Molecular Biology
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Module - 09
Molecular Techniques (Part 2)
Lecture-39 Real Time PCR

Hello everyone. This is Dr. Vishal Trivedi from department of bioscience and bioengineering IIT Guwahati. And in the course molecular biology we are discussing about the different aspects. So in this current module we were discussing about the polymerase chain reactions. And if you recall in the previous two lectures we have discussed about the polymerase chain reactions.

We have discussed about the how the PCR can be evolved from the very crude you know thermal cycler to the very very refined and sophisticated machines and how you can be able to design the or perform the PCR. So we have discussed about the primary designing we discussed about the how you can be able to isolate DNA RNA and all that and how you can be able to perform the PCR. And then at the end when you are done with the PCR you can be able to analyze the PCR also. Now in today's lecture we are going to discuss about the real time PCR.

And the real time PCR is a slight variant of the traditional PCR what we have discussed so far. And it has additional advantage as well. So in the current in this particular lecture we will discuss about the real time PCR and how you can be able to perform the PCR into the your laboratory. So for the first question comes what is the real time PCR and what is the you know advantage of real time PCR. So the first question comes what is the real time PCR and what is the advantage of real time PCR compared to the normal PCR.

So the real time PCR as the name suggests is actually a PCR where you are going to do the amplification cycles and at the same time you are also going to monitor the product. So real time PCR actually combines the amplification of the target DNA and its detection in the single step that is it collects the data throughout the amplification in a real time. Remember that when we were talking about the polymerase chain reactions we were having the four different steps. So we were having the denaturations, annealing, then extension and then that cycle continues. So after at the after at the end of every cycle you are supposed to you know in the in the traditional PCR you will not be able to you know know what is the amount of DNA is being produced whereas in the real time PCR you are actually going to see what will be the amount of product is being developed.

So real time PCR combines the amplification of the gene of interest that is the you know traditional PCR what we are doing also with the intensity of the fluorescence. So this intensity of the fluorescence is directly proportional to the product what is being formed. Remember that when we were talking about additional PCR we are saying that it is you know we are going to run the 25 cycle, 35 cycles and so on. So after end of every cycle it is going to tell you what will be the amount of fluorescence and you can easily correlate that fluorescence to the amount of

product what is being formed. The movement at which the target amplification is observed first mainly define the reactions the time period at which the fluorescence intensity exceed the background fluorescence intensity is called as the cycle threshold or CT values.

As a result a large amount of target DNA the fluorescence signal appear more quickly resulting into the lower CT values. The people sometimes people are not saying the real time PCR they are also using the different words such as the kinetic chain PCR or the quantitative PCR. Quantitative PCR is also been called as qPCR. So there is a slight difference between the traditional PCR versus the real time PCR. In a traditional PCR you are not been able to you are only going to know the end product.

That means after every 25 cycles what will be the amount of DNA is been produced. Whereas in this case you are actually going to see the amplification after every cycle and that is why you it is actually going to give you the real life you know real time monitoring of the product. Now the question comes what will be the advantage of using the real time PCR. So why there is a real time PCR preferred over the fundamental PCR. So the first option is it is going to be a quantitative analysis which means the real time PCR is going to allow for the quantitative measurement of the DNA or the RNA what is present in the sample.

And it is actually going to provide the quantitative results which means it is actually going to tell you okay 10 microgram of DNA is been produced after first cycle 10 cycle fourth cycle like that. Number two is the speed okay. So real time PCR provide the result in a real time hence provide result in a short duration of time. Fundamental PCR requires the post reaction analysis which can be time consuming and so in a fundamental PCR you are actually going to you know run the product onto agarose gel and then you are actually going to you know do the you know transimulator and then you are going to observe the DNA. Whereas in this case it is actually going to give you the real time PCR you know information about what is the product been formed.

Number three the real time PCR it is more sensitive. So real time PCR is more sensitive and can detect the low copy number of target DNA due to the continuous monitoring of the sample. The fundamental PCR is less sensitive because it is actually going to give you the end product. Number four real time PCR is reduced contamination risk. So the closed tube system of the real time PCR reduces the contamination risk due to the minimum post PCR handling.

It is the main concern with the fundamental PCR. Then data accuracy since this is quantitative the data is going to be more accurate. So real time PCR provides the precise and the accurate data throughout the quantification of the frozen signal. Real time PCR is more susceptible to the variation because it depends upon the you know when you are going to run the DNA onto the agarose gel depending upon the many factors your quantitative quantitation could be wrong. Number six it is high throughput.

So real time PCR can be easily automated for high throughput applications making it suitable for the large scale testing. Remember that real time PCR people were using very you know often in the case of COVID testing right because of this feature only that you can actually be able to

you know do like 50 samples 100 samples 2000 samples and so on because it is it can be automated with the help of the high throughput things. And number seven you can actually do a multiplexing. So real time PCR enables the simultaneous detection of the multiple target in a single reaction which is very very difficult to do with the traditional PCR. So because of these seven advantages people are doing the real time PCR because it is giving you more information about your biological samples than the traditional PCR.

