Molecular Biology
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Module - 09
Molecular Techniques (Part 2)
Lecture-38 Polymerase Chain reaction (Part 2)

Hello everyone, this is Dr. Vishal Trivedi from department of biosciences and bioengineering IIT Guwahati and what we were discussing? We were discussing about the different aspects of the molecular biology in the course in this particular course. So, so far what we have discussed? We have discussed about the cellular structures followed by we have discussed about the different types of biomolecules and then we have also discussed about the role of these biomolecules in some of the biological processes such as we have discussed about the cellular metabolism, we have discussed about the apoptosis and death and we have also discussed about the autophagy and we have also discussed about the role of these biomolecules into the cell division and other kind of aspects. Following that we have also discussed about the central dogma of molecular biology and how these biomolecules are very crucial for regulating the different types of molecular processes. We have also discussed about the replications, transcription and translation and we have discussed the replication in prokaryotes and eukaryotes and the other processes also in the both kind of species to give you a comparative study between what happens in the prokaryotic system and what happens in the eukaryotic system. Following that we have also discussed about the some of the molecular techniques.

So, we have discussed about the blotting technique in the previous module and we have discussed about resin blotting, resin blotting and cystin blotting. In the current module we are discussing about the amplification techniques. So, in that amplification technique we are discussing about the polymerase chain reactions and if you recall in the previous lecture we have discussed about the theoretical aspects of these techniques. So, we have discussed how the technique is been evolved by taking the inspiration from the DNA applications.

So, as soon as the author Konberg had discovered the enzymes for the DNA applications and the complete machinery for the DNA applications people have started thinking about modulating the machinery so that it can be done in a better way and that is how we have the scientists have developed the polymerase chain reactions where they are utilizing the different temperatures for performing the different types of events. So, for example, in the initiation you require the melting of the DNA and you also require the addition of the primers and so on and that all been achieved by heating the DNA at 95 degree Celsius for 10 minutes. So, that will be the denaturation steps following that they are actually going to lower down the temperature to annealing temperature so that you can the primers are actually going to go and bind and then they will enter into the elongation phase and there they are actually going to allow the DNA polymerase to go and sit on to the template and they were actually going to do the synthesis of the DNA. They have also discussed that how the different types of components are required and what are the different types of precaution you should take, what is the advantage of the polymerase chain reactions and what is the advantage of using the tag DNA polymerase versus the PFU polymerase and

what are the different types of troubleshooting you can actually be able to do if you encounter the problem of no product or the appearance of the nonspecific products. Now, in today's lecture we are actually going to focus more on the technical aspect of the polymerase chain reaction.

So, in the previous lecture we discussed about the theoretical aspects how you can be able to do all that, but in the current lecture we are going to discuss about the technical aspects so that how you can be able to set up the PCRs, how you can be able to design the primers and so on. So, when we talk about the technical aspect the first thing what comes into the mind is what are the requirement of the polymerase chain reactions and how you are going to set up the polymerase chain reactions. So, as far as the requirement is concerned you require the following reagents, you require the template DNA, you require the primers, you require the magnesium chloride and you require the dNTPs and at the end you also require the tag DNA polymerase. What are I have not written, but you also require a water so that this whole reaction should be of 50 microlitre. So, as far as the template is concerned that template can be vary the amount of template what you are going to add.

So, it could be in the 1 picogram to 1 nanogram for the viral or the short templates whereas, it could be in the 1 nanogram to 1 microgram for the genomic DNA. Remember that the genomic DNA is a large DNA. So, if you are and the PCR is always been depends on the number of molecules, it does not depend on the microgram of the things. So, number of molecules because it is going to amplify based on the number of molecules. For example, if you started with the 10 molecules then it will going after first cycle it is going to be 20 after second cycle it is going to be 40 and so on.

So, it does not depend on the micrograms, it does not multiply the 10 microgram to 20 microgram to 40 microgram to 80 microgram. It multiplies to the number of molecule and that is why it is important that you should take the larger amount because of the larger size of the genomic DNA compared to the viral or the short templates. So, it is you know depends on the moles of that particular template DNA and that is why you see the such a huge difference in terms of the amount of DNA what you are going to use if the DNA is small or the DNA is large. Apart from that you also require the primers. So, you require the two primers one is a forward primer and other one is the reverse primer and the concentration what you require is 0.

