Applied Environmental Microbiology
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Lecture – 44
Drinking Water Microbiology IV

Dear students, so in the previous lecture, we talked about PCR, and how molecular biology methods give us more advantage in comparison to the conventional regulatory method of culturing the microbes, culturing the fecal indicators in lab and getting an idea whether drinking water is safe or not. Today we are going to explore some more of these molecular biology techniques in order to answer the basic two questions we are interested in namely how are we going to manage monitor all the pathogens and how are we going to ensure safety of our drinking water.

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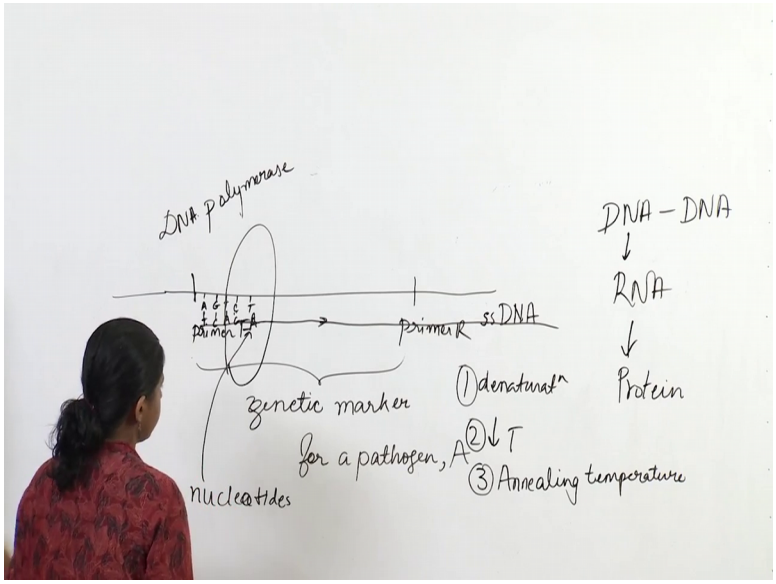

So, before we move ahead I want to give you a quick sketch of what PCR here actually is. So, in the previous lecture I briefly mentioned the PCR is polymerase chain reaction and it is a chemical reaction where we can amplify a single gene, a single copy of gene millions and millions of time and thus get a signal that we can see by our visual tools or maybe even by eye if you are doing gel electrophoresis and staining. So, PCR works in this way.

Let us say we have let us say this is a strand of DNA. So, this is single stranded. And it has atgcd in their own unique signature along it, and then there is another strand. So, now, it is double stranded DNA. I often refer written as ds DNA double stranded DNA. Now, let us say this is the region ok. So, let us say this is the region in that double stranded DNA that marks for a particular pathogen A. And this pathogen could be cryptosporidium could be micro bacterium, could be legionella could be hepatitis a, hepatitis e or any other pathogens.

So, this is the genetic marker. If we can detect this gene we know that this pathogen is present right. So, our PCR what it will do it will undergo different steps to amplify this particular gene element if it is present; if it is not present it will keep trying, but we will never get a positive signal. So, the first step often is denaturation. So, in denaturation we usually by temperature, but we can also do it chemically. We increase the temperature up to high 90s, 95 to 97 and at that high degree high temperature the double stranded DNA separates it goes away from itself the powerful bonds here powerful had the sorry the weak hydrogen bonds here they get broken. And now we have single stranded DNA.

So, now we have single stranded DNA after the first step of denaturation. So, after this step we have oligonucleotides which means some nucleotides join together and make an oligonucleotide. So, it is a short genetic polymer or polymer of nucleotides. So, this genetic this new primer typically we have designed it in a way to be an exact complement to the beginning. And this is primer forward and this is reverse primer.

So, the forward primer is an exact complement of the beginning of this genetic element. So, here for here we have A, then here we will have T; if here we have G, here we will have C; if here we have T, again A; C, we will have G. So, because this is an exact copy it will come and it will make non at this step at the step of annealing, we reduce the temperature. So, temperature goes down in the second step. And the primer anneals to the single stranded DNA ok.