Now what is the principle of the real time PCR? So the principle of real time PCR is to quantitatively measure the amount of specific DNA or RNA target in a sample by continuously monitoring the amplification process in a real time fluorescence. This technique leverage the natural ability of DNA to produce a fluorescence signal when it is amplified. There are two common methods which are being used. One is cyber green dye method and other one is called as the TaqMan probe method. So the cyber green method in this method a fluorescent dye such as cyber green is added to the PCR reaction mixture.

Cyber green binds to any double standard DNA and this generates during the process as the DNA target amplifies more double standard DNA is produced leading to an increase in fluorescence. This rise in fluorescence is directly proportional to the amount of target DNA. However it is important to design the specific primer to ensure that the fluorescence signal correspond to the specific target. So this is exactly what is going to happen. When you are doing a PCR reactions you are going to do the denaturation, you are going to do the annealing of the primers, then you are going to do the extensions and after every cycle you are actually going to have the more amount of double standard DNA.

Remember that after if you start with the one amount one DNA molecule then after first cycle you are going to have so after first cycle you are going to have two copy of DNA and again you are going to have four copy of DNA and so on. And as the amount of DNA is going to increase it is actually going to bind the cyber green. So cyber green is a dye which actually goes and binds to the double standard DNA. So as soon as you have the one amount of DNA its cyber green is going to give you the X fluorescence. If the two DNA molecules are being formed then the two molecules of cyber green is going to bind and that is why you are going to get the 2 X fluorescence and you are going to get 4 X fluorescence and so on.

And that is why you are actually going to get the fluorescence signal proportional to the amount of DNA. And that is what it is going to happen. So once you are going to do the DNA synthesis after DNA synthesis you are going to have the two amount two strand of DNA and the cyber green is actually going to intercalate into the DNA stuck DNA and it is going to give you the fluorescence. So this is the first method. The second method is the TaqMan method TaqMan probe method.

So TaqMan probe method is more advanced method. So in this method apply a specific sequence specific fluorescent probe like TaqMan probe that is designed to bind the target DNA. When the probe binds to the target during the DNA synthesis the DNA polymerase cleaves the probe releasing its fluorescence reporter. The increase in fluorescence is directly proportional to

the amplification of the target sequence. This method offer high specificity because the probe only fluorescence when it is bind to the intended targets.

The real time PCR instrument set a predefined fluorescence threshold and the cycle at which the fluorescence signal surpasses the threshold that is recorded as the cycle threshold or the CT values and the CT value is very very important. So in a TaqMan method what you are going to do is you are going to do annealing. So when you are going to do annealing the your TaqMan probe is actually going to go and bind and when there will be polymerization and the strand displacement what will happen is that this fluorescent dye is actually going to be cleaved by the DNA polymerase and it is actually going to be released into the signal released into the solution and that is actually going to be detected by the detection system and is actually going to be proportional to the amount of DNA what is been synthesized. And this is exactly what you are going to go. You are going to see the increase in the fluorescence signal and then it reaches to the threshold and then it actually goes and reaches to the saturation.

So when it reaches to the saturation then it is actually going to called as the cycle threshold or the CT value. So this is actually going to be CT values. And so this is going to be the maximum fluorescence what it is actually going to achieve. And the CT value is inversely proportional to the amount of target DNA or RNA in the sample like the lower value CT values indicate a higher concentration of the target sample. This means when it reaches to this threshold values it is actually going to reach this point if you are going to have the.

So it takes 10 cycles it can take 15 cycles it can give 30 cycles. So more number of cycles it reaches and crosses the threshold cycle threshold values that means that the lower the concentration of the DNA or RNA was present in your sample. So it is inversely proportional to the amount of DNA then the amount of time it is going to take to reach to the threshold values. The specialized software which process the you know which process the flows and data to calculate the CT value and a standard curve is generated using the unknown concentration of the reference DNA and RNA. This is standard curve enables the determination of the initial amount of the target sequence in the sample.

Now if you want to perform the real time PCR you require a set of reagents you require the set of machines and so on. So let us see what are the things you required. You require a thermal cycler and this thermal cycler is different than the thermal cycler what we were using for the traditional PCR because this thermal cycler should have a detection system so that it can be able to detect the fluorescence. So you require a thermal cycler and you also require a fluorescent measurement system so that it and you can integrate that into the machine so that while it is amplifying the reactions it can also detect either the cyber green method or the TaqMan probe method. Then you require the DNA or RNA as a template right mostly people use the RNA as a template because that RNA is the most desirable thing what you can do but you can also do the DNA also and the target or DNA which can you want to quantify or amplify then you require steep primers you require the forward and the reverse primers you requires the enzymes so you require the two different enzymes you require the DNA polymerase and you also require the reverse transcriptase.

So DNA polymerase is responsible for the synthesis of new DNA strand during the PCR so it is exactly the same as what we were using for the traditional PCR and then you also require the reverse transcriptase so you can use to quantify that RNA which is done by converting the RNA into cDNA and before the process of amplifications. Then you require the fluorescent dye so either a fluorescent dye that binds to the double standard DNA like cyber green or you can use a specific fluorescent probe that bind to the target DNA generating a signal during amplification that means you can use the TaqMan probe method. Then you require the detection system so you require the real time detection system so this system requires the fluorescent measurements during PCR cycle commonly cyber green or the TaqMan detection systems are used. So in many of the time what happen is that this real time detection system and the thermal cycles are actually going to be integrated into each other and that is why it is going to be called as real time PCR machine. And then you also require the PCR tubes or the plates.