1 to 0.5 micro moles of the each primer. Then you require the magnesium chloride and magnesium chloride will be 1.5 to 2 millimolar which is optimal for the tagged DNA polymerase. Magnesium chloride is optional it is not required or it is not something which is compulsory because for example, if you use the PFU DNA polymerase then magnesium chloride may or may not be required. Then we also require the dNTPs.

So, difficult concentration of the dNTP is 200 micromolar of each dNTP which means you are going to require the dATP, you require the dCTP, you require the dTTP and then you also require the dGTP right. These are the four nucleotides what is present in the into the DNA and that is what you require. Then you require the polymerase you can use the tagged DNA polymerase or you can use the PFU. So, tagged DNA polymerase you require into the range of 0.5 to 2 units

per 50 micro reactions.

If you go to other reactions then you are going to require the more amount of the tagged DNA polymerase. And then you also require the water and as well as the polymerase buffer also. Now, one of the most crucial component in this whole reaction is the primers right. The better you and remember that when we were discussing about the troubleshooting we gave you the many types of excuses many types of options how you can be able to improve the product and as well as the accuracy of amplification. And one of the major reason why you are getting the non specific amplification or why you are getting the no amplification is because you have designed the primers which are not correct or you have designed the primer which are not serving the purpose of working as the template or working as a attachment point.

Because remember what is the job of the primer? Primer for example, if this is the 5 prime to 3 prime right and if you add a primer right because you remember that the DNA polymerase has a limitation that it cannot start the DNA synthesis from the nascent DNA. It cannot start from the just by looking at the nucleotide. It requires the some kind of attachment point and that is why you require a primer. So, if you add a primer which is so, then it is going to bind as per the you know the what centric base pairing information right. For example, if you have A here then it is going to be T here and so on.

So, and then this last nucleotide is actually going to provide a starting point for the DNA polymerase and that is how the DNA polymerase will come sit here and it will go into start synthesis of the DNA right. So, if the primers are not good they are sitting in a very random fashion like if they are sitting somewhere here or if they are sitting somewhere here or if they are not sitting at all on to the target DNA they are sitting on to somewhere else. So, for example, if you have another DNA and it may be sitting somewhere here then it may be initiate the synthesis of that DNA because tag DNA polymerase does not know which DNA it supposed to synthesize. So, in that case it only goes to know that wherever I will find the attachment point wherever I will find the binding of the primer I will extend that particular DNA. So, that is why the accuracy and the primer of the DNA or the primer what you are going to design is the most crucial component of the polymerase chain reactions and that is why we are in we are going to discuss in detail about how you can be able to design the primers and how you can be overcome these kind of limitations.

So, as far as the primer is concerned primer is a short DNA stretch that serve as a starting point for the DNA synthesis. In PCR the two primers are required to bind to the each of the single standard DNA you will obtain that after by denaturation flanking the target sequence. These are called forward primer and the reverse primer. They are the pre-mermer R sequence complementary to the sequence in the template DNA where they are supposed to start the synthesis. So, that the in the PCR the primers are the made up of the DNA.

So, they are actually deoxyribonucleic acid whereas, in the case of the DNA replication they are you have seen that we are actually going to use the primers which is being seen synthesized by the primase it is actually RNA. So, that is very very different because in that case you actually

have the machinery which actually is going to remove the RNA and it is actually going to replaced by a short stretch of DNA by the some kind of enzymatic system right. But here you do not have that and that is why you are actually going to put the DNA single standard DNA as the primer right. And you require the two primers forward primer and the reverse primer. Forward primer is for the synthesis of the leading strand or reverse strand for the reverse primer for the synthesis of the lagging strand.

Although I just said in the previous lecture also and I am going to clarify today also that there is no leading and lagging strand because there is no delay in the synthesis of the one strand versus another strand. It is actually going to start simultaneously right. Suppose you have the DNA right once you are actually going to have the denaturation it is actually going to have the two different independent templates and that is how you are going to have the independent finding of the forward primers, finding of the reverse primer and that is how it is actually going to do the synthesis of both the strands simultaneously. Just for the sake of making it analogy with the DNA replication I am using the term as leading and the lagging strand, but there is no leading strand there is no lagging strand in the case of PCR. Now as I said in the beginning also that the primer designing and the quality of the primer is very very important and that is why there is a set rule and set guidelines how you can be able to use the primers or how you can be able to use those guidelines for designing the primers.

