Genetic Engineering- Theory and Applications
Professor Vishal Trivedi
Department of Biosciences and Bioengineering
Indian Institute of Technology Guwahati
Module 3
Basics of Cloning (Part I)
Lecture 10- Polymerase Chain Reaction

Hello everybody, this is Dr. Vishal Trivedi from Department of Biosciences and Bioengineering, IIT Guwahati and let us continue our discussion about the polymerase chain reactions. So in our previous lecture, what we have discussed? We have discussed about the different steps and how the technique has been evolved in a span of couple of decades and how the polymerase chain reaction is been inspired from the DNA applications and how the people have mimicked the similar kind of steps under the invitro conditions to amplify a shorter stretch of DNA using the site-specific primers.

So in the today's lecture, what we are going to discuss? We are going to discuss about the technical aspects of the polymerase chain reactions and in today's lecture what we are going to discuss?

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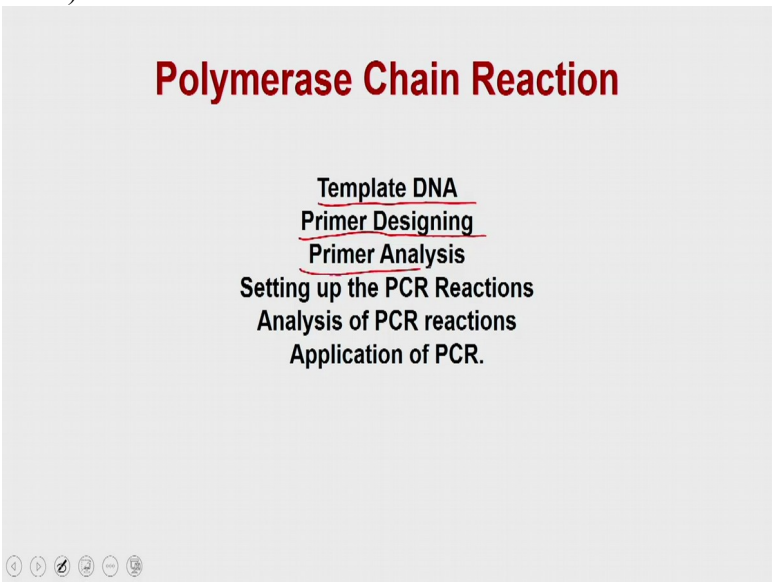

We are going to discuss about the reagents which are important for the polymerase chain reaction such as the template DNA, then we are going to discuss about the primer designing, more in an interactive way so that you will be able to design the primers using the available

software. Then we will also going to show you with the help of one of the softwares, how to do the analysis part. And then at the end we are going to show you how to set up the PCR reactions, how to add the individual components, what are the precautions you should take while you are setting up the PCR reactions.

And they will take you to our lab and we will show you how to set up these reactions. In addition, how to setup the different steps within the thermal cyclers and at the end, we will also going to show you the demo how to prepare and setup the PCR reactions. And once the PCR reactions are over, then we will take out those reaction products and analyse them onto the agro cells and at the end of this lecture, we are also going to discuss about the potential is of PCR in biomedical research or biotechnology research.

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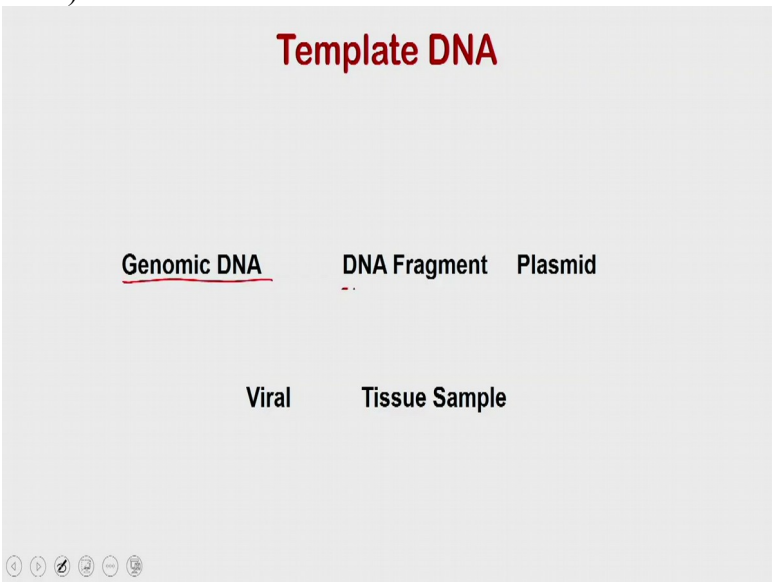

So let us continue with the template DNA. The template DNA as we discussed in the previous lecture could be genomic DNA, DNA fragments which you can isolate from the environment as well as from the biological samples. You can use the plasmids or you can use the recombinant DNA, you can use the viruses, you can use the tissue samples and the only thing what makes a sample good or bad is that the sample what you use in the PCR reaction should not have the mix or it should not be a degraded form because if you are using the site-specific primers and if there is a degradation into your gene of interest, then the PCR amplification may not happen.

And apart from that, when you have a degraded sample, the data disciple sometimes always gives a non-specific reactions as well.

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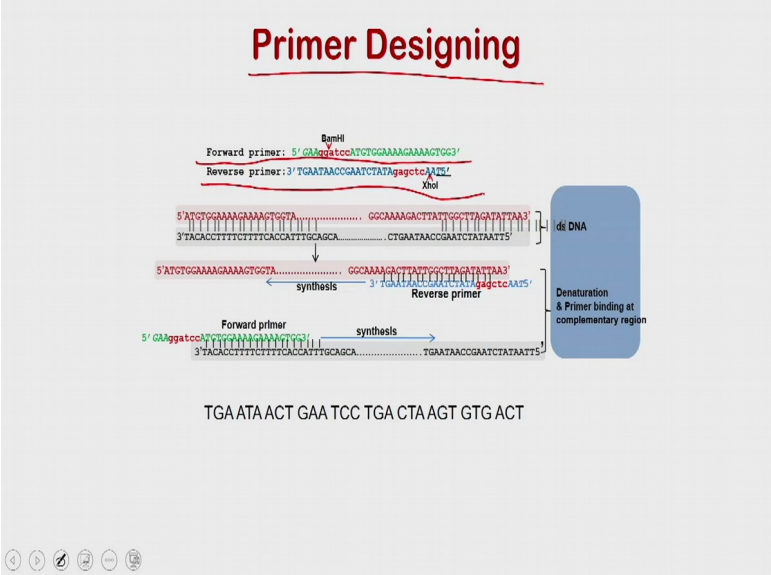

This also we have discussed. So primer designing we have discussed, we have discussed about designing the forward primers as well as designing the reverse primers. So what we have done is we have taken you to our laboratory and where my student, student Banesh has prepared a small movie with the help of softwares. What he is going to show you is that how to prepare or design the oligo primers or oligonucleotides, how to analyse them using the certain softwares under the different parameters.

If you recall, in our previous lecture, we have discussed about the primer length, primer TM, the annealing temperatures, GC content, GC clamps and all these aspects is going to show you with the help of that particular softwares. Apart from that, he is also going to show you the different types of defects which you can encounter into designing of these primers such as the primer dimers or the loop formations and how to get rid of these loops. Suppose you have a loop and that is how the Banesh is going to give you a demo about the primer designing and the analysis of these primer sequences.

Student Banesh starts giving the demo:

Student Banesh: In this video, we will show you how to design primers and analyse for different constituents like hairpin loops and primer dimers, how to analyse these things using various online tools available.

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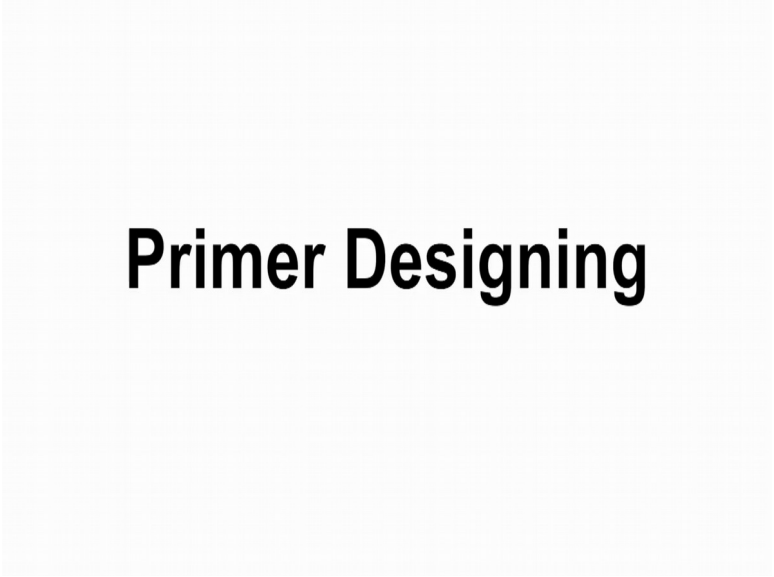

Hello everyone. In this video, I will show you how to design the primers and analyse them.

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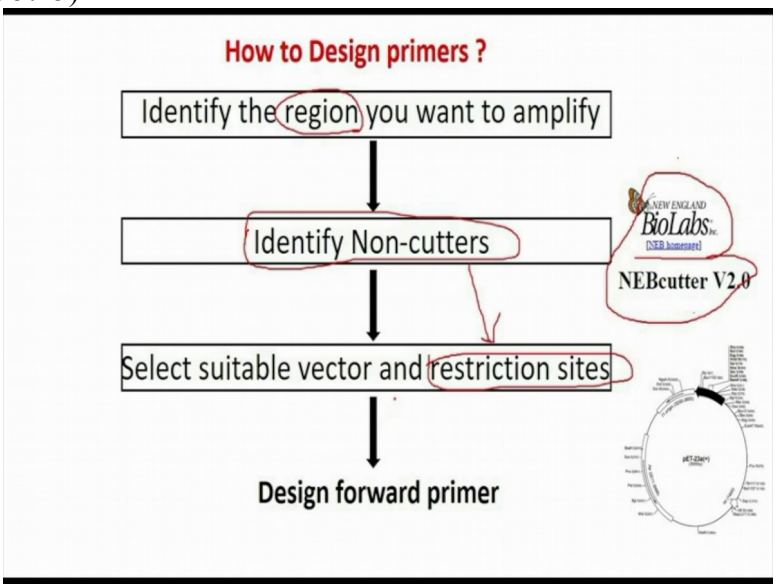

So for designing primers, first you have to identify the region of interest, your region of interest which is you want to amplify from any vector or any sequence. So in second step, you have to identify non-cutters. There are various softwares available but we can use New England biolabs

NEB cutter version 2.O. After identifying non-cutters, you have to select a suitable vector in which you want to integrate this amplified region and suitable restriction sets. You will get suitable restrictions sets from non-cutters. After that, you can go for designing forward primer.

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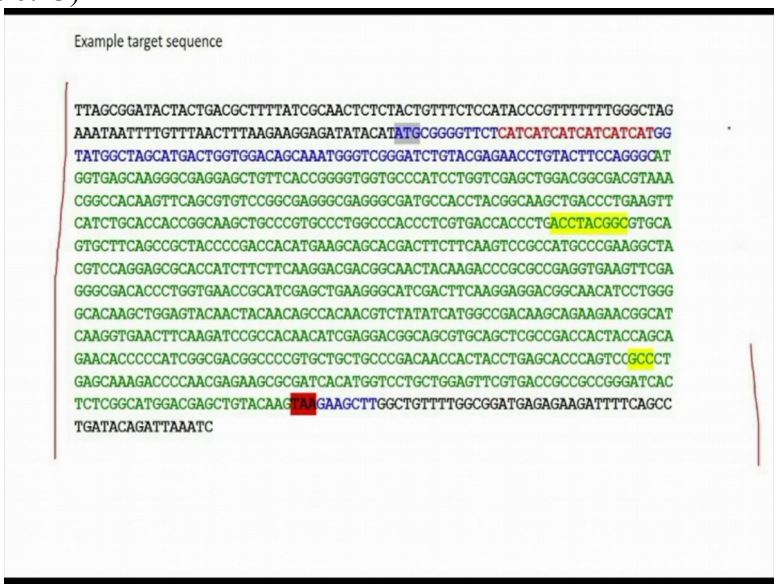

So for understanding purpose, I gave this sequence. So I am using this sequence. I will use this sequence to design the primers and analyse the primers. So this is the whole sequence but I do not want to amplify whole region. I want to amplify the letters, the sequence which is highlighted in green. 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.