Now before moving into how you are going to perform the real time PCR you also should understand how the reverse transcriptase is going to work. So reverse transcriptase are like RNA dependent DNA polymerase so it is exactly the reverse what the RNA polymerase is doing. So RNA polymerase is doing the RNA synthesis from the DNA reverse transcriptase is doing the DNA synthesis from the RNA falls into the category of polymerase enzyme which helps in the generation of cDNA taking RNA as a sample. Reverse transcriptase can perform three activities it can perform the RNA dependent DNA polymerase that uses the single standard DNA as a template to generate the cDNA it can do the RNase H endonuclease activity so it can degrade the RNA strands of DNA-RNA hybrids and it can do the DNA dependent DNA polymerase. So converting the single standard RNA probe into a double standard DNA all these molecular techniques such as real time PCR, RTQ PCR, cloning of cDNA, RNA sequencing frequently uses the reverse transcriptase at requires conversion of RNA into DNA.

So reverse transcriptase has two activities polymerase active site and endonuclease active site and it is actually going to have the three different types of enzymatic activity RNA dependent DNA polymerase, RNase H endonuclease and the DNA dependent DNA polymerase. And exactly this is what it is going to do it is going to take up your single standard RNA molecules then it is actually going to do the polymerization reactions so when the reverse transcriptase is going to work it is going to use the you know the it is going to synthesize the DNA and it is going to produce a RNA-DNA hybrid and you know that reverse transcriptase is also going to have the RNase H activity so it is actually going to degrade the RNA part so it is going to degrade the RNA from the this RNA hybrid and leaving the single standard DNA template and then this single standard DNA template is also going to be converted into double standard activity by the DNA dependent DNA polymerase activity and at the end what you are going to do is you are going to get a double standard DNA template from the single standard RNA template and this double standard RNA template is going to be called as the cDNA or the complementary DNA. There are many more methods through which you can be able to prepare the cDNA but most of the people use the reverse transcriptase because it is easy and it is straightforward. Now once you prepare the cDNA right you can be able to perform the real-time PCR. So there are steps which are involved into the real-time PCR like you are going to have the first step that is the sample

preparation.

Sample preparation means you are going to denature the cells suppose you started with the cell or you have started with the fluid or whatever so you are going to crush the cells you are going to denature the and you are going to extract the RNA or DNA whatever the target molecule you want to quantify and then you are going to extract the DNA or the RNA. If you are going to extract the DNA then it is directly going to get into this reactions. If you are going to start with the RNA then it is actually going to have the additional step of converting the DNA into RNA into DNA with the help of the reverse transcriptase. Then you are going to have the primer and the probe designing so it's going to you are going to design the primers and probe and all that and then you are going to set up the reactions. We are going to set up the body real-time PCR reactions where you are going to have the template DNA or cDNA.

You're going to have the cyber green you are going to have all other kinds of you know the reagents what you are going to add then you are going to set up the amplification cycles you are going to do the flows and monitoring and then you are going to have the threshold detections and then ultimately you are going to do the data analysis. Now each step in each step like for example, in the step number 1 you are going to have the sample preparation. So, you are going to extract and purify the RNA or the DNA whichever the molecule you want to you know isolate or detect from the biological sample both quantify and quality are important factor for the accurate results. So, you are actually going to isolate the DNA and RNA then you are going to check the quality of the molecule by running it on to the agarose gel. So, in the case of DNA you are going to run the regular agarose in the case of RNA you are going to run the denaturating agarose gel.

Once that part is done and you have cleared the QC part it is called as you know you are going to pass the quality control. Then you are going to have the primers and the probe design. So, the primers are designed that will bind to the region complementary to the target sequence. Probes are also designed which are highly specific for the target regions and the primers and probe play an important role in the quantification of the sample. So, once you are done with this you are going to set up the reactions and you are going to prepare the PCR reactions that will contain the cDNA template.

Some cases if you are isolating the DNA then you are going to have the DNA then you are going to put the forward and the reverse primers, you are going to put the fluorescent probes or the dye then you are going to put the DNA polymerase, reverse transcriptase, buffer solutions and dNTPs and then you are going to set up the amplification cycles. So, amplification cycle more or less remain the same as what we have discussed for the regular PCR that you are going to have the denaturations, then you are going to have annealing, then you are going to have extensions and so on and this will continue after the extension it will again go for denaturation and so on. So, exactly the same as what we have discussed for the traditional PCR. And then it is going to have the source and monitoring.