Primers so, first is what is the length of the primer? So, oligonucleotide between 18 to 25 bases is a ideal length which is long enough for the adequate specificity and short enough for primer to bind easily to template at the annealing temperature. This means 18 to 22, 24 is the ideal length if you go little shorter to this then the DNA will not going to bind to the specific site. It is actually going to lose its specificity because that small stretch of DNA probably will find a compliment DNA into the multiple places. For example, it may actually be you know 4 nucleotides suppose you make a primer of 10 nucleotides right if you make a primer of 10 nucleotide then that 10 nucleotide by chance could be present in any part of the genome and that is how it is actually going to compromise the specificity. So, that is why it has to be a certain length so, that that particular complete stretch may be unique for that particular stretch of DNA, but it may not vary to other site.

Now the other question is if you make the very long DNA right if you make a very long primer then the primer will actually going to have the many kind of problems right it may have the secondary structures in within the primer then and it may also have the problem of provide giving you the non specific amplifications because very big like long like for example, if I make a primer of 50 base pair then I am actually you know giving more sequence and I am giving the that particular type of sequence which are not required. So, that those additional sequence additional base pairs are actually going to form the different types of secondary structures and the secondary structure could be problematic in terms of allowing the primer to anneal. The second point is that this long stretch of DNA may be actually finding difficulty in annealing very you know precisely to that template for example, it may anneal like this for example, it may anneal like this right. So, some of the DNA is finding the complementity the other strand is finding the difficult to anneal by the time it actually finding the you know the complementity this portion has already

been annealed right. So, our other example is that it may actually anneal here small stretch and the rest is leaving like this and then goes although it has the complementity here, but the kinetics of binding of this region is slightly faster than kinetics of this that is why it may not get chance to bind and then this portion also has a faster kinetics.

So, it actually goes and bind. So, that is why you are going to have this kind of you know loop or hair pins and that may or also going to affect. The other point is also the cost right because this is actually going to increase the cost because if you are designing a primer of 50 base pair. So, it because the primers are actually going to be synthesized under the in vitro DNA synthesis reactions and that is going to be supplied by a commercial vendor. So, if you are unnecessarily putting another 26 base pair then you are supposed to pay for those synthesis and you are supposed to pay for those primers.

The second point is about primer melting temperature. So, primer with the melting temperature in the range of 52 to 58 degree Celsius generally give the best result. The GC content of the sequence gives a fair indication of a primer Tm. The two primers should be prepared in such a way that the Tm difference should not be more than 2 degree otherwise it will give you the poor annealing efficiency. So, primer first point is going to take care of the annealing.

So, first point is going to take care about the complementarity and it is going to be good for but second point is very very important. So, second point is that if you are going to have the annealing temperature in the range of 52 you know 52 to 58 then when you are going to lower down the temperature the primer will have the better affinity and it is going to anneal right. And that you can actually be able to achieve by keeping the very significant amount of the GC content. I will discuss in detail why you supposed to have the GC content because the GC content is also very important in providing the stability to that interaction because you know that the G is interacting with the C with the 3 hydrogen bonding and it is stronger compared to the AT interactions right. So, AT is having 2 hydrogen bonding and then you can actually be able to calculate the melting temperature.

So, there are 2 formulas if you are having a primer length which is less than 14 then you can use the Tm formula of this like 4 degree into number of G and C in the Tm in the primers plus 2 into number of A and T in the primer. But if it is length more than 13 nucleotide then you can use the this formula for calculating the Tm values. Now the third point is about primer annealing temperature. So, you require the 2 parameters one is primer and melting temperature and another one is the primer annealing temperature. So, too high Ta will produce the insufficient primer tabular hematization results in the lower PCR product yield whereas, the too low will lead to the non-specific product caused by a higher phase pair.

And how you are going to calculate the primer annealing temperature? So, you can actually be able to calculate using this particular formula and Tm for where the Tm is the primer is the melting temperature of the primers and Tm of the product is the melting temperature of the product right. So, that means, that the synthesized product right. So, the synthesized DNA because the synthesized DNA is also going to have the affinity for the template right. So, that also you can

be able to calculate the melting temperature for them. So, you can calculate the melting temperature for the primers you can calculate the melting temperature for the product and then you put it into this particular equation and it is actually going to give you the annealing temperature for the primers.

