard DNA that you will ultimately generate just started with 500 molecules, you are getting the 10 to the power of 8 numbers of molecules after 20th cycle. So, question number 2 is now coming to the concentration, if you start with 10 Nano molar that is the concentration of double standard DNA sample.

You have started with and the DNA has a molecular weight approximately 10 kilo Dalton. Dalton is almost similar to the gram per mole, the normal molecular weight for organic compound that you are out of kilo Dalton means thousand Dalton. So, that means, equivalent to 10000 Dalton that is a molecular rate of the DNA sample, you started with 10 Nano molar.

Nano molar basically means 10 to the power - 9 molar and if you do 10 cycles of PCR, then what is going to be your final concentration, again very easy of course simple.

So, we started with 10 milli molar I am just giving you the simplified examples, you can carry on with number of problems, you can even formulate your own problems into 2 to the power 10 that would be your final concentration. So, that is equal to 10 2 the power 10 that is equivalent to 1024 Nano molar. So that will be coming down to roughly 10 micro molar. Micro molar means 10 to the power - 6 molar. This would be the final concentration of your double standard DNA after 10 cycle, just 10 cycle.

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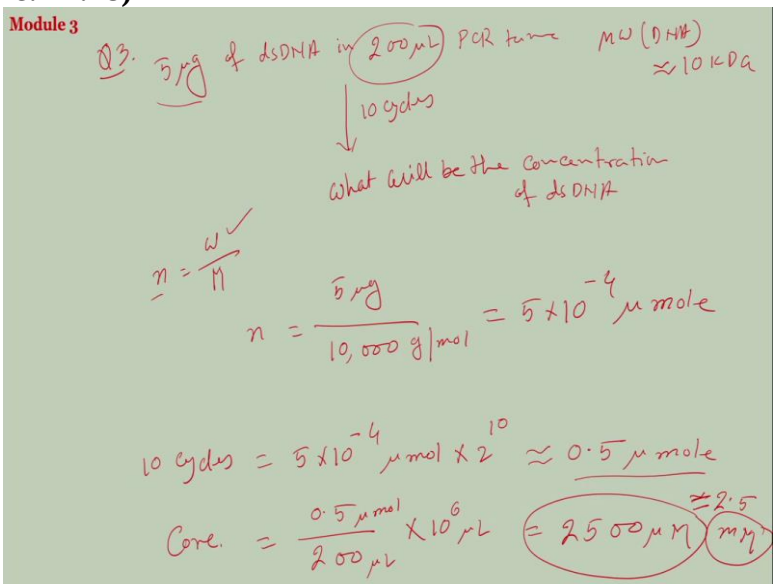

A third kind of problem maybe that you have started with now in terms of weight 5 micro gram of your double stranded DNA. Initially that you started with in 200 microliter volume 200 microliter PCR 2 total volume is 200 microliter and the double standard DNA has a molecular weight of the DNA. I am still keeping it 10 kilo Dalton and after 10 cycles question is what will be the concentration.

Not what anymore the concentration of the dsDNA after 10 cycle, how to calculate that. So, in order to do that, of course, you have to start with the 5 micrograms is equivalent to how many moles or how many micro moles. So, you know, n equals w / m your basic equation. So, this is number of moles. This is the weight of the compound that you have taken molecular weight.

So 5 microgram divided by 10000 Dalton or you can think of it is equal to gram per mole. So, this will be your n. That means, in terms of your micromole is will come up. So, this would be equivalent to 5 into 10 to the power I have calculated already actually in the - 4 micromole that would be your n now it becomes easy. So, the 10 cycles after 10 cycles means your concentration would be 5 into 10 to the power - 4. Micromoles into 2 to the power 10 number of copies would be there. So, that is coming as almost equal to 0.5 micromoles.

So, that is a number of moles micromoles that you are having. So, in terms of concentration you have started your final volume was 200 microliters so, concentration would be 0. 5 / 200. So, this is micro mole this is micro liter if you want to have in terms of molar quantity you have to multiply it by 1 litre that means 10 to the power 6 microliter that would come out as so, litre will be cancelled.

So, it will have micromole basically, you have 2500 micro molar I hope I have done it correct. Yeah, micro litre micro litre will cancel 2500 micro molar will be the final concentrations or this is equivalent to your 2.5 milli molar. So that is how you can make a lot of combinations.

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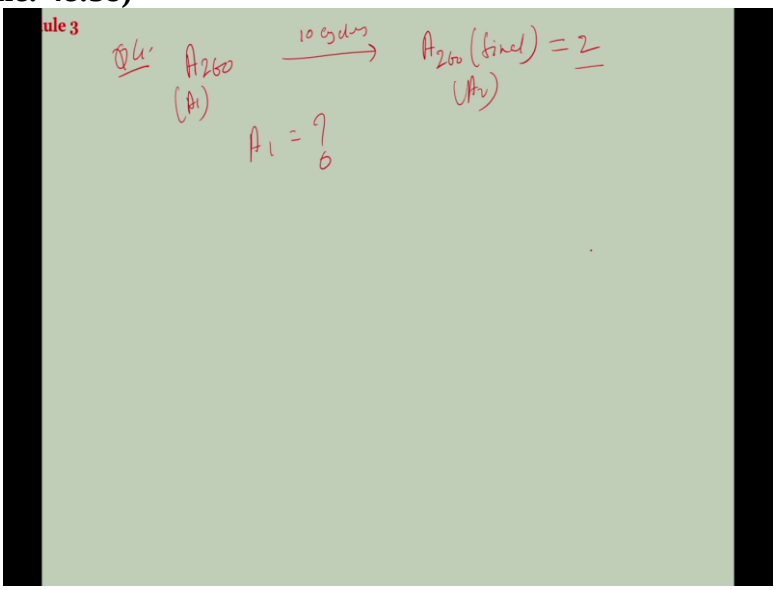

I will just write another question, which I will solve in the next class that let us say you have started with absorbance, A 260 at the beginning and then after 10 cycles whatever number of copies you have got, that has showed the final absorbent at 260 nanometre as 2. 2 was the absorbance at 260 nanometre after the 10 cycles. Now, the question is, what is A 1 so, this is A 1 if this is you are a 2, then how can you calculate A1. And you can use all the other

parameters like the molecular weight and the concentration whatever it is, as was given in the earlier question. Thank you.