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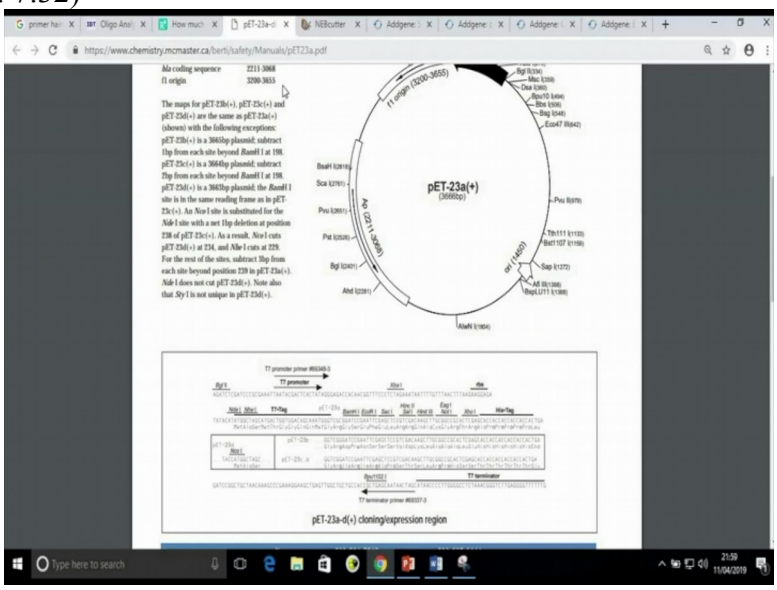

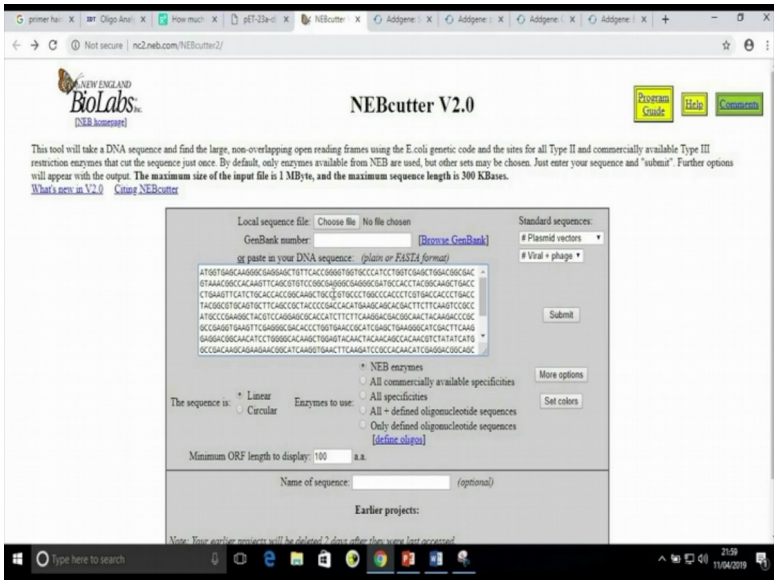

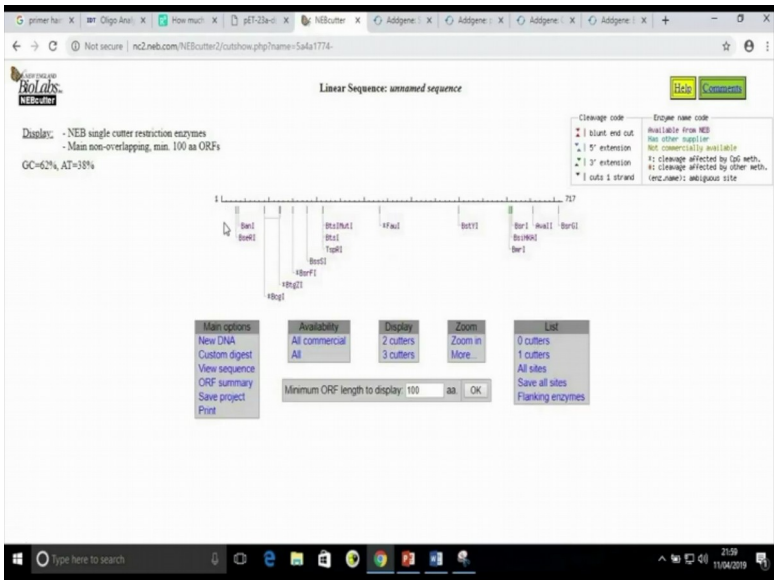
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Enzyme name code
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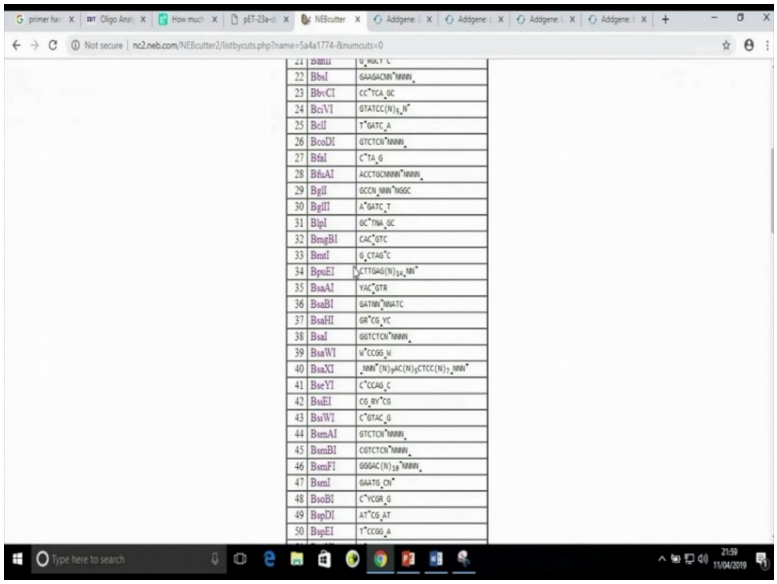
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23 BbvCI CC^TCA_GC
24 BciVI GTATCC(N)5_N^
25 BclI T^GATC_A
26 BcoDI GTCTCN^NNNN_
27 BfaI C^TA_G
28 BfuAI ACCTGCNNNN^NNNN_
29 BglI GCCN_NNN^NGGC
30 BglII A^GATC_T
31 BlpI GC^TNA_GC
32 BmgBI CAC^GTC
33 BmtI G_CTAG^C
34 BpuEI CTTGAG(N)16_NN^
35 BsaAI YAC^GTR
36 BsaBI GATNN^NNATC
37 BsaHI GR^CG_YC
38 BsaI GGTCTCN^NNNN_
39 BsaWI W^CCGG_W
40 BsaXI _NNN^(N)9AC(N)5CTCC(N)7_NNN^
41 BseYI C^CCAG_C
42 BsiEI CG_RY^CG
43 BsiWI C^GTAC_G
44 BsmAI GTCTCN^NNNN_
45 BsmBI CGTCTCN^NNNN_
46 BsmFI GGGAC(N)10^NNNN_
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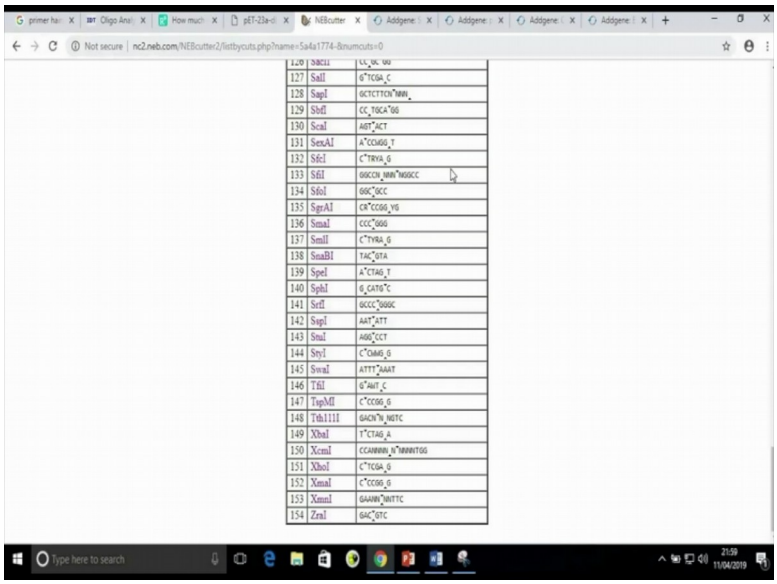

So just copy the sequence, paste here and I will ask submit. So it will analyse the sequence and give non-cutters. These are the enzymes cutting inside the sequence. But we are interested in which are non-cutters. So that means, you can see here, non-cutters. So just click here. It will give you a number 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.

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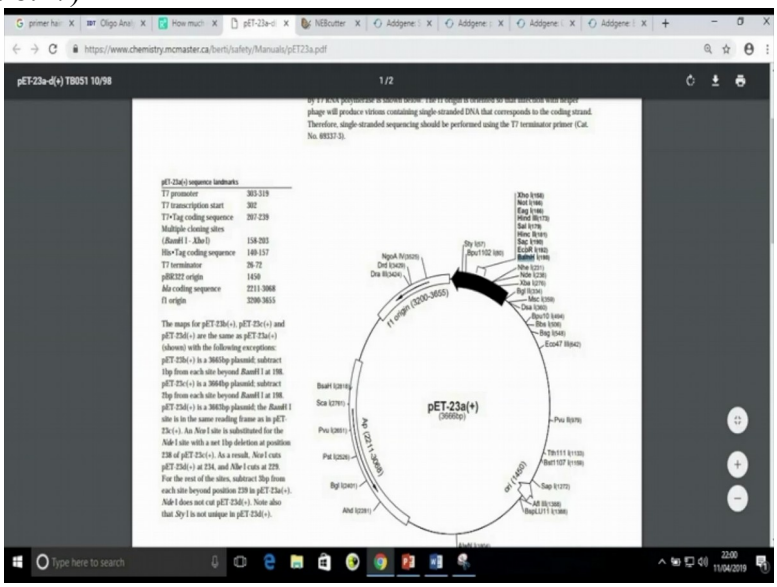

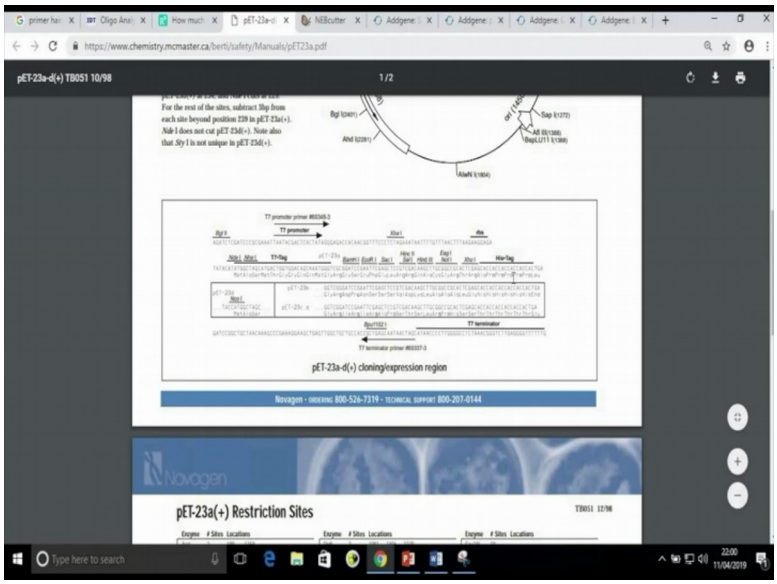

So for that purpose, I have selected for ease of understanding, I have selected pET 23 vector. So you can see, this is the vector map. So this is the 5 prime side, this is the 3 prime side. M terminal and this is the C terminal side. M terminal means forward primer, C terminal means reverse primer. So I can use BamH1 in forward primer and Xho1 in reverse primer. This is the detailed map.

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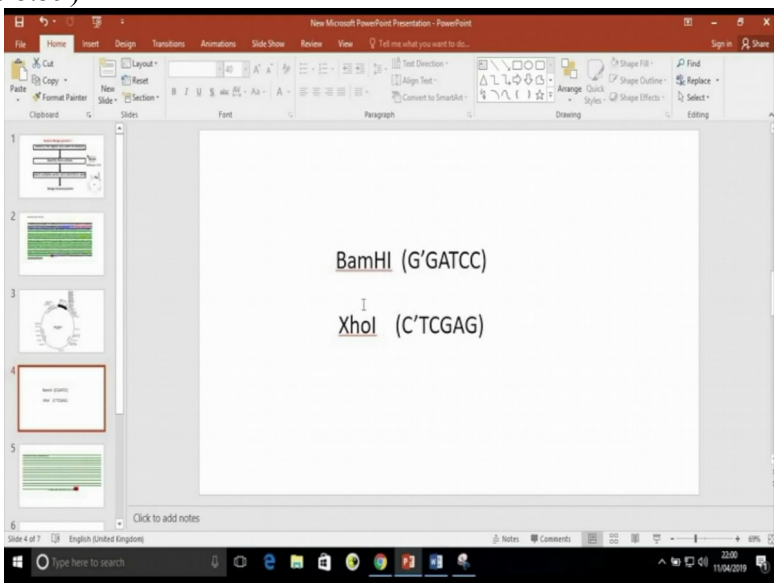

So I have identified two restriction enzymes. That is BamH1 and Xho1. So I can use these enzymes in forward primer and reverse primer. So after identifying restriction enzymes and the vector, we will go for designing forward primer.