Then the fourth point is very very important then you supposed to have the GC content the number of G's and C's in the primer as a should be in the range of 40 to 60 base pair. And the GC content is very important because it is actually as I said you know in the previous slide itself that G is making a base pair with C with the 3 hydrogen bonding compared to the A to T because T A to T is always responsible for having the 2 hydrogen bonding. So, because of it is strong the binding of the primer to the template is going to be strong right it is going to be very strong and that is how it will allow the DNA polymerase to go and sit and do a efficient synthesis. It is going to give you the efficient initiation and once the initiation start then there will be no doubt that you are going to get a PCR product. But if this is very weak for example, if you have lot of AT and G those kind of sequences then what will happen is that the primer will be binding primer will still be binding because the 2 base pairs are still good enough right.

But they are not good enough right they are not efficient they are not going to provide the efficient initiation and because of that the primer RNA polymerase or DNA polymerase may start, but it may take time because it has to sit on this double standard DNA and then it has to actually extend this strand right. So, this for this reason only it has to be a GC content. Now apart from that you also should have the GC clamp right. So, it is not important that you should have a GC content you also should have that the corner and the corner you should have the G G and C kind of sequences. So that the corner should be intact ok even if this region is not having the lower affinity it does not matter because this portion can be taken care.

But if the lower if this stretch like the corner of the DNA is also coming off right it is because of the lower affinity or some other reason then it is will be a problem. So that is why you should have a GC clamp on the corner and as the GC forms a strong bond with then the AT the number of GC content at the 3 prime end of the primer should not be more than 3 otherwise it will result in a non specific binding at the region where the G and C are abundant. So, this is this is very important that that you should have a GC clamps to hold the primers. And then we have you are supposed to design the primer. So, I have taken an example of this you know.

So, for example, if this is your gene for which you are going to have the amplification right or you want to design an amplification. So, apart from the GC content you are supposed to have the you are not supposed to have a GC clamp for example. So, if you have a primer where you have the you know the G G G like that sequences at the corner then it is actually going to have the problem because it may actually flip on to this and it actually can make a loop like this. So, it is going to form a clamp. So, as the GC forms a stronger bond than the AT the number of GC content at the 3 prime end of the primer should not be more than 3 otherwise it will result in a non specific tight binding at the region where the GC are abundant.

So, this is very important that you should not have the GC clamp you should have not have the

more than 3 nucleotides at the corner because otherwise the binding is going to be very strong and it will result into the non specific tight binding at the region where the GC are abundant which means it is actually going to guide the binding of the primer into any sequence where you have this complementary sequence. And in so because this sequence does not match this sequence is not matching with this, but this sequence is matching and because it is at the corner it will allow the DMA primer to go and sit. So, this is a non specific template this is not a specific template, but it will still be get amplified and that is why you are going to have the non specific amplifications. Now, let us take an example of this double standard DNA. So, this is the double standard DNA which you are interested to do a PCR.

Now what we are supposed to design a forward primer and you are supposed to design a reverse primer. When you want to design the primer what you require is you require an information about the restriction enzyme because most of these primers are going to use for cloning purposes. So, when you design the primer you are also going to ask a question whether I want the primer for sequencing purpose. Remember when we were discussing about the Maxine Gilbert method we are saying that you can actually be able to use the primer that is how you can be actually you know design the four different types of reactions A reaction, G reaction, C reaction and D reactions and so on. So, whether you are designing a primer for the sequencing purpose or whether you are designing a primer for the cloning purpose.

90 percent people are designing a primer for the cloning purpose. So, if you are requiring if you are designing a primer for sequencing purpose then the requirements are different then you do not require the restriction enzyme, but for the cloning you require following things. You require a restriction enzyme because this is the restriction enzyme what you are going to use for cutting the fragment and then you are getting going to paste it into your vector of interest. So, you require the information of the restriction enzymes then you also require the you know the sequence information sequence of the template right and as I said you know that is the one of the limitation of the PCR. So, for example, this is the stretch of DNA and I want to clone this into PET23A ok.

This is just an arbitrary example it could be any other vector. So, it could be a one of the vector which is where I would be interested to clone this right. Now, the first thing what I have to see is I have to go through with the PET23A multiple cloning sites and I have to look for the restriction enzyme what I suppose to use right. Restriction enzyme what I can use right. So, I will have a list of restriction enzyme which are present in the NCS and then I have to also look at the sequence of my template right and I have to list out the non cutters right those restriction enzyme which are not cutting the sequence right and then I can use non cutters from this list and then I can choose the enzyme.