So, you are going to have the real time PCR. So, that is you do not need any kind of steps to be done that will eventually be done by the machine and it is going to measure the intensity of

the cyber green method or the intensity of the fluorescent dye from the Taqman probe method and then you are going to have the threshold detections. So, the predefined threshold level of fluorescent is set and that is actually been done by the experience ok. So, you will know that what will be the threshold values for this particular gene product or so. The cycle at which the fluorescent surpasses the threshold level is recorded as the cycle threshold or the CT values and the CT value is inversely proportional to the initial concentration of the sample which means if the CT values are low, then your concentration of molecule is concentration of molecule is high is very high because then that is how you are reaching to the CT value at very very low cycle number. Then you also have the 6 number that is the data analysis.

So, there are softwares available which detect the fluorescent signal and generate a standard curve which used as a reference which used as a reference DNA RNA concentration which allow the determine the initial concentration of the sample. And now once you are done with all these you can actually going to get the fluorescent data right the pattern how you are going to your how the fluorescent signal is moving into this and then you can actually be able to do the data analysis. So analysis of real time PCR. So in real time PCR the most common graph method is the amplification plot which usually represent the accumulation of PCR product over the course of the action. So this is what it is you are going to get of in on this side on the x axis you are going to have the cycle on the y axis you are going to have the fluorescence and it is going to have the multiple phases like the initial phase you are going to have the baseline then you are going to have the threshold values then you are going to have the plateau.

So in the x axis represent the PCR cycle or the time each PCR cycle involves the denaturation annealing and extensions y axis represent the fluorescence cycle detection by the instruments right and then you are going to have the amplification curve. So each sample or target gene is represented by the specific curve on the graph these curves show how the fluorescence signal increases over cycle a steeper slope indicate a higher amount of target DNA. So this pattern of this slope is also going to give you an idea what could be the amount of DNA present right because if the pattern is sharp if it is slowly moving then you are actually having the very low concentration of the target DNA. If it is moving very fast or steep then basically it is you know your amount of DNA is very high. So amplification curve has three phases the initial phase then you are going to have exponential phase and you are going to have the plateau.

So in the initial phase it represent the early phase of the cycle PCR cycle not enough amplification of cDNA to produce a significant amount of flow the cycle just started with denaturation of double standard DNA to separate the single standard DNA. So in the initial phase it is just going to prepare the machine or prepare the system for starting of amplifications. Then in the exponential phase there will be a you know there will be of amplifications and every after every cycle it is going to be double right. So in the exponential phase the flow sense signal increases with each cycle and it is going to be double after every cycle. So from it forms a well defined sigmoidal curve which indicates that the PCR reaction is proceeding efficiently.

Then during this phase the CT value is typically at the middle of the phase which is used to determine or quantify the initial concentration of the target DNA. The slope should be steeper

which tells the efficiency of the reaction while the linearity of the slope represent the doubling of the amount of DNA. Then you have the plateau phase at this phase the system is going to get saturated and you are going to get the plateau. So the plateau represent the point where the majority of the target DNA has been amplified. The curve loses its linearity since it is not the suitable for precise quantification of DNA.

And then you have the threshold line typically set the level where above the threshold baseline with flow sense the cycle at which the each curve crosses the threshold is called as the cycle quantification or the cycle threshold. The lower the QHC value higher the initial amount of target DNA. As it is known that CT value is inversely proportional to the initial concentration of graphs from the graph it can be concluded that the CT1 is smaller than the CT2. That means the DNA concentration in a small sample 1 is bigger than the DNA concentration in the sample 2. And that is what you are going to do the analysis and based on that analysis only you are going to say which gene or which gene product or which sample has more amount of DNA and less amount of or less amount of DNA.

So you can actually be able to compare the two samples. So this is all about the theoretical aspects of the real time PCR what we are going to do and what we have actually understood that what are the different steps you can be able to perform what are the requirements and how you can be able to perform. Now if you want to do the real time PCR in your laboratory you also need experimental feedback. You also require the practical demos so that you can be able to understand how it can be performed. So in the if you see the steps first step is how you are going to prepare the sample right. So in the first step you are going to denature the cells and you are actually going to prepare the RNA right.

And we have prepared a small demo clip so that you can be able to get acquainted how you can be able to isolate the RNA. Remember that in the past we have discussed about the RNA isolations whether it is using the quality affinity chromatography or whether we have used the other methods. So we have prepared a small demo clip and it is actually going to explain you how you can be able to prepare the RNA from the sample. Hello everyone in this video I will be discussing how to isolate RNA from the clinical samples.

So first step is to add the trizol in the samples. So this trizol that I have already added in it this trizol contains 40 percent of phenol, guanidine thiocyanate, ammonium thiocyanate and sodium acetate buffer. So after the addition of trizol we will add 200 microliter of chloroform in it. So I have added the chloroform in it then I will gently mix the solution. So until it turns milky. So after that I will centrifuge it at 4 degree Celsius for 15 minutes at 13000 rpm.

Now the centrifuge step is done we will collect this upper transparent layer from it and without touching the middle interface layer and transfer it to a new tube. Then we will add equal volume of isopropanol into this. Then gently mix the tube by simply inverting it. Now we just incubate this tube at the room temperature for 15 to 20 minutes. Now the incubation is done we will just centrifuge it for 15 minutes at 4 degree Celsius at 13000 rpm.