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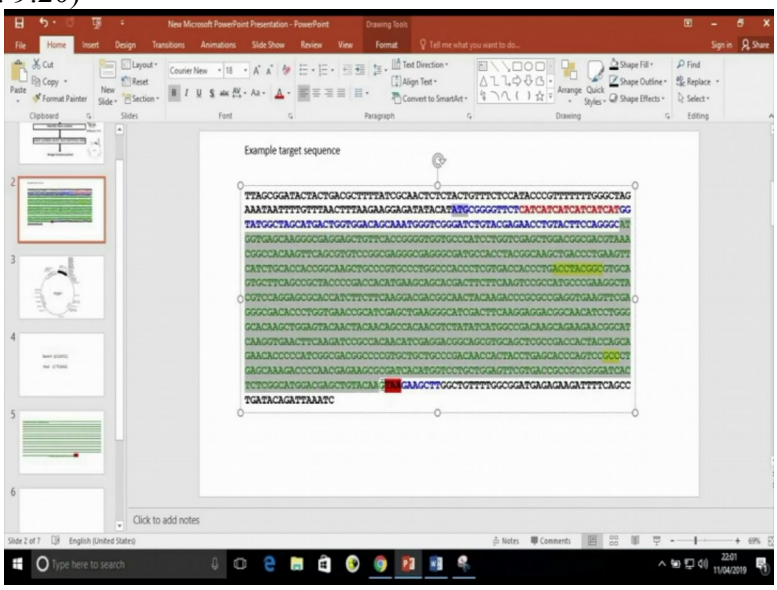

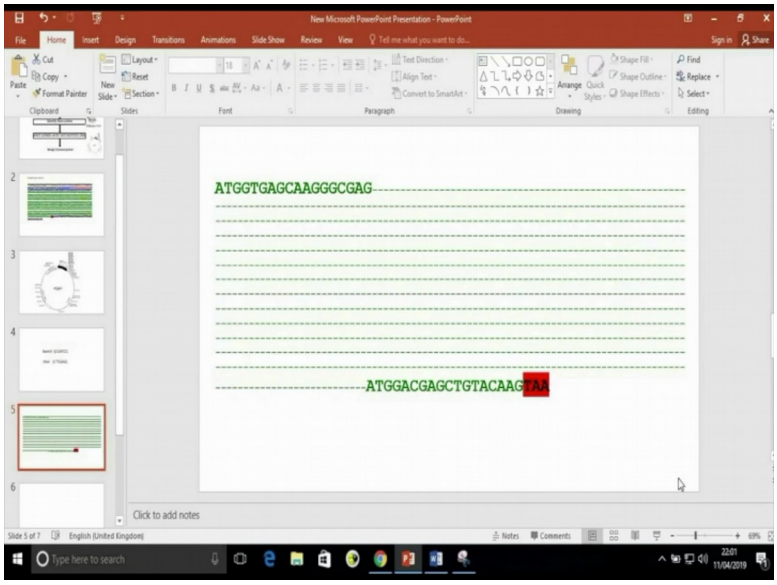

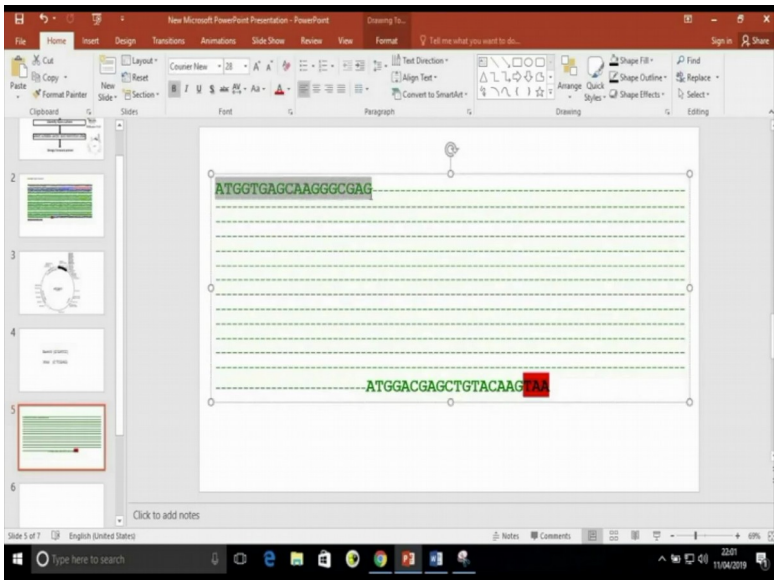

So we will take this sequence. I want to amplify from here to here. So I will copy this sequence here. So for designing forward primer, it is very easy. You have to take the sequence whatever you are getting up to 15 to 20 bases you can take as it is. So if you want to insert a restriction enzyme, suppose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

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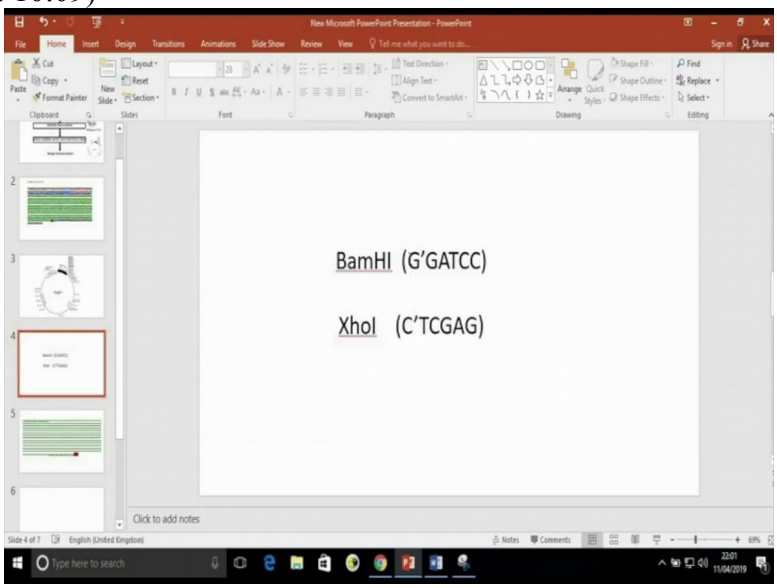

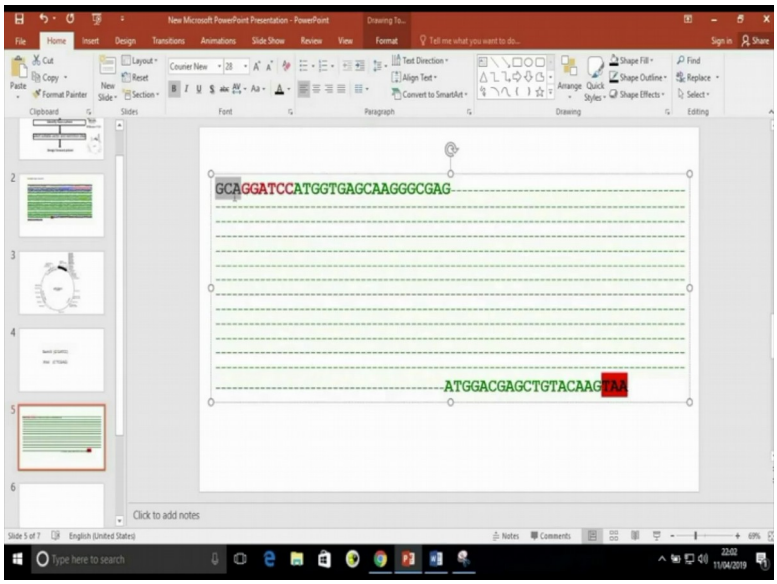

So this is the sequence for BamH1. Here it cuts. So I can use this sequence here. So this is our restriction enzyme. Here it will cut. So we cannot simply give like this. So there should be some more basis, extra basis we have to add in the 5 prime side. So I will use, so this sequence I will use. So now, this is 5 prime to 3 prime side. So this is our forward primer is ready. So after designing this forward primer, we have to analyse this sequence, so this primer.

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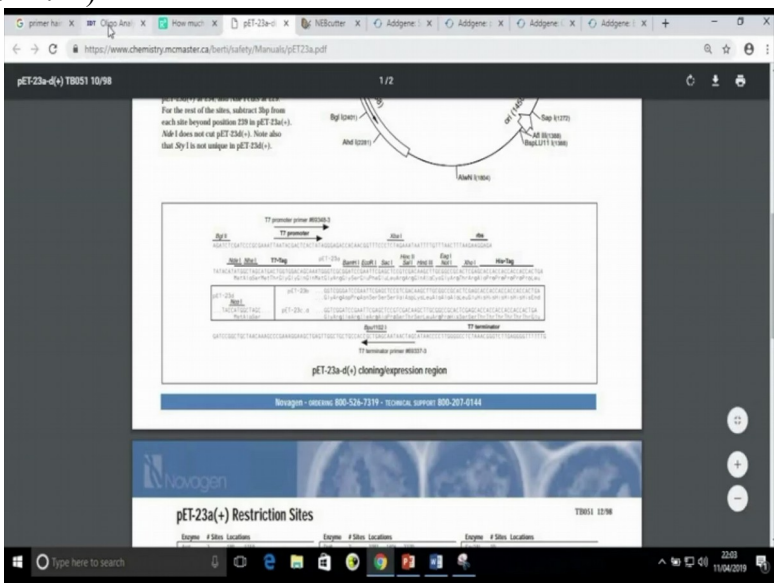

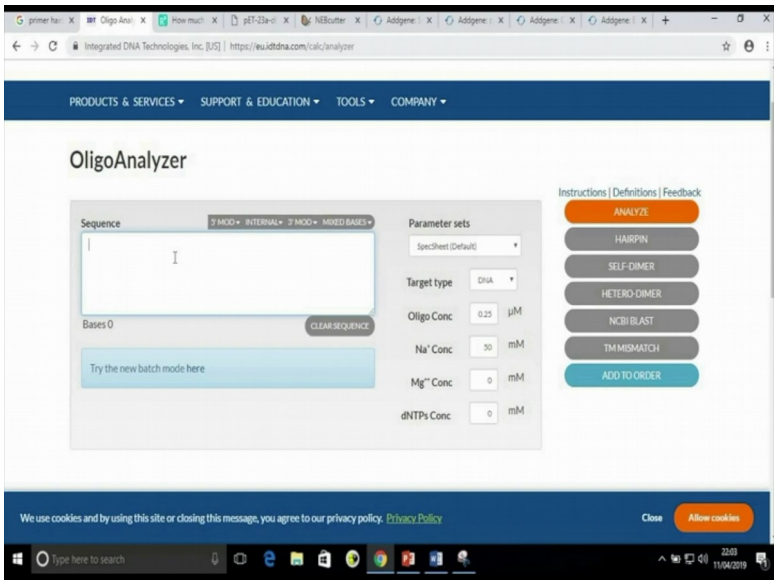
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PRODUCTS & SERVICES
SUPPORT & EDUCATION
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Instructions | Definitions | Feedback
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DNA
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0.25
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Mg++ Conc
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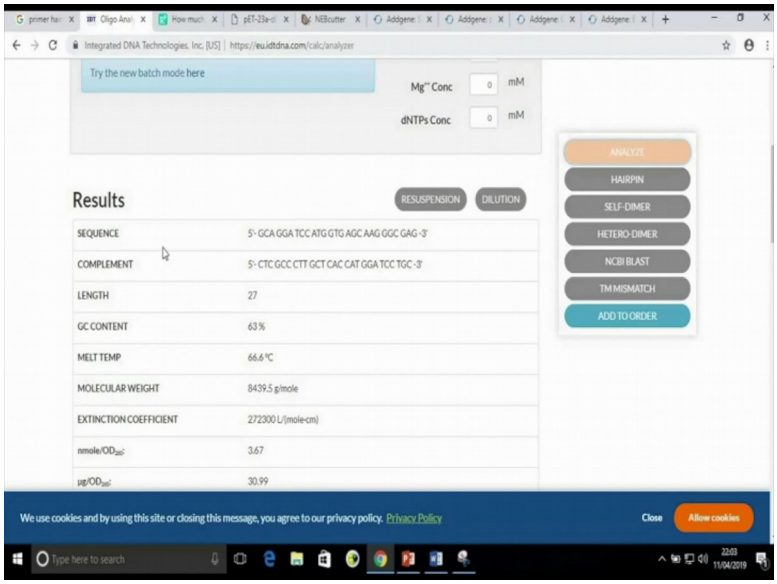
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Results
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DILUTION
SEQUENCE
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COMPLEMENT
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MELT TEMP
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MOLECULAR WEIGHT
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EXTINCTION COEFFICIENT
272300 L/(mole·cm)
nmole/OD260
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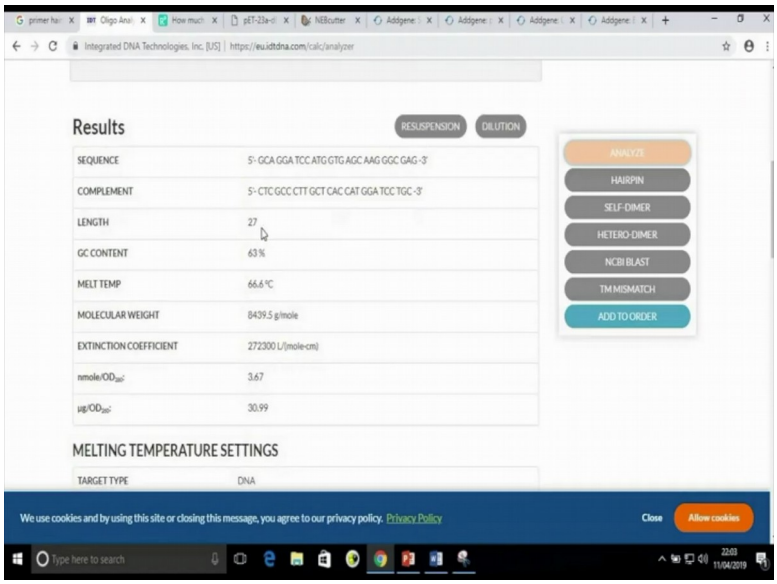

So what I will do is I just copy this sequence and will use OligoAnalyzer software which is specially designed for this purpose only. I will paste the sequence. Just ask, analyse. So here also, you can see there are so many options are there like you can analyse hairpin loop, self dimer, hetero dimer. So these are the general details. What is the length and GC content, built-in temperature, molecular weight. So these are normal details.