Now, at next step a third step we make our temperature perfect for annealing to happen. Now, at annealing temperature, we have this polymerase, which is a giant protein or enzyme whatever you call it DNA polymerase. Now, DNA polymerase is an enzyme that is required for DNA to replicate. So, remember the central dogma of microbiology then we make another copy of DNA from an existing copy of DNA, it is called replication ok.

And then when we make DNA from DNA to RNA, remember what it is called transcription. And then when an RNA is converted or used as a template for making protein this step is called as translation. So, DNA to DNA replication is catalyzed by DNA polymerase, and there multiple other proteins that involved, but here our interest is in DNA polymerase.

So, this DNA polymerase comes here and in our PCR reaction. So, all this is happening in a tube in a in a 0.5 in a yeah in a really small tube you know the typical volumes of this reaction can range from 10 micro liters to 20 micro liters or 30 micro liters. So, it is really small tube. And in this we also put nucleotides. So, we put our nucleotides here. And if then so this primer has stuck, now this DNA polymerase because it is noticing here we had double strand, but here we have single strand, it wants to move in this direction, and it wants to make a complementary chain because that is what it does that is what it is working.

So, next if we have t it will consume a nucleotide and join it here. So, we have a and this way so and so forth from this direction it will complete as far as it can before it drops away. So, what we get in the this is first cycle my dear students. all right students.

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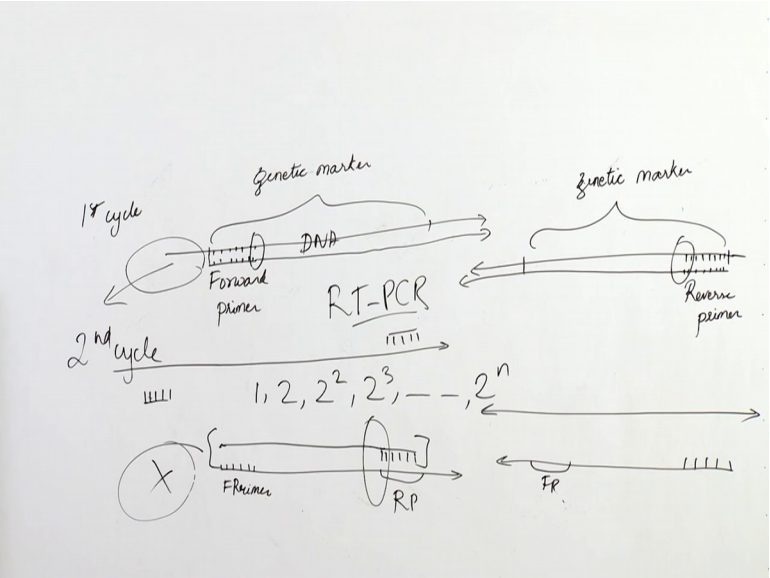

So, let us return to where we were in PCR. Now, I have drawn, I have redrawn these diagrams to give you an idea of what is going to happen in the next cycle. So, in the first cycle, the DNA the genetic DNA was denatured by increasing temperature by using

some chemicals. And then after that the primer that is present in a chemical reaction matrix came and attached because it perfectly complements the beginning or the ending of the genetic marker which is the gene that we are interest to the part of the gene that we are interested in. So, we have two kinds of primers in any PCR reaction. Whereas, forward primer and the other is reverse primer ; and both are perfect complements to certain portions of genetic marker. Reverse primer ends attaches to the end part; and the forward primer towards the forward part as the name suggests.

Now, once they have attached we have DNA polymerase. It comes here and it completes the sequence on and on and on. Similarly, the DNA polymerase will come here and to complete the sequence on and on and on yeah. So, this is the first cycle ok. And then again in the second cycle, we will have denaturation. So, in second cycle, we again have denaturation. So, they separate now where we had earlier two copies of one copy of DNA right; the one of the single strand, the other of the single strand. Now, we have two copies of DNA right because this and this is one that and that is one.

Now, in the next cycle what we have at the beginning of cycle is something that looks like this. So, after denaturation we have, so they will denature. So, we have this now. So, here we have the original DNA left and then we have the newly formed genetic the new the newly formed DNA that made by DNA polymerase. Now, in the seconds now in here also we have to we have the original DNA that goes in both direction and then we have our the newly formed DNA, and here is the reverse primer. And somewhere here the forward primer can fit and somewhere here the reverse primer can fit.