So, for example, in this case I have chosen that I will use the BAMH1 for the fiber primer and I will use the XHO because looking at the MCS you will be able to know which one which enzyme is in the front and which enzyme is on the 3 prime end. So, I can use the BAMH1 in the 5 prime end and I can use the XHO in the 3 prime end ok. So, this means the gene is going to be placed between the BAMH1 and the XHO1 ok. So, this is your DNA this is the DNA you are going to have the restriction enzyme in the front you are going to have restriction enzyme in the bottom.

So, that when you are going to digest this with BAMH1 versus XHO you are going to have the BAMH1 site here XHO site here and then you are going to have the similar kind of cutting for the vector also and that is how it is actually go and insert into the vector.

Now for this what you require is you are going to have the simple thing ok. What you can just do for forward primer it is not an issue forward primer what you have to do is you have to just take the 3 prime end whatever the sequence you require. So, you can take the 15 to 20 nucleotides from the 3 prime end right you can just take the in fact what you can do is you can just take the 5 prime end ok and then you can just add the. So, you can take the 5 from the 5 prime end ok and then you put the restriction enzyme in the front ok. Now this is your primer ready this is your forward primer ready ok and you can write the name you can just give some name like F1 or you can say F1 X gene like that ok because it is important that you should give the better name.

So, that you should be able to you know get this primer at a later point. Then for the reverse primer what you are going to do is you are going to generate the 5 prime end of this. So, you are going to write the 5 prime end of this and you are going to write the 3 prime end to this and you are going to write the complementary sequence to this and then you are going to do the same you are going to put the restriction enzyme here you are going to put the restriction enzyme 1 that is the Bamach 1 and the other one you are going to use restriction enzyme 2. And you can finally, you what you are going to do is you are going to do lot of quality testing you are going to look at whether the primers are annealing with each other or not whether there is a primer dimer is forming or not and so on. So, all these I have done or we have done it in a demo video.

So, that the students are actually going to explain you how you can be able to do the design primer manual method and as well as the software based method because there are so many software available where you are just going to put your gene and they will actually going to give you the you know the simple examples of the these are the potential forward primer and potential reverse primers. So, you can get the readily you can get the sequences. So, for designing primers first you have to identify the region of interest your region of interest which you want to amplify from any vector or any sequence. So, in second step you have to identify non cutters. There are various software available but we can use New England Biolabs NEB cutter version 2.

0. After identifying non cutters you have to select a suitable vector in which you want to integrate this amplified region and suitable restriction sites. You will get suitable restriction sites from non cutters. After that you can go for designing forward primer. So for understanding purpose I gave this sequence.

So I am using this sequence. I will use this sequence to design the primers and analyze the primers. So this is the whole sequence but I don't want to amplify whole region. I want to amplify the letters the sequence which is highlighted in green. So I want to amplify starting from here to here. So now the question arises what are the non cutters? So you want to amplify this region and integrate into another vector for that you have to identify which are non cutting restriction enzymes.

So what I will do I will copy this sequence into NEB cutter and identify what are the non cutters. So I just copy the sequence paste here and I will ask submit. So it will analyze the sequence and give non cutter. These are the enzymes cutting inside the sequence.

But we are interested in which are non cutters. So that means you can see here non cutters. So just click here. It will give a number of enzymes which will not cut inside the sequence. So once getting this list we have to identify in which vector you want to integrate your amplified region. So for that purpose so I have selected for easy of understanding I have selected PED 23A vector.

So you can see this is the vector map. So this is the 5 prime side. This is the 3 prime side. N terminal and this is the C terminal side. N terminal means forward primer. C terminal means reverse primer.

So I can use BamH1 in forward primer and Xho1 in reverse primer. This is the detailed map. So I have identified two restriction enzymes that is BamH1 and Xho1. So I can use these enzymes in forward primer and reverse primer. So after identifying restriction enzymes and the vector we will go for designing forward primer.

So I will take this sequence. I want to amplify from here to here. So I will copy this sequence here. So for designing forward primer it is very easy. You have to take the sequence whatever you are getting up to 15 to 20 bases you can take as this. So if you want to insert a restriction enzyme.

Suppose I want to insert a restriction enzyme. This is the sequence as it is given from this whole sequence. So I want to insert a restriction enzyme that is BamH1. So this is the sequence for BamH1.