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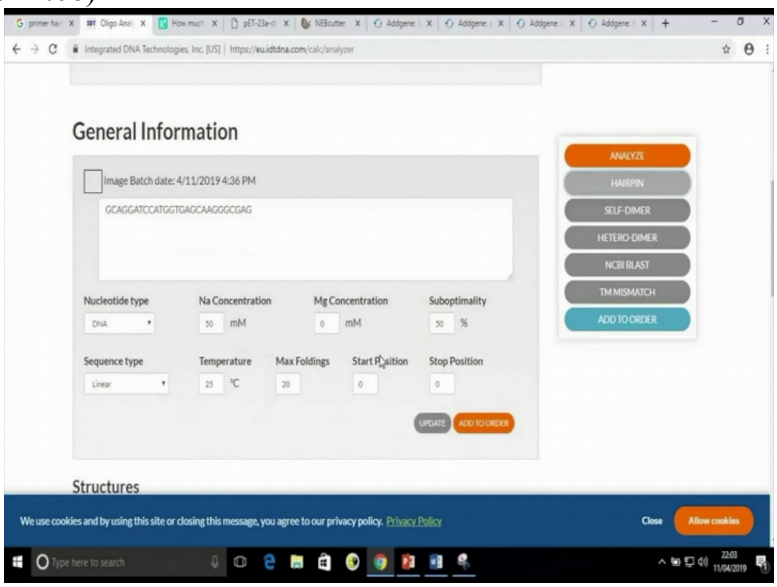

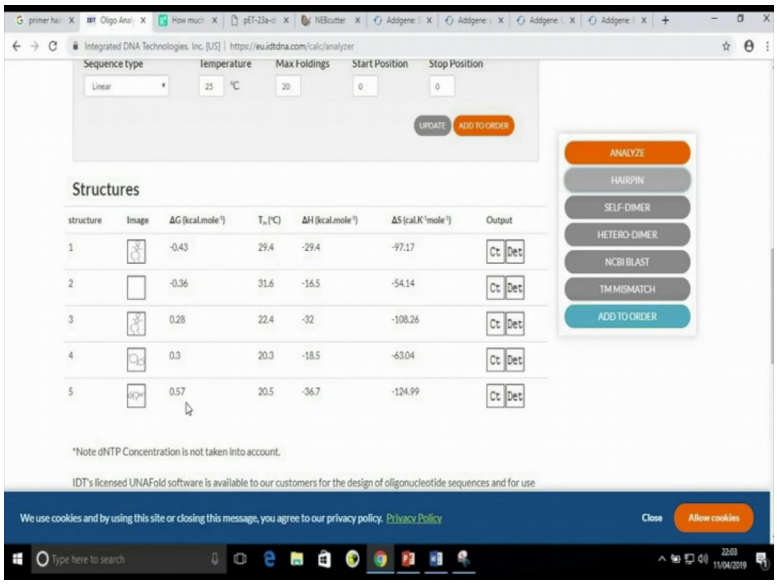

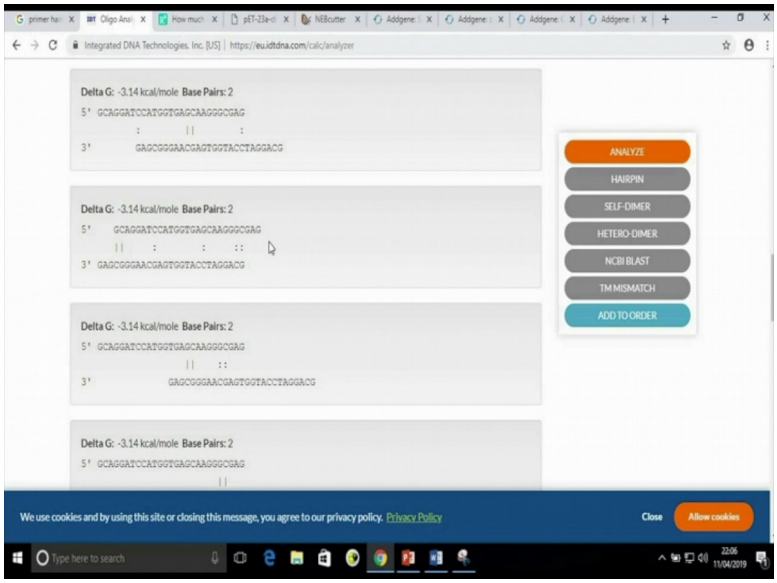

I will go for hairpin loop. Is there any hairpin loops? So we can see, there are a number of hairpin loops. We can see, different different structures predicted by the software. So if you want to explore this thing, you can explore. Only 2 bases, 2 bases it is forming and the Delta G value is minus 0.43 kilocalorie per mole. So this is fine. Up to minus 10 kilocalorie per mole is fine. Those hairpin loops broken during the amplification process but above that, above minus 10 kilocalorie per mole cannot be broken.

So in that case what we will do? Either we redesign the primers or we will add 5 percent, 1 percentage butane or 5 percentage DMSO. These are, these chemicals disrupt the (())(13:29) so

that the application will be fine. So next, I will analyse for self dimer. Is there any self dimers and what is the maximum Delta G. So this is forming continuously 5 bases. It is because of the restriction sets. So those are restriction sets, those homo dimers forming 2 restriction set, can be broken.

There is no issue but other than that, this is also because of restriction set. But other than that, we have to look carefully. So is there any continuously 4 or 5 bases forming this homo dimer, then it is very difficult. These interactions can be broken easily. So here is some of the consecutive base pair is not it. These are very weak interruptions. So they can be broken, so other than that, there is no significant self-dimers. So this sequence can be used.

And for hetero dimer, predicting hetero dimer, you need a 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 taken as the sequence 15 to 20 bases as it is from sequence 8 but here, we have to take complementary sequence, not 3 prime to 5 prime or 5 prime to 3 prime sequence.

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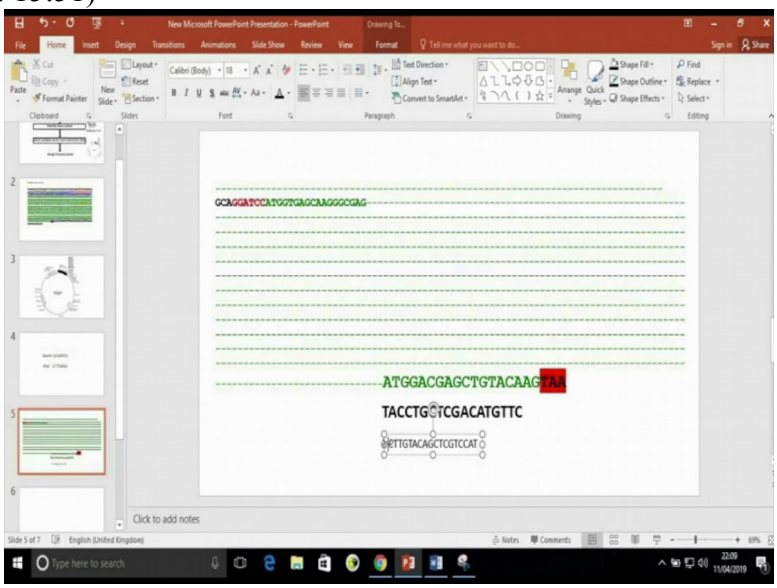

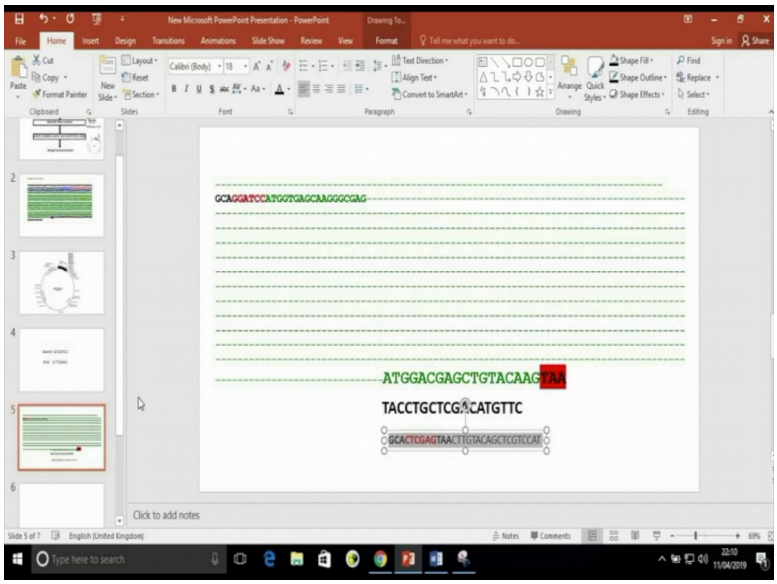

We have to take complementary to this one. Say this is the sequence we got from here. So what is the complementary to this one? So just I will add here. So this is the complementary to this particular sequence. So as you can see, this is we have to keep from this direction, 5 prime to 3. So I will take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

So in reverse primer, we wanted to insert Xho1 set. So this is the restriction set. As usual, we can use, we have to insert T8. So this is the restriction set we added. We can add franking regions in between, franking spaces before this restriction set. So now we got our reverse primer. So we have to go through same procedure like what I have shown in case of forward primer.

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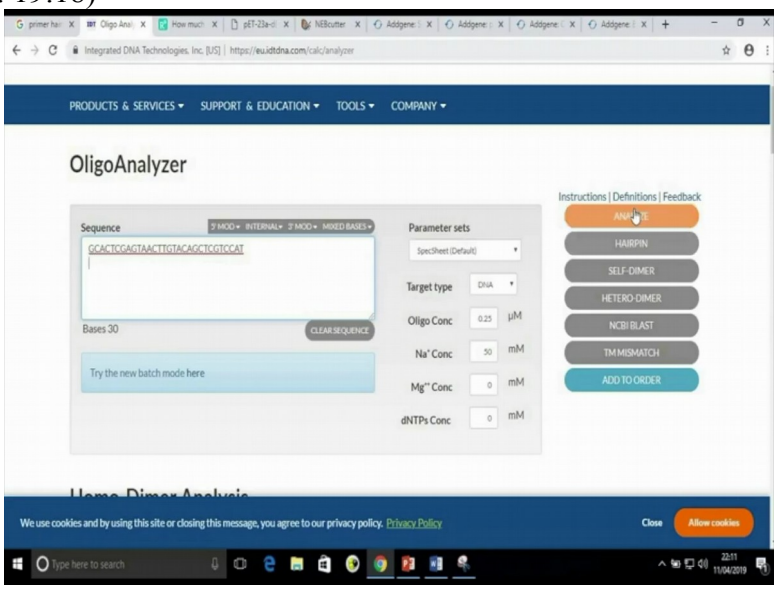
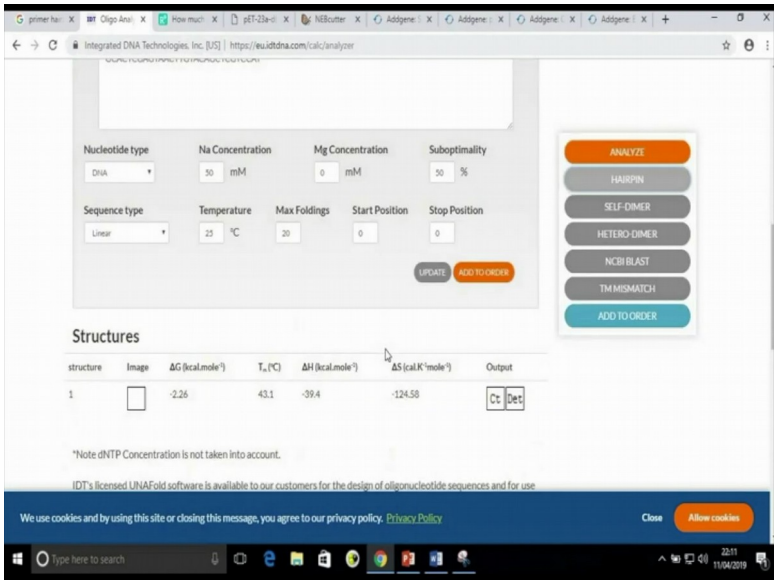
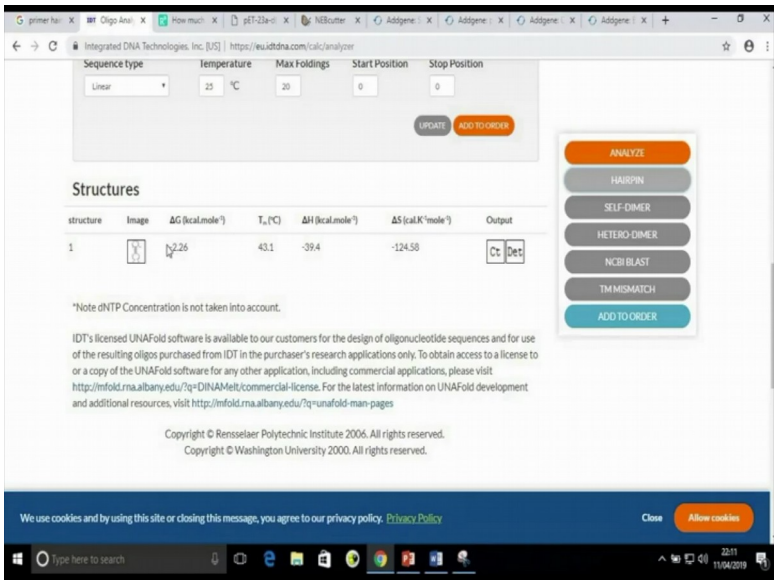
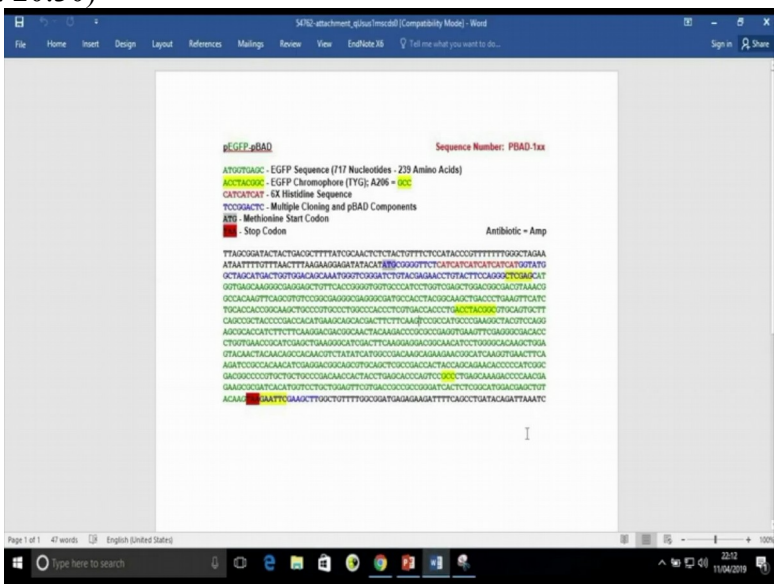