So, now what will happen here is that this is perfect complement to reverse primer. So, reverse primer will come here and it will fit here. And then DNA will attach and here we can either have reverse polymer come or we can have a reverse primer come or we can forward primer come. And either of them will attach and this will undergo similar in cellular replication. Now, this is interesting. So, notice here in the first cycle we had forward primer at attach here. So, all the DNA that should have been in this end of the original molecule has been lost, there is nothing here. So, we have forward primer at the starting and then we have a long length of DNA.

Now, when reverse primer attaches, DNA polymerase will come, nucleotides will come, and it will come here. And by the time, it reaches the end of forward primer the DNA

polymerase will drop of it because there is nothing more to extend nothing more to replicate. And thus the new fragment that we have created here is the exact length of our genetic marker. So, initially we had so at the end of PCR, most of the and remember from one we are making two, from two we are making four, so this is following series like this and so on and so forth. Thus we will have at the end of n number of cycles will have a much higher number of these genetic markers only genetic markers then the original that had extra DNA.

So, most of what we will have is our the genetic marker of interest, this is how PCR works. There are certain limitations we cannot quantify it. And we have to concentrate the sample extract the DNA as we talked about in last time. We also have problem of PCR inhibitors these are certain metals and certain other compounds that inhibit PCR reaction from happening. And then we have another issue may be this bacteria from where I got this DNA was already dead. So, it was not a danger, but its DNA was intact and now I am getting a positive signal. And the other thing is this kind of reaction requires cost and expertise.

So, the now notice here that all this is happening by DNA. So, my starting template is DNA, but let us say there are certain microbes which are RNA based microbes you know viruses for example, some of them are RNA based Ribo oxy nucleic acid based viruses. Now, in order to detect them first I have to convert RNA into DNA. So, I do an RT PCR step which is reverse transcriptase stat now.

Remember from DNA to RNA is called transcription right, so RNA to DNA when we are going in the reverse direction, its reverse transcription. So, we do a reverse transcription make a reverse transcript reverse transcribe copy of the RNA, so we get our cdna which is complementary DNA. And then it undergoes this PCR reaction. And it is referred to as RT PCR; our reverse transcriptase PCR. So, this is reverse transcriptase PCR.

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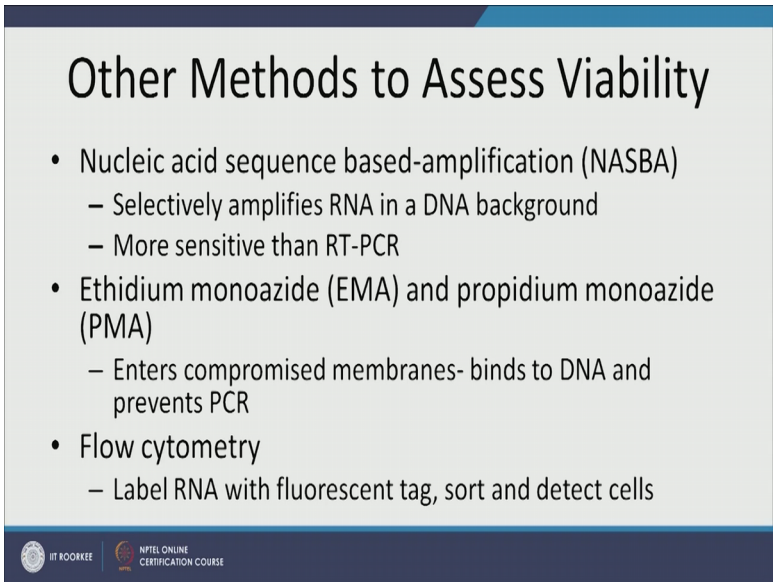

And then they other methods now here is the thing I mentioned that one of the limitations of PCR is that I cannot tell if my original my starting DNA was from a live cell or from a dead cell there are certain methods to assess the viability of the pathogen that we are detecting. One is that we can do we can selectively amplifier RNA. Now, if you remember for a pathogen if it is alive then it is replicating right; and it is also translating its genetic code into proteins that it requires. Thus it should have some amount of RNA present in it right.