Here it cuts. So I can use this sequence here. So this is the this is our restriction enzyme here it will cut. So we cannot simply Q like this. So there should be some more bases extra bases.

We have to add in the 5 prime side. So I will use. So this sequence I will use. So now this is 5 prime to 3 prime side. This is our forward primer is ready. So after designing this forward primer we have to analyze this sequence.

So this primer. So what I will do is I just copy this sequence and I will use aligo analyzer software which is specially designed for this purpose only. I will paste the sequence. Just ask analyze. So here also you can see there are so many options are there like you can analyze hairpin loop, self-dimer, hetero-dimer.

So these are the general details. What is the length and GC content, melting temperature, molecular weight. So these are normal details. I will go for hairpin loop. Is there any hairpin loops? So we can see there are a number of hairpin loops.

We can see different structures predicted by the software. So if you want to explore this thing, you can explore only two bases, two bases it is forming and the delta G value is minus 0.43 kilocalories per mole. So this is fine. Up to minus 10 kilocalories per mole is fine. Suppose hairpin loops are broken during the amplification process, but above that, above minus 10 kilocalories per mole cannot be broken.

So in that case, what we will do? Either we redesign the primers or we will add 5%, 1% B10 or 5% DMSO. These are these chemicals disrupt these loops so that the amplification will be fine. So next I will analyze for self-dimer. Is there any self-dimer and what is the maximum delta G? So this is forming continuously 5 bases.

It is because of the restriction sites. So those are restriction sites. Those homodimers forming due to restriction site can be broken. There is no issue. But other than that, this is also because of restriction site. But other than that, we have to look carefully. So is there any continuously 4 or 5 bases forming this homodimer, then it is very difficult.

These interactions can be broken easily. So here are some of the consecutive base pairs. These are very weak interactions. So they can be broken. So other than that, there are no significant self-dimers. So this sequence can be used. And for heterodimer, predicting heterodimer, you need complementary sequence with reverse primer, like reverse primer you need.

So that we will discuss later on. So we got our forward primer here. So it is very easy to generate forward primer. But in case of reverse primer, it is somewhat difficult because not in terms of predicting things, it is somewhat tricky. So what I am saying is, here we have sequence. So in case of forward primer, we just take an as-t sequence, 15 to 20 bases as-t from sequence. But here we have to take complementary sequence, not 3 prime to 5 prime or 5 prime to 3 prime sequence.

We have to take complementary to this one. Say this is the sequence we got from here. So what is the complementary to this one? So just I will add here. So this is the complementary to this particular sequence. So as you can see, this is, we have to keep from this direction, 5 prime to 3.

So I will take like this. So what we have to do is, we want to insert a restriction site here. So we can insert a restriction site here directly. So in reverse primer, we wanted to insert Xho1 site. So this is the restriction site.

As usual, we can use, we have to insert T here. So this is the restriction site we added. We can add flanking regions in between flanking bases before this restriction site. So now we got our reverse primer. So we have to go through the same procedure like what I have shown in case of forward primer. So just I will copy and paste here and analyze the reverse primer.

So is there any hairpin loop? Only one hairpin loop that is within the range of delta G. So there is no issue and self-dimer. So we can see here continuously 4 bases are forming. In this case, we have to either change the sequence or remove some of the bases. We can ignore those restriction, those dimers forming through the restriction site.

So next heterodimer, we have to analyze. For heterodimer, we need forward primer. Just copy, paste here and calculate. It will give is there any heterodimer.

This is because of restriction site. This is also because of restriction site. This can be broken. Those which are at the end of the sequence, they can be broken but which is in the middle if those bases are middle, it is very hard to disrupt those interactions and our amplification will be not good. So there is no amplification literally. Other kinds of interactions will be broken easily.

These are weak interactions. So this is how we can prepare, design the primers and analyze the primers. We have done all these processes for designing forward and reverse primers. But instead of doing manually, we can do it online. We just have to submit the sequence and it will return the forward and reverse primers. These are some of the tools available online for freely but there are commercial tools also available like algo 7 vector NTI primer firmware.