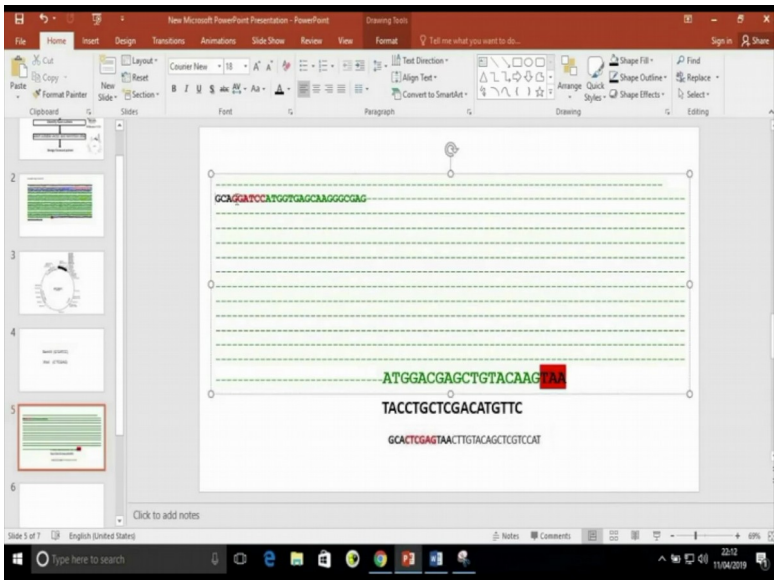


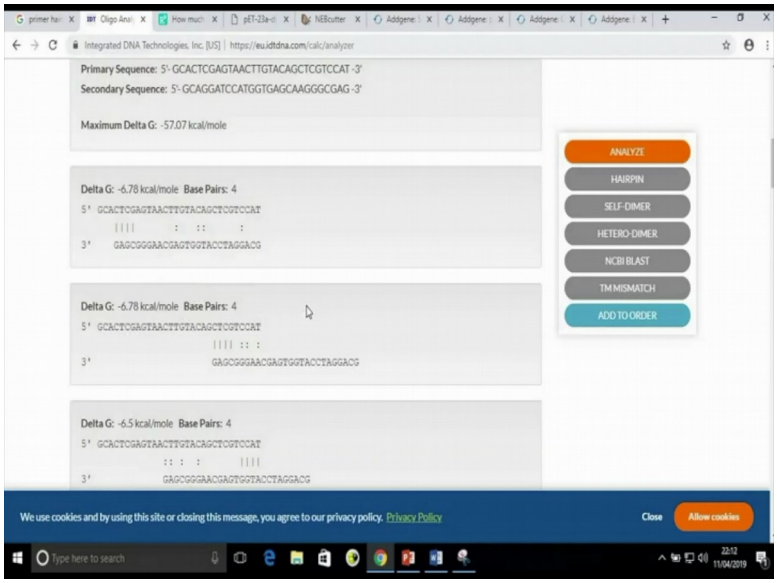


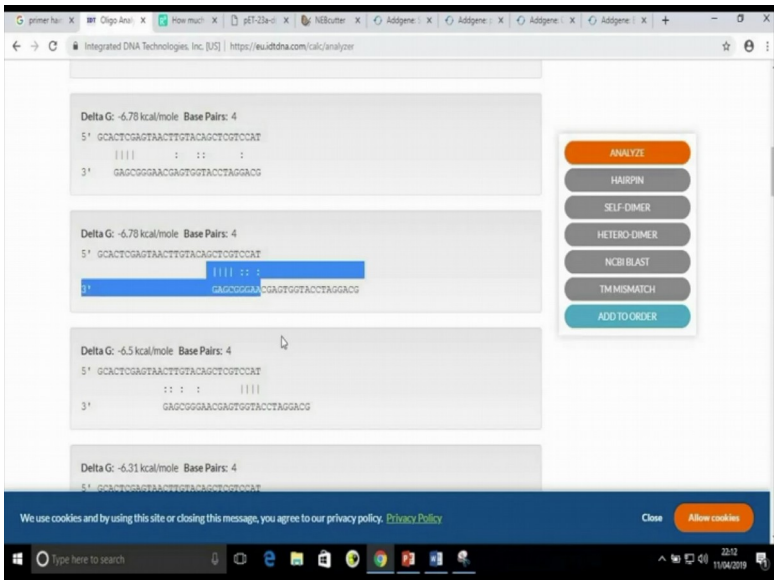
So just I will copy paste here and analyse the reverse primer. So is there any hairpin loops? Only one hairpin loop that is within the range of Delta G. So there is no issue and self-dimer. So we can see here continuously 4 bases are forming. In this case we have to either change the sequence or remove the some of the bases. We can ignore those dimers forming through section set. So next hetero dimer, we have to analyse.

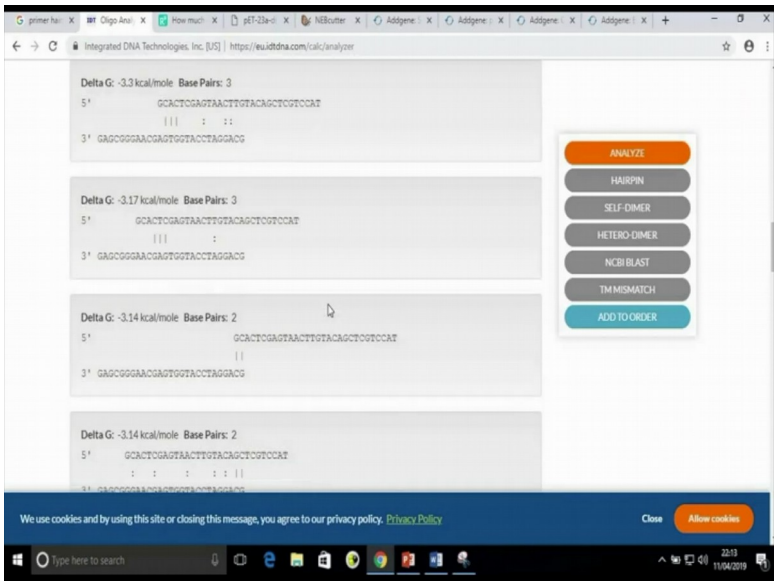
(Refer Slide Time: 20:30)




New Microsoft PowerPoint Presentation - PowerPoint
GCAGGATCCATGGTGAGCAAGGGCGAG
ATGGACGAGCTGTACAAGTAA
TACCTGCTCGACATGTTC
GCACTCGAGTAACTTGTACAGCTCGTCCAT
Click to add notes

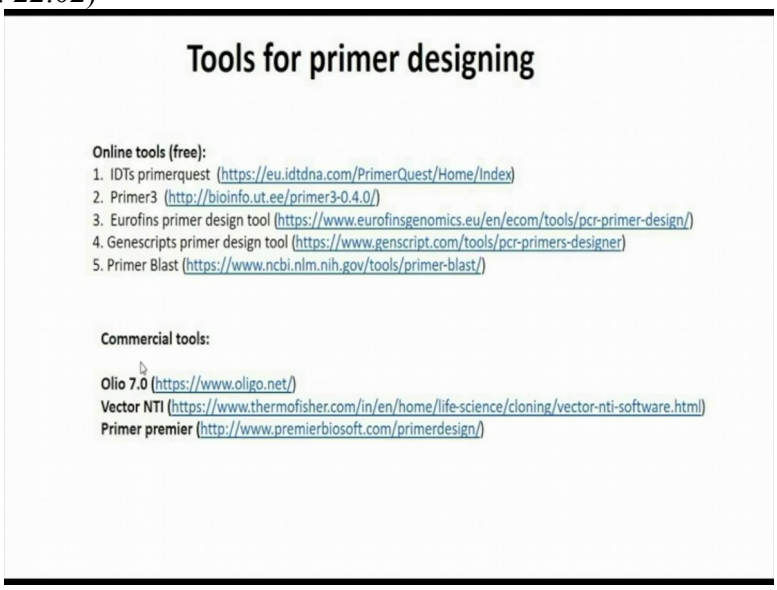

Primary Sequence: 5'- GCACTCGAGTAACTTGTACAGCTCGTCCAT -3'
Secondary Sequence: 5'- GCAGGATCCATGGTGAGCAAGGGCGAG -3'
Maximum Delta G: -57.07 kcal/mole
ANALYZE
HAIRPIN
SELF-DIMER
HETERO-DIMER
NCBI BLAST
TM MISMATCH
ADD TO ORDER
Delta G: -6.78 kcal/mole Base Pairs: 4
5' GCACTCGAGTAACTTGTACAGCTCGTCCAT
3' GAGCGGGAACGAGTGGTACCTAGGACG
Delta G: -6.78 kcal/mole Base Pairs: 4
5' GCACTCGAGTAACTTGTACAGCTCGTCCAT
3' GAGCGGGAACGAGTGGTACCTAGGACG
Delta G: -6.5 kcal/mole Base Pairs: 4
5' GCACTCGAGTAACTTGTACAGCTCGTCCAT
3' GAGCGGGAACGAGTGGTACCTAGGACG
We use cookies and by using this site or closing this message, you agree to our privacy policy. Privacy Policy
Close
Allow cookies





For hetero dimer, we need forward primer. Just copy, paste here and calculate. It will give is there any hetero dimers? This is because of restriction set. This is also because of restriction set. This can be broken. Those which are at the end of the sequence, they can be broken but which is middle, the bases are middle, it is very hard to interrupt those interruptions. And our amplification will be not good. So there is no amplification literally. Other kind of interactions will be broken easily. These are weak interactions. So this is how we can prepare, design the primers and analyse the primers.

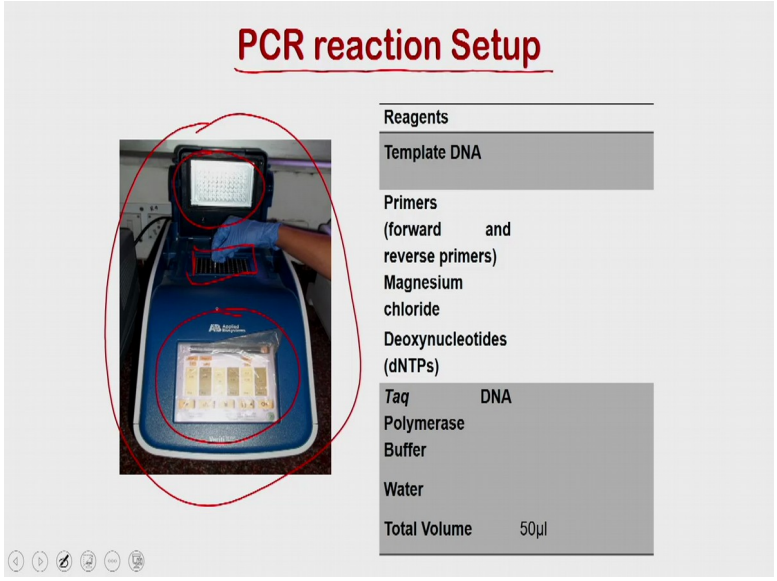
(Refer Slide Time: 22:02)



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 Olio 7, vector NTI, primary premier. So if you are interested in this software or you can just go through these sites and submit your sequence, you will get your primers.

Demo over.

(Refer Slide Time: 22:41)



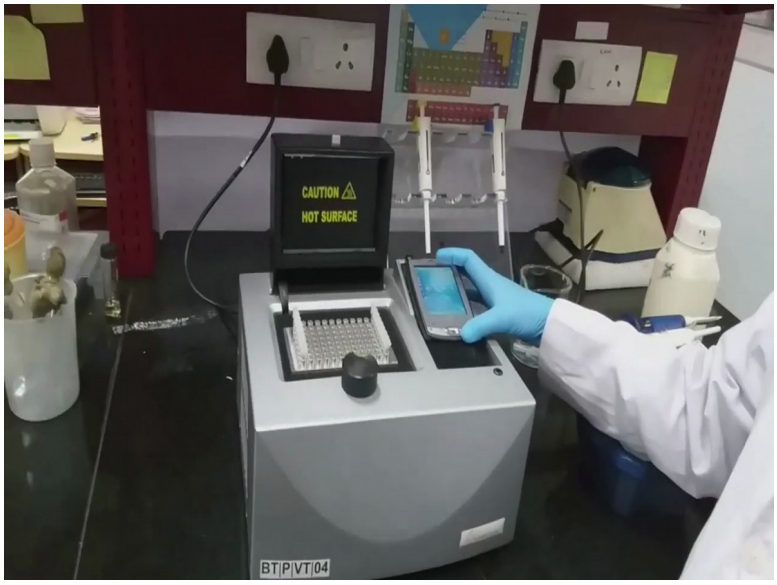
And then subsequently, we are going to show you how to setup the PCR reactions. This is a typical thermal cyclers. We have already discussed the different components of thermal cyclers and what we have done is we have also prepared a very small movie from the thermal cyclers what is available in our laboratory and in that particular small clip, what students are going to show you is about the different components which are present in a typical thermal cyclers such as the reaction purchase or the place where you are going to keep the reactions or the heating block which does not allow the evaporation of the Sample and the different types of buttons what are available for different types of operations within the machine.