Now, this RNA if I detect RNA then I know that cell was active. Why would it be so because RNA usually is short lived. So, if RNA is present in our sample and we detected we assume that the cell is active and our life obviously,. So, we can do reverse transcriptase PCR or we can do this RT-PCR or we can only amplify RNA in the DNA background which is nucleic acid sequence based amplification.

And the other thing is we have chemical such as ethidium monoazide and propidium monoazide, EMA, PMA. If we add them to our sample it will if there is a cell microbial cell whose cell membrane has been compromised whose cell membrane has degraded which is the which is what happens right after cell dies. Then these compounds will enter through the compromised membrane, and they will bind to DNA and they will inhibit the PCR they will not read PCR here to happen. So, we would not get a false positive. So,

there are other methods in which we can assess the viability of our pathogens that we are detecting by a PCR.

The other option is flow cytometry and this is really promising something I am interested in working myself. In flow cytometry, we can what we can do is we can label we can flow add a fluorophore to our RNA molecule so a fluorescent tag. And then the flow cytometer will tell me how much is present and then it can sort if it is something like fax sorter we it can sort the cell, so it can remove pathogens from the rest of the sample. So, again I can actually quantify the pathogens I can know whether they are alive; obviously, because the fluorophore attached to RNA, this is a really promising technique.

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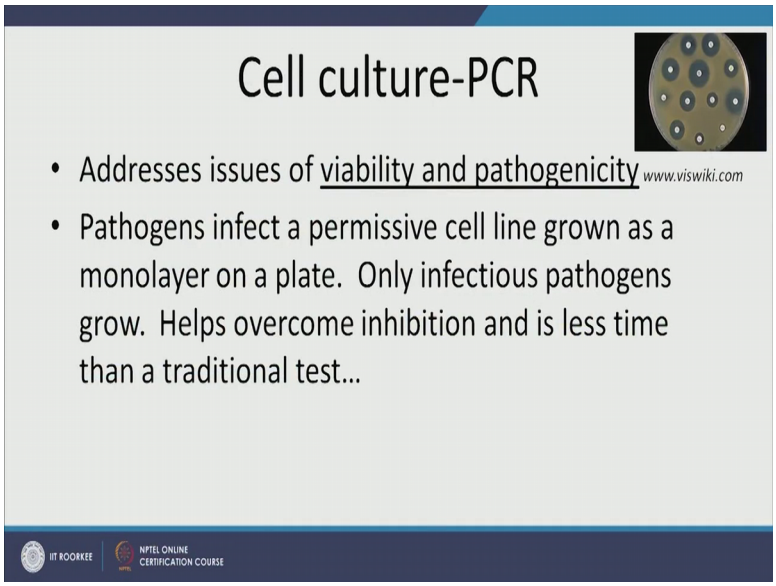

The other is cell culture PCR this is a wonderful technique which will address the issue of both viability and pathogenicity. What it does is it works in the principle of virus detection. Mostly, we have a we make a cell line that the pathogen usually in facts for example, if pathogen infects intestines will make an intestinal cell line and then we will put the water sample. And if we see plucks in it then we know oh wow there are pathogens present.

So, only infectious pathogens grow. So, maybe there are some pathogens that are viable, but they are not pathogenic. So, we might have certain kind of micro bacteria that can cause diseases, but some micro bacteria are benign. So, we do not want to be detecting mycobacterium and then panic about it. So, this will not allow that because only those

micros will be detected that can grow that are alive and that are pathogenic. This is believed to be it was proposed in 2009 to be very valuable to drinking water industry.