Now after the centrifugation we will just discard the supernatant and wash the pellet with 70 percent of ethanol. As the RNA quantity is very low we cannot see this pellet. So we will just blindly add the 70 percent ethanol.

Just try to detach it from the bottom. Just mix it two three times. Now after the mixing we will again centrifuge it for 5 minutes at 8000 rpm at 4 degree Celsius. Now we will just discard the supernatant and let the RNA pellet dry out and let the ethanol to evaporate for 5 to 10 minutes. Now as you can see this ethanol is completely evaporated. Now we will just re-suspend the RNA pellet into the QPSD water.

Here I am just re-suspending it in 20 micrometer. I am mixing it with a pipette so that all this RNA get diluted in this. Now we just incubate this RNA samples at 60 degree Celsius for 10 minutes. Now after this heating we just quick chills the RNA at ice for 5 minutes. Now the RNA isolation step is done. We can just quantify the RNA how much RNA we have just isolated and then we can just use for this like for cDNA preparation.

So now you have understood how you can be able to isolate the RNA from your samples. Once you isolated the RNA you are actually going to set up the reactions. And we are not getting into the detail of primer and probe designing because that we have already discussed when we were discussing about the traditional PCR. So primer designing is exactly the same but once you have isolated the RNA your second part would be that it gets converted into cDNA because you are not going to put the RNA into the reactions you are going to put the cDNA into reaction and that will be your second part. So that also we have prepared a small demo clip where the students are going to show you how you can be able to convert the RNA into the cDNA and you are going to set up the reactions.

Now as the RNA isolation is done in this video I will be showing you how to prepare the cDNA from the RNA. Now for that we need a I have prepared a I will be preparing a 10 microlitre of reaction and in 10 microlitre I will be adding 1 microlitre of random primers to prepare the cDNA. This is a random hexa what I will be using for this. For the cDNA preparation everything should be at a it should be after the primer I will be adding 2 microlitre. I have 10x reverse transcriptase buffer so for the 20 microlitre of reaction I will be adding 2 microlitre.

Now 5 microlitre is water. This is the RNA so I am using 3.2 microlitre of RNA because I have quantified the RNA that we have isolated and for 1 nanogram of RNA we will need 3.5 microlitre of RNA. Now the reaction which is done we will just set it up in the PCR. So, we have 2 standard PCR cycles first is 10 minutes for at 25 degree Celsius next is incubation or extension time for 2 hours at 37 degree Celsius and finally, we will just inactivate the whole reaction at 85 degree for 5 minutes. We just confirm the volume all the confirm the steps and keep the tube in the PCR.

Now we will try the block and we just start the reaction. Now as you can see it will take 2 hours and 15 minutes to complete the reaction and after that our cDNA is prepared. Now once you have prepared the cDNA you are going to set up the reactions. So, you are going to put the cDNA into the reactions right you are going to set up the RT-PCR reactions you are going to set the

amplification cycles you are going to see how the fluorescent monitoring is working or not and you are also going to set up the threshold detection right and these steps also we have prepared a demo clip so that you can be able to get familiarized with the how you can be able to perform the real-time PCRs and how you can be able to set up the reactions once you have the cDNA. In this video I will be showing you how to do the real-time PCR of just of the cDNA that we have recently prepared. So, my targets will be GAPDH and some virus primers like in this case I am using NDV and N primer.

So, for all the reactions I will be doing in the triplicates. So, as you can see these are the tubes for the real-time PCR and I am using 3 tubes 3 sets for a single sample. So, this is the sample and I will be amplifying with the cyber green. So, for a 10 microliter of reaction I will be requiring 5 microliter of cyber green. So, I am going to add 5 microliter in each tube. So, I am going to add 5 microliter of cyber green, 1 microliter of PCR sorry primer, each of the tube.

This is GAPDH, this is a virus primer. So, I am going to compare this amplification of viral RNA with the internal control of GAPDH. So, this is the sample, 1 microliter of primer, I will add 3 microliter of water to make the old reaction up to 9 microliter. Now, I have added 9 microliter of buffer which contains cyber green, primer and water. Now, the most crucial step is to add the cDNA which is 1 microliter.

So, in 1 microliter of cDNA I will be adding 5 nanogram of cDNA. So, to by adding equal amount of cDNA which is 1 nanogram, 5 nanograms sorry into each well I will be ensuring we have equal amount of RNA. So, that we can compare if we can compare the virus modulation or virus replication inside the samples. Now, the reaction is complete. So, I will just close the lid.

The reaction is setup, we will just put the these tubes in the real time machine. Now, we just setup the reaction of real time. This is the point studio real time machine and we will just setup complete run. Now, we will just fill up the data. So, we have just done the experiment test 1, this is the block type. We are calculating comparative C value, CT value and we have used cyber green for the amplification.

Just click next. This is the this is the PCR cycle. The total volume will be 10 microliter as you can see. This is the cold stage 15 degree Celsius for 2 minutes, then 95 degree Celsius for 10 minutes step 2 and final PCR stage or amplification stage it will be at 95 degree Celsius for 15 seconds and 60 degree Celsius for 1 minute and this complete cycle will be done for the 40 times. The final step is melting curve which is 95 degree Celsius for 15 second and just 60 degree Celsius for 1 minute and final is 95 degree Celsius for 1 second.