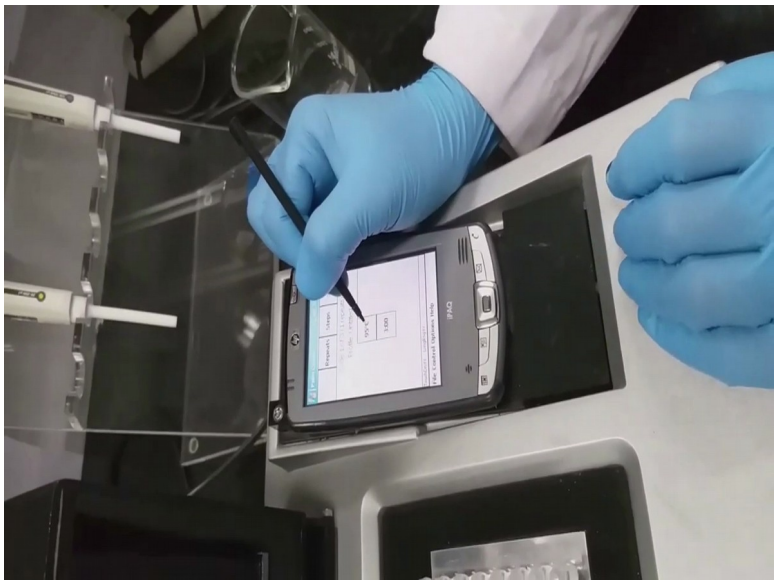
Apart from that, the students would also show you how to setup the PCR reactions. So the purpose of these videos or the demo is to show you how to perform these experiments.

Student starts a demo:

In this video, we will be demonstrating how to setup a PCR reaction and analyse the results using (())(23:51). PCR are polymerase chain reaction. It is a widely used molecule biology technique to amplify a particular segment of DNA. It is also deployed in biomedical research and forensic medicine. The main application of this polymerase chain reaction is chlorine. To setup a PCR reaction, we need template DNA, site-specific primers, DNTP mix, nuclease free water and tack polymerase. For a 50 microlitre reaction, in a typical concentrations of 10 to 100 nanograms of template DNA used and 5 picomoles of each primer will be used.

(Refer Slide Time: 24:50)





This is a earlier version of thermal cycler which contains display unit where we can observe the parameters and change the parameters. This is a heart shield, this is Sample holder and inside there is a peltier system which can maintain the temperature fluctuations. For setting up a PCR reaction, initial denaturation at 95 degrees Celsius, 3 minutes and these steps we will use 30 repeats where initial denaturation will be 30 seconds and annealing it, extension. Extension time should be given, 1 minute per KV.

And here, final extension should be given, 10 minutes and hold it, 4 degrees Celsius.

Demo ends.

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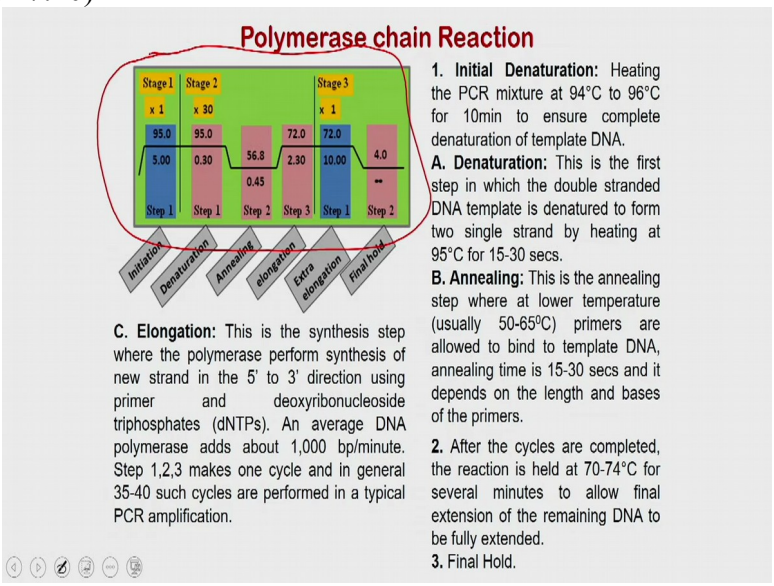
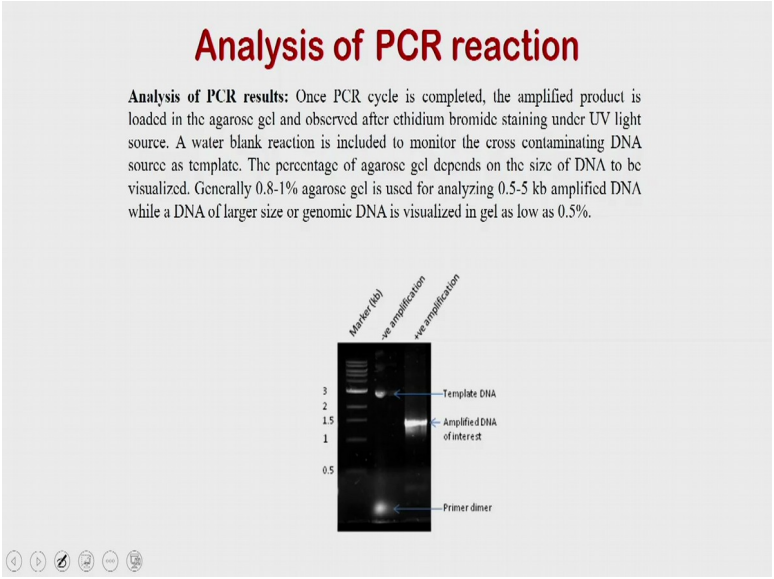


Now moving on to, 13th is about PCR reaction. So not only that they will let you to discuss the said PCR reaction, they will also let you to discuss about how to set up the cycles onto the machine and how to operate them and this operation could be, the machine what we have in our laboratory may not be the machine what is available in your institutions or in your setup, but the more or less, the steps are remain constant whether it is a machine from the one company or the other company or only the few the patterns may be different or few, the way you have to operate these machines may be different. But the overall basic principle remains constant.

(Refer Slide Time: 28:29)



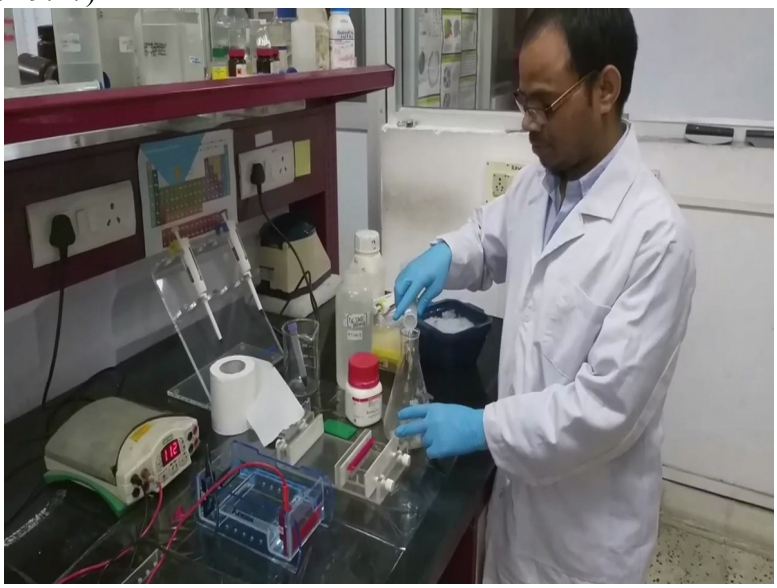
Then at the end, the Banesh and his team is going to show you how to analyse the PCR product. So what we were supposed to do is, once the PCR is over, then you are going to take out the Sample from the PCR machines, then you have to cast a agarose gel and agarose gel, while you are casting the agarose gel, you have to take a lot of precautions and then while you are casting, you can add the dye which is called the ethidium bromide. So ethidium bromide is an inter-collating agents and inter-collates into the DNA and that is how it gives a fluorescence into the DNA.

So once the gel is ready, you can load your Sample into the wells and you can run the samples and visualise it under the UV trans-illuminators and you can take the images. Once you take the images, the students will also show you how to analyse these DNA, both for the size as well as for the amount of DNA variant present in the O gel.

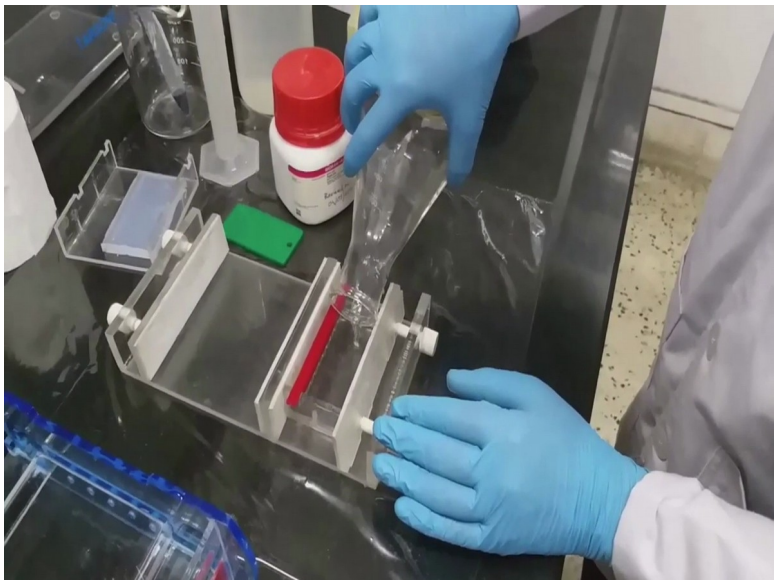
Demo begins.

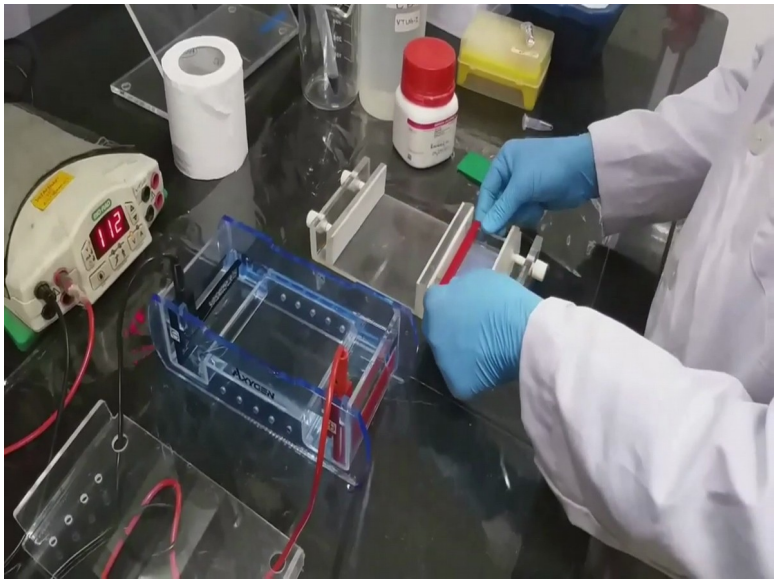
Once the PCR reaction is completed, we have to analyse the results for amplification.