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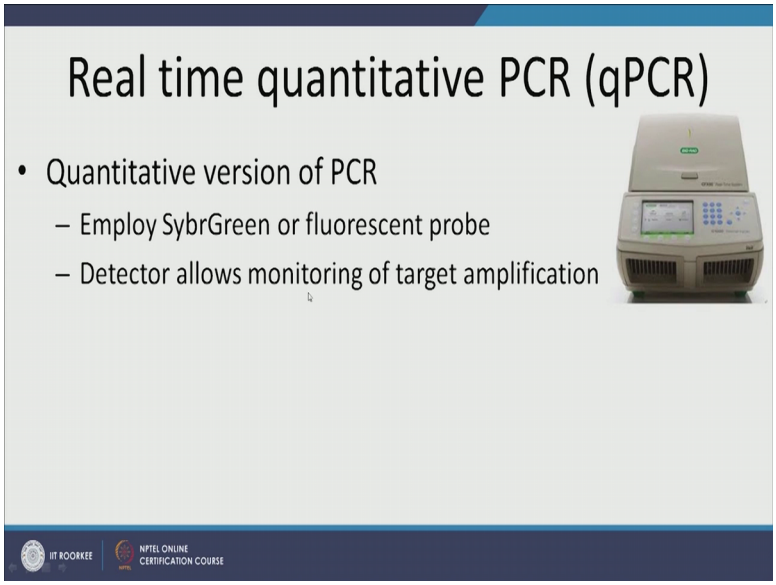

Next is real time quantitative PCR. So, this is referred to as QPCR some people call it RT PCR again which is real time PCR, but it confuses with reverse transcriptase PCR which we talked about few minutes earlier and thus I refer to it as QPCR. Now, in QPCR what we do is it is a quantitative version of PCR.

So, coming back to our PCR at every step we undergoing replication, and there is some efficiency you know not all of them will make two copies, but most of them would. Now, if it every step I could get a signal that would tell me how many gene copies I have now I can go back and I can calculate what amount of starting genetic material was present, this is the basic principle behind QPCR.

So, let us take a look. They either employ probes like they are fluorescent probes or they employ SybrGreen our similar dyes like evergreen and many dyes these are the most popular one. And it allows us to monitor target amplification. So, as our target gene is amplifying and increasing in number at each step I will get a signal, at each step I will now these many targets are present now these many, so I can track it I can plot it and then I can get my starting value. These are extremely sensitive I can get up to ten copies. Remember infectious dose for some pathogens is ten copies. So, this is the closest we get. And it gives the unit is gene copy, it tells me how many gene copies are present per

ml. And it gives you result in nearly one hour. Most of the QPCR reactions are one hour or less.

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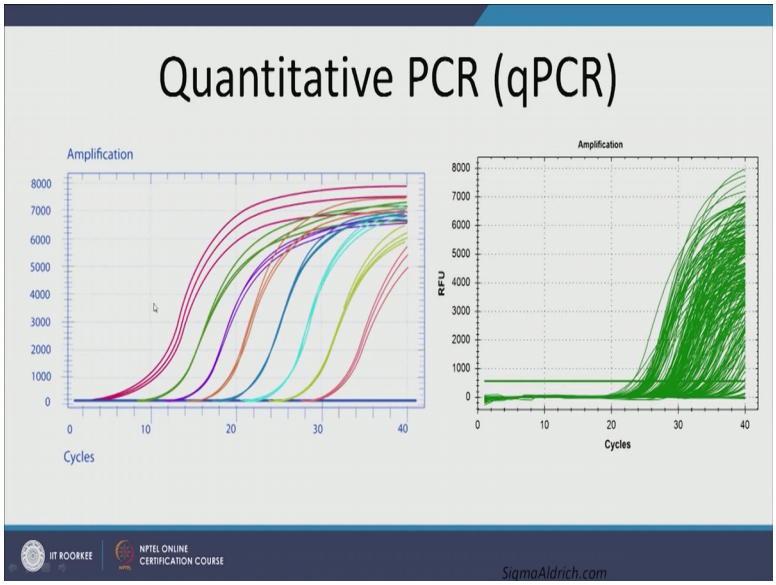

So, this is this is a typical picture. And ideally you will see really beautiful plot. So, this is a standard curve actually on the left; on the y-axis you have amplification. So, signals for how many copies are present so giving an idea of amplification; on x-axis, your number of cycles. So, initially the growth is small then the growth is exponential grows really fast in a somewhere in the middle that becomes linear. And the reason for linearity if you remember logistic growth is because now it is running out of nucleotides is running out of other things it require such as primers, and then it plateaus because it has reached the maximum amount of copies it could make for the gene. And we use this plot. So, at every step, at cycle 1, cycle 2, cycle 3, cycle 10, cycle 20 at every step I get a signal and using that we make this plot.