This is the dissociation step. So, this is the complete run complete cycle. We just click next. We do the advance setup. As you can see as I have already discussed, I have two different targets.

First is gap DH and next is NDV NDV N and we have only one sample. So, this is test sample. Test sample we just tell the machine or tell the software that which well we have used. So, I have

placed my tubes in the sixth lane. So, all these things are these six things are test sample.

First three are for gap DH and next is the NDV. We just hit next button. We just see if there is something in the machine.

It will show here the run number. So, this is the run number. Just click this. I will save here. Now, the reaction is started. So, it will take around 96 minutes to complete. So, this is the amplification plot. This is the number of cycles that we have put from as you can see this 0 to 40 cycles and this is the reaction threshold.

From here we will see this sample are getting targeted hits or not. So, these are the samples. Now, it will take 96 minutes to complete. Now, this is it for the real-time PCR. So, after this 96 minutes, we will just get the CT values. For that, after that we can just compare the gap DH and the NDV and CT values and see if there is an amplification of NDV and gene or not.

That is it for the review. Now, we have what we have discussed so far. We have discussed about how you can be able to prepare the RNA. Once you prepare the RNA, how you can be able to generate the cDNA from the RNA and then how from cDNA how you are going to take the cDNA and put it into the RT-PCR reactions, how you are going to set up the RT-PCR reactions, how you are going to set up the amplification cycles, how you are going to monitor the fluorescence and threshold. Now, once you are done with the threshold detections, you are going to get the data actually and you are going to get the fluorescent data. Now, at the last step, you are actually going to do the data analysis and data analysis so that you can be able to calculate the CT values.

You can be able to know what is the number of cycles in which you have crossed the CT values and so on. So, that also we have prepared a small demo clip which will actually going to explain where the students is actually going to explain how you can be able to compare the expression level of the two different genes from a particular two different samples. So, in this analysis, they have taken the GAPDPH as the housekeeping genes and it is not going to change from one sample to another sample. So, that is actually going to be used for the background corrections or for equalizing the two samples and then they have also tested the other gene for experimental purposes. So, this is the analysis part. So, as I said at the end of the PCR reaction, we will get a CT value and then we have to compare how the expression of that gene is modulating due to the virus infection.

So, you can see I have already arranged the data. These are my samples, mock, these are treated or we can say virus infected and these are the repeat samples for second gene. So, one gene I am having GAPDH as a normalizing control and second I am having a p53 gene. So, in this case, we want to see how this p53 gene is getting modulated due to virus infection. So, suppose if I am having a control sample and the second one I am infecting the cells with the virus, so some proteins will go down regulated, some protein will go up regulated.

So, we want to see what is exactly happening to this p53 at the mRNA level. So, these are the

CT value and the method that we are going to follow to calculate the full change is this one. Analysis of relative gene, the method name is 2 raised to the power minus delta delta CT method. So, you can read this paper about the analysis. This paper is having almost 160,000 citations.

So, you can see, you can take a photo of that paper as well if you want or you can write the title. So, this is the analysis of relative gene expression data using real-time quantitative PCR and that this method. So, in this method to calculate the full change, we need to calculate this 2 raised to the power delta delta CT. So, in a minute, I will tell what exactly this is.

So, as I said, we have a CT value. So, you can see the CT value is 19.7, 19.365, 20.459. So, in all the three cases, this is almost similar. So, we will calculate a mean CT value, average of these three values. So, this will copy and we will calculate the CT value for each for all the cases.

Now, we have a mean CT value, say 19.8, this 25.8, then we have to calculate a delta CT. Now, this is mean CT value. Now, we will calculate delta CT, difference of CT value. So, which difference? So, in a single sample, we have two genes GAPDH and a P53 gene and we have two CT values. So, we will calculate the difference between these two.

So, for every difference, we will have a two value, this one and this one. So, first I will use the value of control, this minus, this value. Now, we have a delta CT. Similarly, we will calculate the delta CT for the treated samples.

So, this is delta CT. Now, we will calculate delta delta CT. Delta delta CT is now we are using, as I said, we are using GAPDH as a normalizing control. So, suppose I am giving an infection and some of the protein, as I said, is going down regulated. So, the cells will probably die. So, the number of GAPDH mRNA in all the cells will be different.

So, we want to normalize the expression of all the proteins using a GAPDH. So, see, the GAPDH is, in this case, it is 19.8, but in the infected one, it is 25.

8. So, you can guess in which of the cells the GAPDH expression is more. Obviously, 19.8, but we want to normalize it. So, if we are getting the value of GAPDH in this case, P53 is 25. But in the infected one, the GAPDH is 25.8 and this is 29.

5. So, we can't say if the virus is getting, due to virus infection, the value of P53 is down regulating or up regulating. Can you guess it? No, we can't say for sure. Because in both the cases, the GAPDH is different.