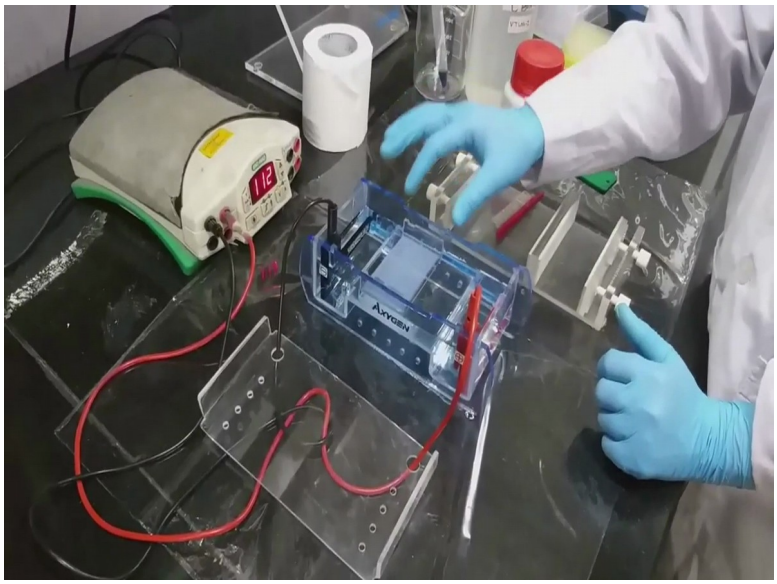
(Refer Slide Time: 29:47)




PCR



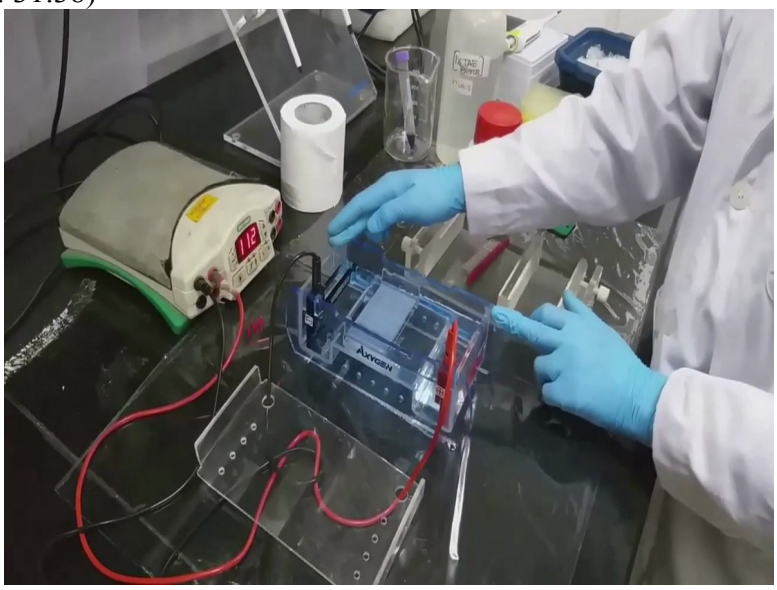

ACCUGEN

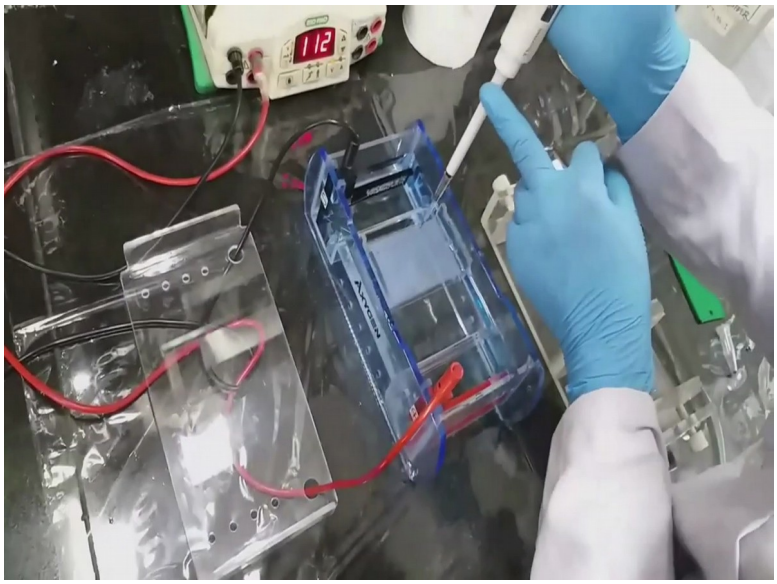


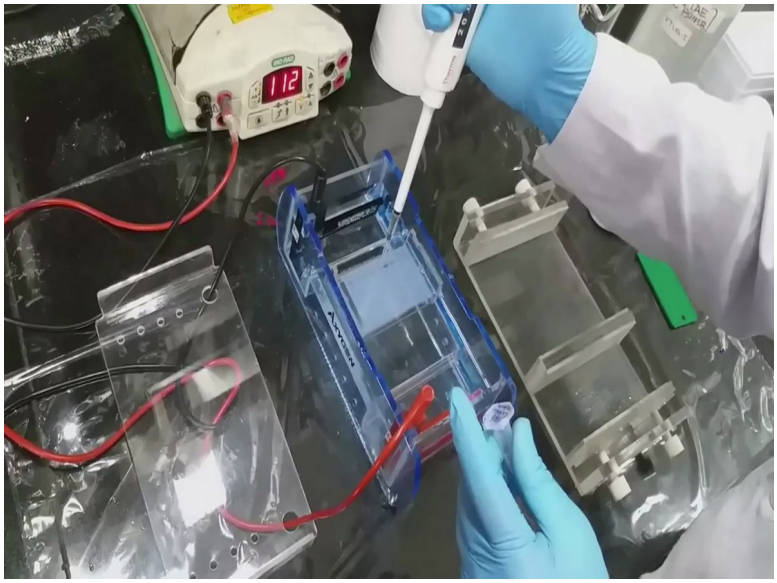
Further, we need agarose and DAE buffer. 1st we have to weigh agarose and mix with the DAE buffer. It will not dissolve easily. So we have to heat it in microwave oven until it gets dissolved. Now agarose got dissolved in DAE buffer. We have to like it hold on up to 50 degrees Celsius. Now before pouring, we have to add ethidium bromide for decation purpose. Now the gel got solidified.

We have to take out the gel and keep it in the electroporotic apparatus. We have to gently remove the comb, loose the nuts and keep the gel in the apparatus. Make sure that the buffer is submerged in the gel. We have to fill the remaining part with 1X DAE buffer.

(Refer Slide Time: 31:38)

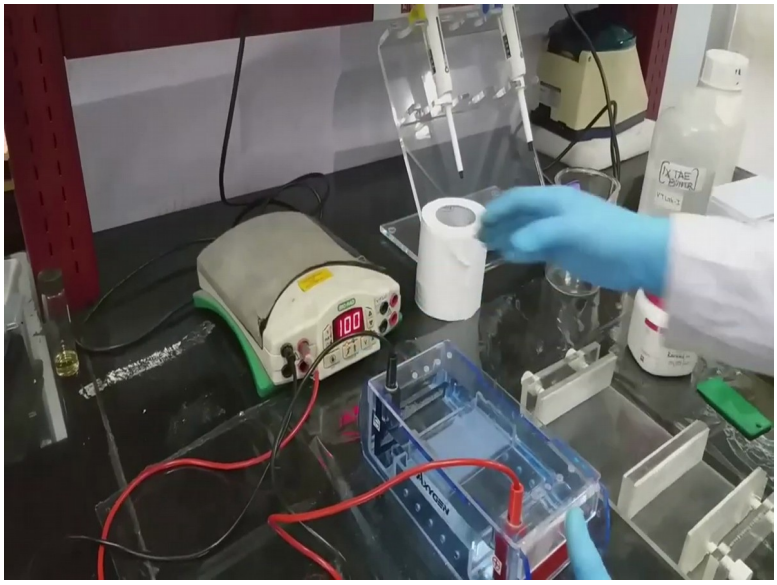






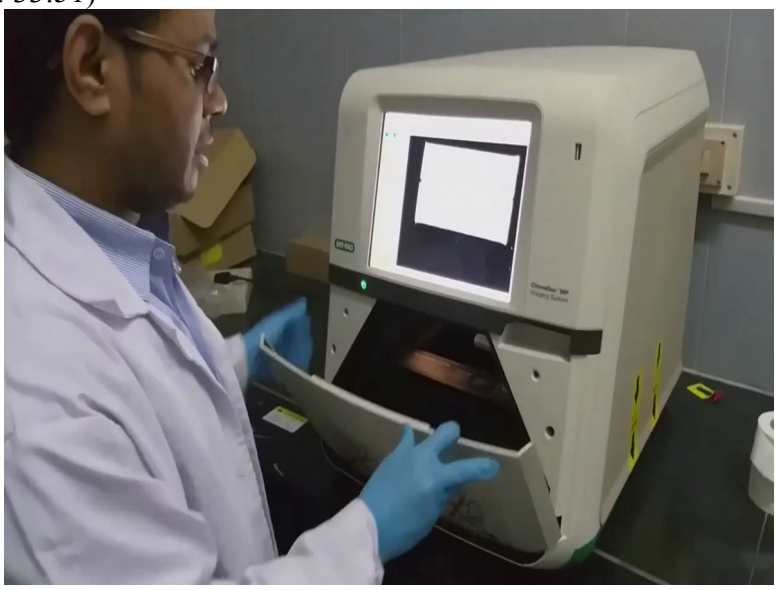
Generally for analysing the DNA samples, we will use agarose gel electroporosis. This is the powerpack and this is the electroporotic apparatus. This is a negative electrode and this is the positive electrode. We can change the voltage from here. For loading of sampling, we have to mix PCR reaction mixture with 5X loading dye.

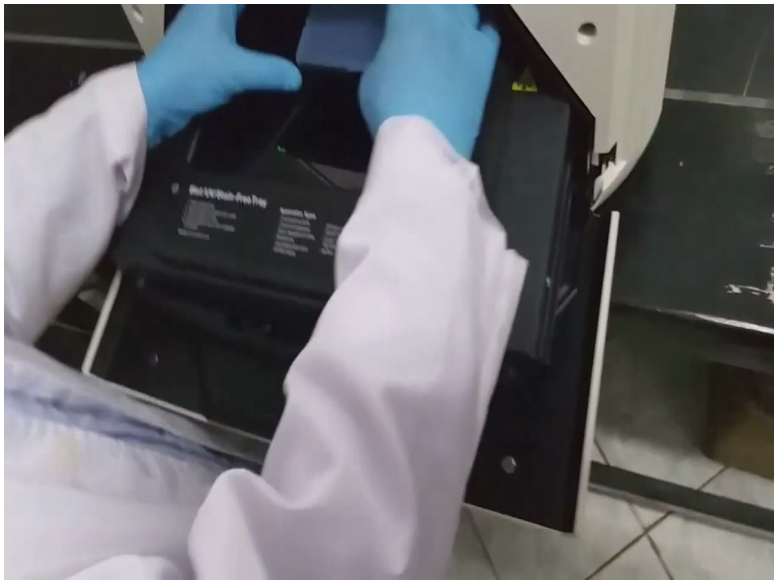
(Refer Slide Time: 33:21)



When the loading is over, we have to cover the electroporotic apparatus with the lid and we have to adjust the voltage, then start (())(33:44).

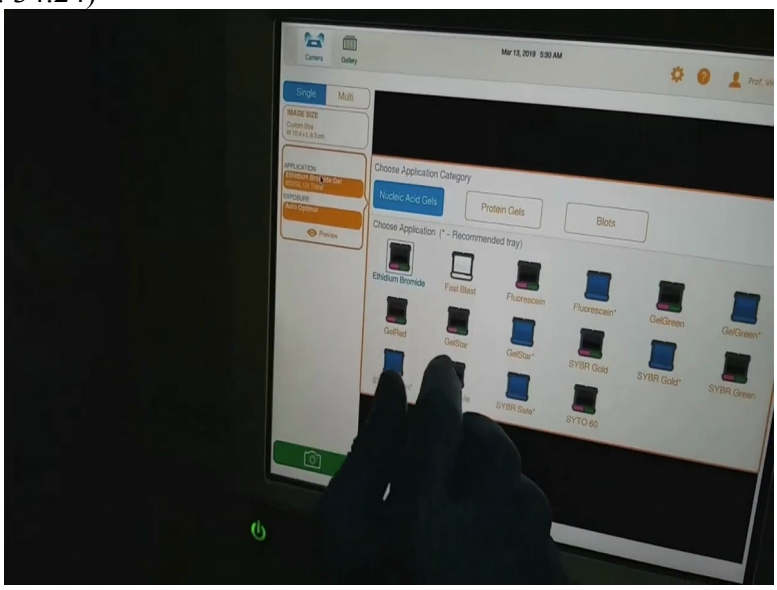
(Refer Slide Time: 33:51)

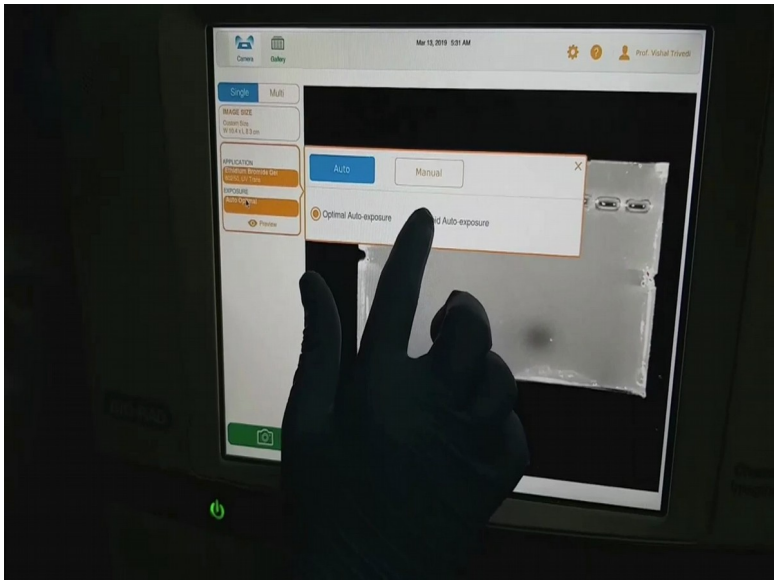


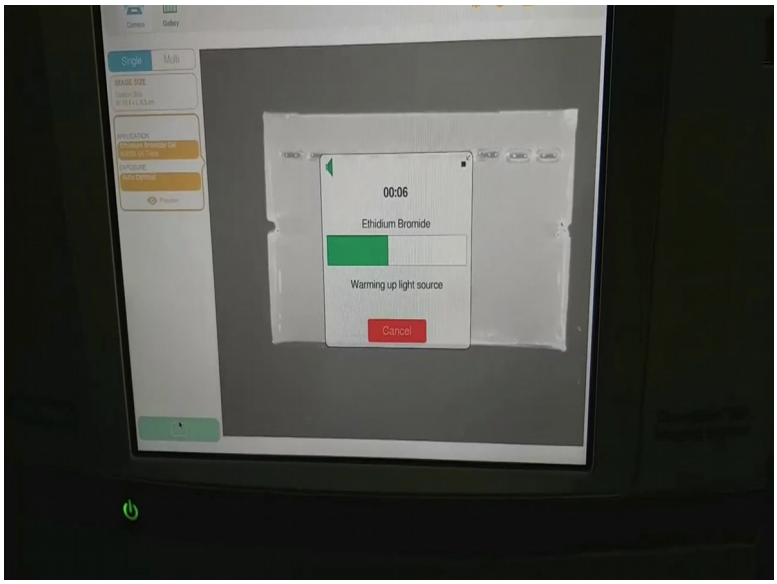


After the agarose gel electroporosis we have to visualise the amplified product. This is the chemiduct MP where we are going to visualise the amplified product. Now we have to keep the gel, then close the thing.