So, these are standards in and our samples may lie anywhere between the standards and usually make standards so that they lie between the standards um. So, these this beautiful clarity you would not see between these standards, you will see something like this on the right side. Most of my QPCR curves have looked like this and in fact if I only look at the standards it will probably look like this again.

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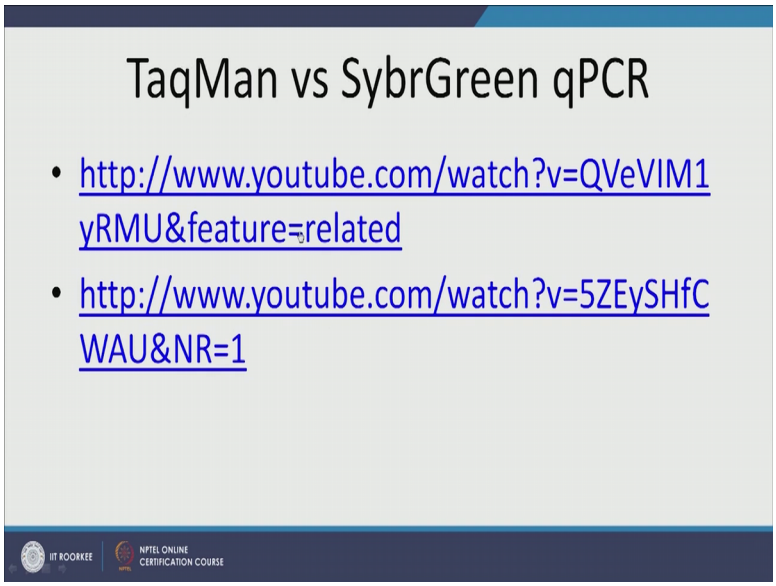

So, I want you to look go through these two YouTube videos an d this link that I have shared in this slide and look at what is the difference between TaqMan and SybrGreen. I will briefly go through TaqMan and SybrGreen QPCR to give you an idea of what is the general differences, but visualizing it using these YouTube videos would really help make things more clear for you. So, let us start with TaqMan.

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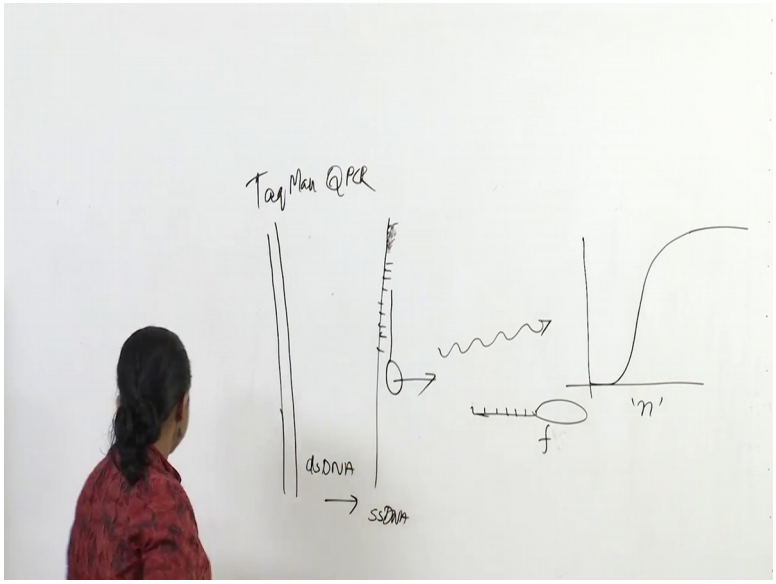

So, let us say this is my starting genetic material, and it is really long double stranded DNA. Now, my dear students you should remember that in real life double stranded

DNA will never look like this remember it is a double helix. So, it will in it is in cartoons its often represented like this, but for just ease of drawing it here, I will I will draw it like two parallel lines.

Now, we are starting with TaqMan. Now, in TaqMan quantitative polymerase chain reaction, what TaqMan does is after ds DNA double stranded DNA undergoes denaturation and becomes single stranded DNA. There we in the reaction we have added a probe. Now, this probe has some sequence ATGC sequence and it has a fluorophore. So, it is ok. So, this is a sequence and this is a fluorophore, fluorophore will give a signal a fluorescent signal that can be picked up by any our highly sophisticated optical device.