Suppose we are getting the same GAPDH here. So, in both the cases, we are getting 19.8 value for GAPDH. And then we are having the P53 at 25 and then 29. Then we can clearly say because of this virus infection, the P53 is going up or down? Not up regulated, down regulated because the CT value is increasing.

So, it is down regulating. But in this case, we are not having a similar GAPDH. It is slightly

increased. So, the GAPDH is also less. So, we need to normalize this value to this value.

So, we can compare the expression of P53. So, this is normalization. First is calculating the delta CT. Then we will calculate the delta delta CT. Delta delta CT will normalize by subtracting this value with this value only. So, I will repeat it again. Delta CT is the difference between the mean CT value of two genes from the same sample.

Difference between the mean CT of two genes from the same sample. So, we calculated the difference between here and this. And this delta delta CT is normalization basically. So, we are going to normalize this value with this only. So, that's why I am subtracting the same value.

But in this case, we are not going to normalize. We are going to simply use subtracting this value with the test one. So, here we have minus 1.6. Now, the fold change is as I said the method is 2 raised to the power delta delta CT.

Now, as you can see we got our delta delta CT. So, we will raise the power. 2 raised to the power minus of delta delta CT. So, you can see fold change is 1 which means we have successfully normalized the GAPDH value because it's coming 1. But we have to calculate the fold change for this. So, you can see it is coming 3. So, which means that if we are normalizing the value in the mock treated, the expression that we have normalized is 1.

But in the treated one, it is 3 times. So, it is up regulating the gene, the p53 gene. But initially when we saw the value, we could see that 25 or 29, we could see that the value was getting increased. Because of this gap, we are able to see this is not decreasing, the expression is higher as compared to this one. So, this is how you plot a graph. So, in the mock control, you will have value 1 and in the treated one, you can see this is 3.

So, suppose if I am changing the value and decreasing the value, suppose I am 22. So, this is 21 and this is 22.5. So, you can see this is how this value is increasing. Now, this is 655 times of the one.

And say if I am decreasing this value, say 35, this is also 35 and this is 35, 24.5. So, you can see now this value is 0.08, which means the virus down regulated the gene. So, do you have any doubt? So, this is the analysis part. Now, as Sir was mentioning about the COVID-19 or say like you generally went to, suppose you went to a doctor and you gave your sample for the testing, like if you are COVID positive or not. So, what will you do? You will collect the samples and will run the PCR and at the end, you will get a CT value.

Now, as I said, we are setting up 40 reactions. So, out of 40 reactions, at which cycle we are getting the blood pressure? In this case, 25. In this case, 90. So, suppose if you are COVID positive, then you will get a CT value lesser than 40. So, suppose you are getting 40 value of 35 and my friend, suppose I am getting a value of 35 and my friend is getting a value of 29. So, the doctor will say that guy is more infected with the virus because the CT value is less and I am less infected.

So, this is just telling by the CT value if you are infected or not. But if you want to see how the gene is getting modulated to the infection, so we can do this. At the end, we will get a fold change and you can plot and you can just simply publish your data, whatever you are getting at the end. So, this is experiment number one. So, we have to, as I said, this is a quantitative data.

So, we have to repeat the same experiment with biological replicates and at the end, we will get a three-fold change. Suppose in this case, as I said, we are getting fold change of 3.

08. In the next experiment, we will get something around 3. In the next one, suppose we are getting 2.0. So, we will take average of all the three experiments and then we can do the statistical analysis and then we can see our data. We can say that our data is significant and this is the final value.

So, this is the analysis part. So, if you have any doubt, you can ask now. Housekeeping changes. So, if you don't have a doubt, we can end this session. Thank you. So, this is the analysis part. So, as I mentioned in my earlier session that at the end of the PCR action, we will get a CT value.

So, these are the CT value that we got and this is the MOF control and the CT values are there. And this is the virus infected sample. So, I am using two genes, a GAB-DH as a normalizing control and my TANF gene, a p53 gene. So, in the analysis, we are going to see after infection what is happening to the p53 gene expression. It is up regulated or down regulated due to the virus infection. The method that we are going to follow is this method, analysis of relating gene expression data using 2 raised to the power minus delta delta CT method.

So, you can take a picture if you want of this paper and read it thoroughly. So, at the end of this analysis, we will be calculating this value. So, I will be explaining this what exactly delta delta CT is. So, now we have a CT value.

So, you all know what the CT value is already. So, you can see that we have three values. So, for that we need to calculate the delta delta CT. So, first we will calculate the mean CT value of all the values.

So, first we will calculate the average. This is the average. I will copy, cut, copy and paste it here. This is the average of p53 gene. Then we will calculate the mean CT value. After that we will calculate the delta CT value. Delta CT value means difference of CT value. So, if you want to see the expression of p53 gene, we want to, first we have to normalize the expression.

Because you can see that in the first case our CT value is 19 in GAPGAT and in the second one it is 25. So, can you guess like in which of the cases the expression of GAPGAT is more? In the first one. So, obviously this is very much high. So, the expression is less because of virus infection. So, if you want to see the expression of p53 gene, what exactly is happening with the p53 gene, you have to normalize it with a caret here.