So if you are interested in these software, you can just go through these sites and submit your sequence. You will get your primers. In this video, we showed you how to design forward and reverse primers and how to analyze for non-cutters and what are the restriction sites we can use based on non-cutters and how to integrate our occupied product in which regions like we have to observe the vector if we want to integrate our gene of interest. So hope this will help you to advance your work. Now once you design the primer right, so once after this demo probably you will be able to design the primer on your own and you can be able to test these primers under the in silico PCR. So, you can actually be able to do that by using the some of the in silico tools what are available on to the web.

So I hope you could have been understood the process. Now let us think about the what could go wrong and what are the different problems what you are going to face. So one of the primer one of the major problem what you are going to face is the primer secondary structures. So primer secondary structure arise as a result of intra or intramolecular attraction between the primer or the other primer which eventually reduce the yield or amplification at the availability of single standard primer will be limited to the PCR. The various types of primer secondary structure are as follows for example, you can have hair pins, you can have dimers, you can have repeats and the runs.

So hair pins are the loop structure formed by the intramolecular interaction within the primers. Optimally a three prime end primer with a hair pin with the delta G of minus 2 kilocalorie and an internal hair pin with the delta G of minus 3 kilocalorie per mole is tolerated generally. Then we have a primer a primer is a structure formed from the double standard like structure which is formed by the intermolecular interaction between the two primer. If the interaction is formed between the two homologous or same sense primer it is called as self-dimer whereas, if the

interaction is formed between the two different primer then it is called as cross primers. Optimally a three prime end self-dimer with a delta G of minus 5 kilocalorie per mole and an internal self-dimer with a delta G of minus 6 kilocalorie is tolerated generally. So, dimer is going to be formed between the two primer for example, this is a forward primer and this is going to be a reverse primer.

So, they may actually have a complement between them and they may actually have like binding like this. So, they may have a binding like this they can have multiple options like if they can have a binding like this and so on. So, this is the small stretch if it is having a small stretch and if it is a having a delta G in the range of minus 5 kilocalorie to minus 6 kilocalories then it can be broken down when you are going to have a very high temperature, but if it is a very tight binding and if it is having the. So, this is the primer dimers between the primers it could be also that forward primer itself is binding to the forward primer. For example, you can have a forward primer binding to another forward primer because you have some sequence which is complementary to each other or you can have like this I can have complete binding right in the middle actually and so on.

So, this is can be well tolerated also if you have you know the bind the delta G in the range of 5 to 6. Then you also have the repeats and runs. So, repeats are the consecutive occurrence of dinucleotide whereas, runs are continuous stitches of signal nucleotide. A maximum number of repeat and run accepted is the 4 dinucleotide and the 4 base pair respectively. So, repeat and runs are also going to have the similar kind of problem that they are actually going to create the hairpins and all those kind of problems.

Then you can have the primer template homology. The primer should be designed in such a way that there should be no homologous within the template other than the target site. This will result in a non specific binding and the amplification. So, this is just a few examples of the primer secondary structures. So, you can have the for example, if this I have synthesized a forward primer and I have synthesized a reverse primer and this is the primer sequences.

So, then I have to analyze them whether they are forming the loops or hairpins and those kind of errors. So, for example, in this case what you see here is this is the actually the hairpin what is being formed and it is very strong because it has been bound by the two different interactions. So, this is the GC interactions and this is the AT interaction and that is very very problematic because and in if you want to have this and why it is happening because it has a very strong you know the intermolecular primer-dimer formations. So, if I have this and you will see the delta G delta G is in the range of 9.

47 which is above the 0.6 the number above to the 6. This means this is cannot be broken down this is very very strong it cannot be broken down even if you have increase the temperature. So, in that case I have an option of either using the some other stretch of the of the template DNA or I have to modify the primers. For example, I have modified the primer and then now I have broken down that and what you see here is delta G is still you know in the range of minus 2 right. So, it is still there is a interaction it is still there is a 3 nucleotide what is binding to the corresponding you know the templates, but this is well tolerated this is going to be interaction this is going to be

bind and this kind of interaction you do not have to worry about. So, this is the way you supposed to you know vary the you are supposed to analyze the primer sequences you are supposed to check for these kind of things and there are software available there are software available for doing this and that is how you can be able to do the primer designing and other things.

So, this is all about the technical aspects what we have discussed we have discussed about the primer designing what kind of troubleshooting you are supposed to do and what are the how it is actually going to impact the PCR amplification and other things. So, with this I would like to conclude my lecture here in our subsequent lecture we are going to discuss some more aspects related to molecular biology. Thank you. Thank you.