So, this sequence is perfect complement of a part of the sequence of the pathogen. So, the perfect complement of it exists here. So, this will come the moment the denaturation has happened, this will come and stick here and this is it ok. Now, when the amplification happens and when the forward primers and usually this is attached to the forward primer by the way the sequence is usually or used a part of it is used as forward primer. So, when the amplification begins this fluorophore is dropped and it releases a signal; a fluorescent signal a light signal that can be picked up by your sophisticated camera.

So, every so if we have more copies of our starting copies at any time t, we have more copies of the target, more of this probe with the sequence and the fluorophore will attach and every time there is a reaction, you know every time there is amplification its signal would be given. And the intensity of signal would be proportional to the number of cop target copies we have gene copies. And thus we can track the signal with number of cycles. And number of cycles, so we can track the signal and it will look something like this. It will look something like this. So, and we can assume that this is pretty much how our gene copies are also behaving, this is how TaqMan QPCR works.

Now, let us take a look at SybrGreen QPCR. SybrGreen is a dye and evergreen works in the same way these dyes have a tendency to attach themselves to double stranded DNA. So, the moment you put your primers your nucleotides and your genetic material in your tube small micro centrifuge tube or in the well plate that moment the SybrGreen will go and attach to every double stranded DNA that it can attach all righty.

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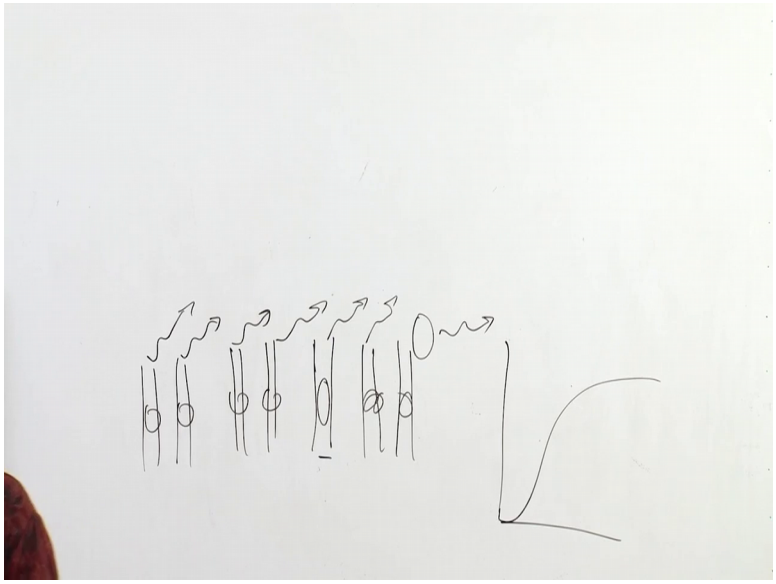

So, when this is double stranded DNA and SybrGreen sits here, it attaches to it. So, when denaturation happens, it is released, and it gives signal. So, you can notice SybrGreen is not very specific, but as our number of gene copies increase. So, we have more fragments of double stranded DNA. So, we have more place for SybrGreen to attach and when all of these undergo denaturation, our signal strength increases. And just like in case of TaqMan, we can correlate this intensity of signal we get with the number of gene copies at any amplification step.

And this is how used to calculate that calculate the starting quantities of your genetic material. In your homework this time, I will share a small example for you on how we calculate the starting amount of QPCR if we know the fluorescence or the signal that we capture at two different steps of QPCR.