(Refer Slide Time: 34:24)







We have to select here, application, nucleic acids, ethidium bromide, exposure, optimal exposure or we can select manual also. Then we will acquire the images. Now we can find here, this is the DNA ladder, this is the PCR amplified product. We can transform it into, transform or save this image into JPEG.

Demo ends.

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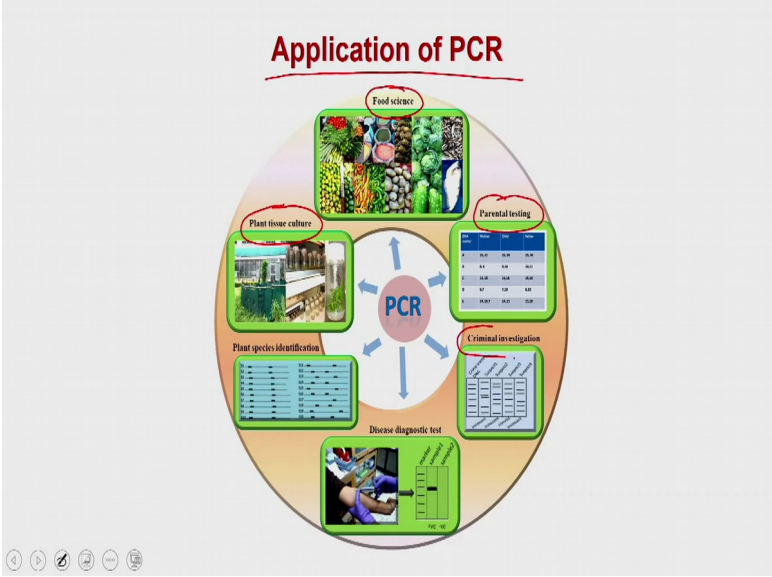
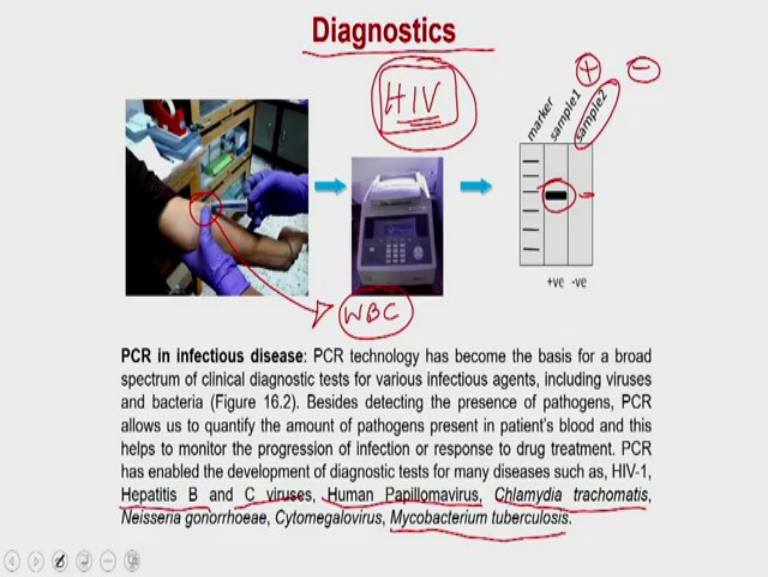


Now at the end we would like to discuss about the application of PCR in different fields. So the PCR has the vast applications, whether it is a food science, whether it is a plant tissue culture or it is a parental testing. Means the all sort of genetic investigations or whether it is a criminal investigations or whether you are going to use the PCR for the diagnostic purpose or whether you will use the PCR into identifying a plant species or characterising a plant species that this is actually the particular plant species because many times, the molecular markers what you are going to use to identify a particular gene or particular plant is more specific compared to the (()) (36:04) evidences.

(Refer Slide Time: 36:10)



So let us discuss few of them. So PCR is extensively being used for diagnostic purpose. In this particular example, we have taken an infectious disease which is called as the HIV and what you are supposed to do is suppose a patient is infected with HIV or it is a sample where you have to detect, so what you are supposed to do is first you extract the blood from the patient and from the blood, what you are going to do is you are going to form the PCR analysis for the HIV virus.

So you have the site specific primers which you can use to amplify the viral DNA and if the sample is going to show you a PCR product for the virus DNA, then the Sample is considered to be positive. If it is going to show you negative result, then it is going to be negative for the particular disease. It could be the HIV, it could be viral diseases such as the hepatitis B and C, it could be human papilloma virus, it could be Mycobacterium tuberculosis and so on.

That you cannot use the PCR-based diagnostics for any organism, you can use it for bacterial species, you can use it for viral species, you can use it for fungal species as long as you will be able to characterise the particular type of molecular marker or particular type of DNA sequence which is specific to that particular organism and that may not be present in other organisms. So as long as you have that specific sequence, you could be able to identify that particular Sample by doing the PCR amplification. So the way you have to do is analysis is very much similar that you are going to get the organics and then you will do the PCR amplification.

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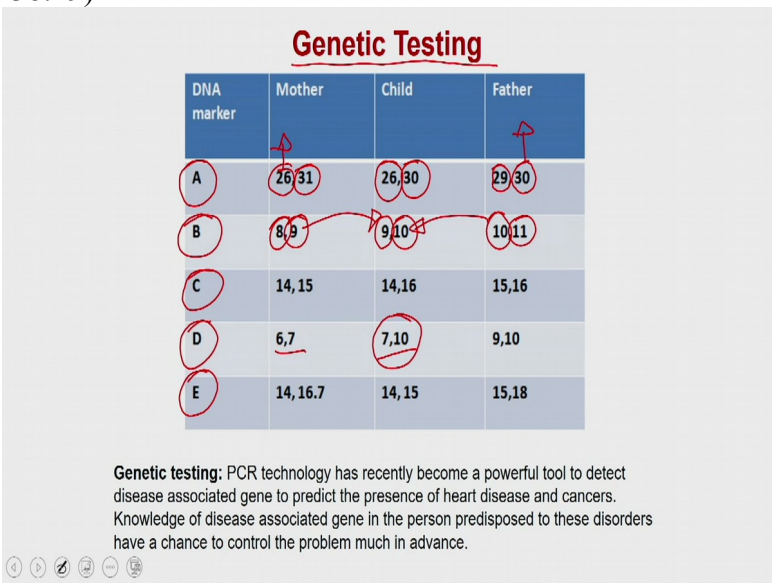

Now in many of the cases, the parental DNA or the parental testing is also one of the major reason or major area where the PCR is contributing into the biomedical applications. So with the help of the PCR, you can be able to assess whether a child is belonging to the given parent or not. You can see that for that particular kind of analysis, what you are supposed to do is, 1st you have to characterise the DNA marker what is present in that particular family and once you characterise these DNA markers, what you have to do is you have to see whether those DNA markers are present in the child and as well as into the mother and father.

So what you see here is that the marker A is present in mother, 26, 31. Whereas in the case of father, it is 29 and 30. So you can imagine, if it is a child of these 2 mother, then the child should have the combination of these DNA markers of mother or the father. So you can see that the child is having a marker which is of 26 and 30, which means it is getting the 26 from the mother and the 30 from the father. So that means it is potentially being a child of these 2 mother and father.

Similarly, you can see that the mother has the marker for 8, 9 whereas the father has for 10 and 11 and the child has 9 and 10 which means the 9 it is getting from the mother and 10 it is getting from the father which means it is actually being a potential child of these 2 parents. Similarly you can see the all other combinations where the child is showing the markers which are of the

mixture of these 2 parents. So the PCR is a very very powerful technology to detect the diseases for genetic testing as well as for the parental testing.

For example, if somebody is prone to the cancer because it has a BRCA gene, that BRCA gene PCR amplification also can be done to predict whether a particular person is predisposed to the breast cancer or not using the this PCR technique.

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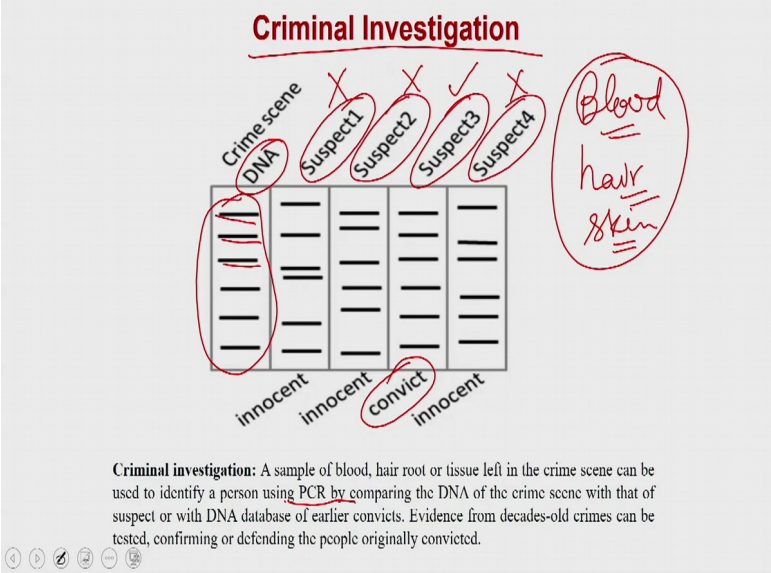

Then the PCR is a very very powerful technique for criminal investigations. In the criminal investigations, what you have is you have the blood, hair or the skin sample from the side of crime and from any of these sources, you will get the cells. Means, you will get the DNA and once you get the DNA, what you can do is you can do a PCR amplification and generate the particular type of pattern. So what you can do is you can see that from the crime scene, you got the DNA which is giving a pattern like this.

Now what you are going to do is, you are going to analyse the blood of the suspects which means in this case, we are showing the 4 suspects. Suspects number 1, 2, 3 and 4. And what you can see is that the suspect number 1 is showing a pattern which is not completely matching with the crime, the DNA what you got from the crime scene. Similarly for the suspect 2, the pattern is not matching with any of these DNA what you got from the crime scene and the suspect 4 is also not matching.

But what you see is that the suspect 3 is showing a DNA pattern which is exactly matching with the DNA what you got from the crime scene which remains this particular guy is the potential convict and the potential criminal who probably have done this particular crime. And the PCR mediated evidences are very very strong, very very reliable and they are being tested on several time or several attempts. So that is why, the judiciary system as well as the criminal system is utilising and relying on the PCR mediated investigations and the result what you get is very very reliable.

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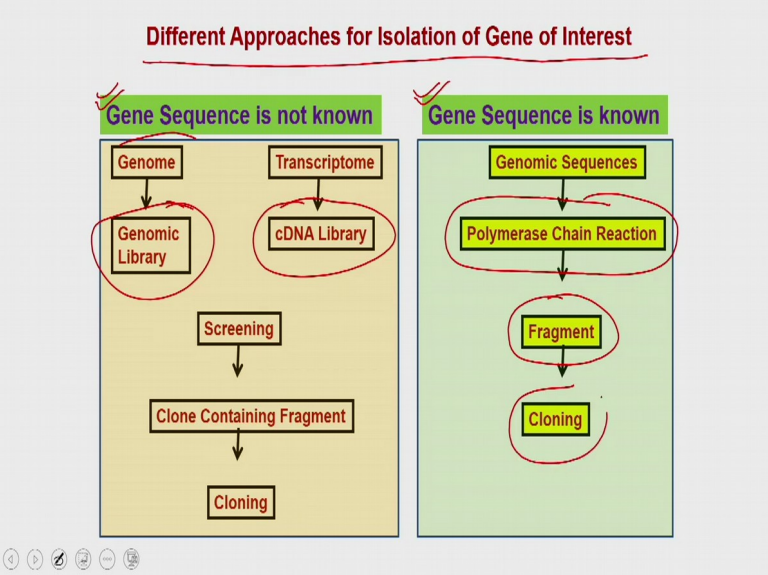

With this, we have concluded our discussion about the isolation of gene of interest. What we have discussed so far? We have discussed that the gene sequence can be isolated under the 2 different aspects, whether the gene sequence is known or whether the gene sequence is not known. Okay. And in both of these cases, when the gene sequence is not known, you can have the flexibility of using the genomic library or to the cDNA library and subsequently, you will get the desired gene fragments and that can be sub cloned and can be used for downstream applications.

Similarly, if the gene sequence is known, you can use the PCR as a technique to get the DNA fragment and then these DNA fragments can be cloned into the expression vectors for the downstream applications. So with this, I would like to conclude our lecture here and in our subsequent lectures, we are going to discuss about the how this particular fragment what you got

from the after isolating the fragment from the either genomic library, cDNA library or utilising the PCR as a technique, how to clone this particular fragment into your desirable transforming agent such as the plasmids. So with this, I would like to conclude our lecture here. Thank you.