The word normalization means, can you guess like this is the CT value is 25. Around 25. In this case, the CT value is 35. So, these are the values. So, can you guess which of the cases the expression of p53 gene is more? In the first one. Now, you are guessing just because of these values. But normalization means like first we have to normalize with a single gene.

In this case, the GAPGAT is different. But if the GAPGAT is 19 or 20, in that case we can say the p53 expression is same. But due to the difference between the CT value in these two things, we can't say like the expression is more in which case. So, we have to normalize or we have to cancel out the difference between these two values of GAPGH of both the samples. So, we have mean CT value as we have calculated, then we have to calculate the delta CT value.

Delta CT value as I said is a difference between the CT. Which difference? The 19 in this 25. So, we will calculate the difference. So, first I will take 19 minus this value. Now, this is delta CT value. Similarly, we will calculate a second CT delta CT for infected samples.

Now, this is delta CT. Then we have to calculate delta delta CT which means difference of delta CT value. Now, how many delta CT values we have? Two. So, we have to normalize it this one. So, first as I said we will be normalizing the value of expression. So, what we have to do? We have to subtract these values with this one only because this is control sample and we want to normalize it.

We want to see what exactly is happening in this case. So, we make the value of P53 expression as 1 in this case and then we will calculate the full chain in this case.

Are you getting my point or you are simply saying yes? Repeat. Yes. Okay. So, first we calculated the CT value. Okay. Then after the CT value we calculated delta CT. Difference between the CT value. Now, you can write the definition of CT value delta CT.

Delta CT is difference between the CT value of two genes from the same sample. Difference between the CT value of two genes within the same sample. So, this is delta CT. Difference between this value of P53 and GABH from the MOB treated. And in this and other case, difference between the CT value of P53 with GABH is the treated one. Next, we have to calculate the delta delta CT.

So, as I said we are going to normalize the expression of P53 as 1. So, for that we need to subtract the same value with the same value only. This value with same value. So, the delta CT value will be 0. In the next case, now we are going to see the expression.

So, for that we need to normalize with the first one. So, we will subtract the value of delta CT with the MOB treated one. Okay. So, the delta CT is now you can write the definition of delta delta CT.

It is the difference of delta CT value between two samples. Okay. Between the two samples.

In this case, as we are normalizing, so we subtract it with the same value. But in this case, we are not normalizing, we are calculating the, we are checking the regulations. So, that's why we are subtracting with the control one.

Now, the fold change. So, as you can see from the paper, this is 2 raised to the power minus delta delta CT. Now that we got this delta delta CT, we will calculate the fold change.

What the fold change is? 2 raised to the power minus of this value. Okay. But the fold change is 1. Why we are getting the fold change as 1? Because we normalized it to 1. Okay. So, if we calculate the fold change for treated one, we are getting 3.408. So, in the initially, initially you were saying the expression is more in this case in the control one.

But after analysis, what we got? The expression is more in the infected one. So, for graph, the mock treated one will be 1, the treatment will be 3.08. So, what we got at the end, the virus infection is increasing the expression of p53 by three fold. Okay. So, this is the expression. Now, as I mentioned earlier, like we generally prefer the real-time detection method.

Like if you remember, you can recall this thing, like you went to a doctor and you got your real-time PCR if you are COVID positive or not. So, what he did, what he or she did, he took your samples from your cheese and they did the real-time PCR and at the end, they got the CT value. Now, the CT value that we got was ranging from, was lying between from one to 40 because we are running the reaction for 40 cycles. So, suppose I am getting the CT value of 35 and my friend is getting a value of 32 or 30.

So, which of us are having more infection? 32. Obviously, but if you want to analyze the expression of a particular gene in both the cases, then we have to use the GANs. And this is the analysis method and this is how you calculate the whole gene and you can plot a graph and then publish it. This is a replicate first. We have to again do the same experiment, biological replicate and at the end, we will get a similar value.

Then we can take the average and see how significant our data is, then you can publish it. Okay. Do you have any doubt? We are isolating the mRNA from the cells, then we are reverse transcribing it to the cDNA and we are using that cDNA as a template in this reaction. We are not isolating it for the, no we are not removing anything.

This is one simple, one step, yes. Okay. So, after the end of the analysis, you could have understood that how you can be able to perform the real-time PCR. You are going to prepare the samples preparations, you are going to isolate the RNA, you are going to prepare the cDNA, then you are going to put the cDNA into the reaction mixtures, you are going to put the, you are going to set up the amplification cycles. All these depends on the type of products you want to develop and then from that you are going to get the data and then that data is actually going to be analyzed thoroughly. So, that you can be able to know which protein or which gene is being amplified or it is actually, you know, showing the lower expression. So, this is all about the real-time PCR.

We have discussed, what we have discussed, we have discussed about the basic principle, why we are using the real-time PCR, what is the advantage of using the real-time PCR and so on. And at the end, we have also discussed about the experimental aspects. So, we have prepared, we have shown you the couple of demo videos, how you can be able to perform the real-time PCR in your laboratory. So, with this, I would like to conclude my lecture here. In our subsequent lecture, we are going to discuss some more aspects of molecular biology. Thank you. .