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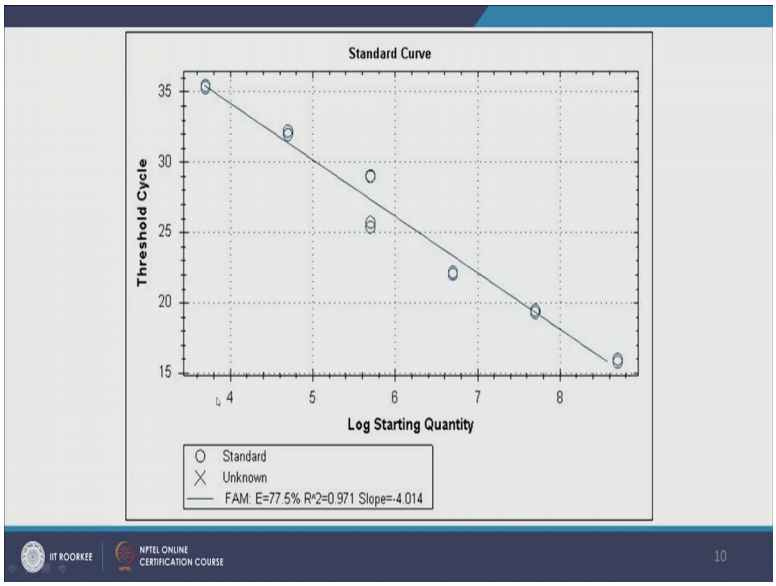

So, us so remember I talked about calibrating the standards. So, standard curve could look something like this, this is a typical standard curve. So, on x-axis, we have log starting quantity. So, we started with 10 to power 3, ten to power of different quantities I see they are not exactly 10 to power 4, 10 to power 5, but little lesser than that. So, we to be run these QPCR in triplicates in this point they agree really well here not.

So, much here in other points actually really well so, a this linear fit is made and then we get our standard curve, and everything is calibrated to this. Now, notice here there is a word on y-axis called threshold cycle. The threshold cycle is plotted here and that is our CT line and when I share to you the solution for an example question in the homework I will explain this to you better.

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So, now let us move on to automatic symptomatic systems for detecting our pathogens using molecular biology tools in dear students here is a taq talk and this is a link for the taq talk, and I highly encourage you to go and look at this link. So, this is an assistant professor at the time he was giving his talk here assistant professor in Kansas University.

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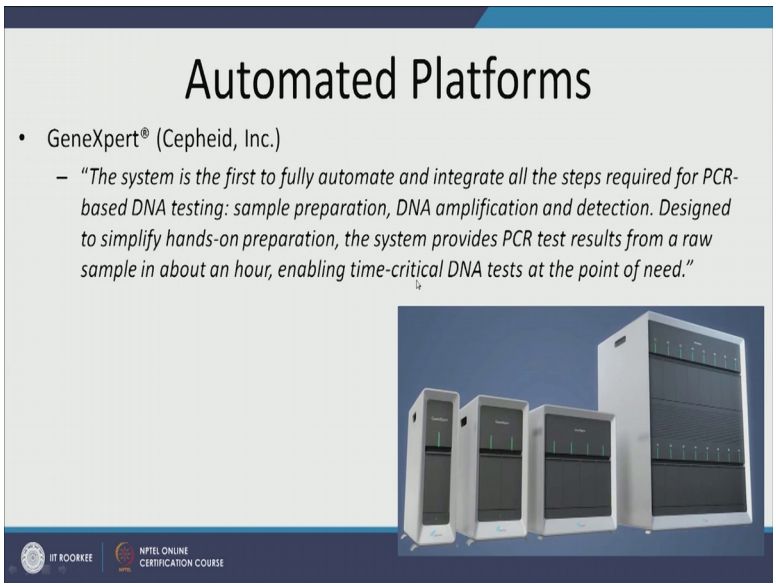

And he was talking about an automatic system for detecting pathogens referred to as lab on chip. So, basically it is a chip you put your water sample, and to give you signal and let you know what pathogens are present and perhaps in what quantity. So, this was a

groundbreaking research and very very useful for environmental monitoring and public health. So, please go ahead and watch this YouTube, there will be certain questions in your homework this week from this.

The other automated platforms include gene expert which is all in one solution for our pathogen detection. Just put your water sample it will do the sample preparation DNA amplification and detection. And then it is very important actually because it does everything really quick in one hour, which is usually the time taken for QPCR, it will do and it will tell you it will do its analysis and tell you what pathogens are present. This is very very important in critical places, such as ICUs hospitals and in terms in cases of rapidly spreading infectious diseases.

Now, to revisit the general challenges of molecular methods, so molecular methods come up come with lot of advantages they have low detection quantity they are easy to do if you have the right skill set. And they also have higher specificities and along with higher sensitivity, and they we can actually directly target the pathogen. Instead of looking into indicator organisms, but they do compare some of the challenges.

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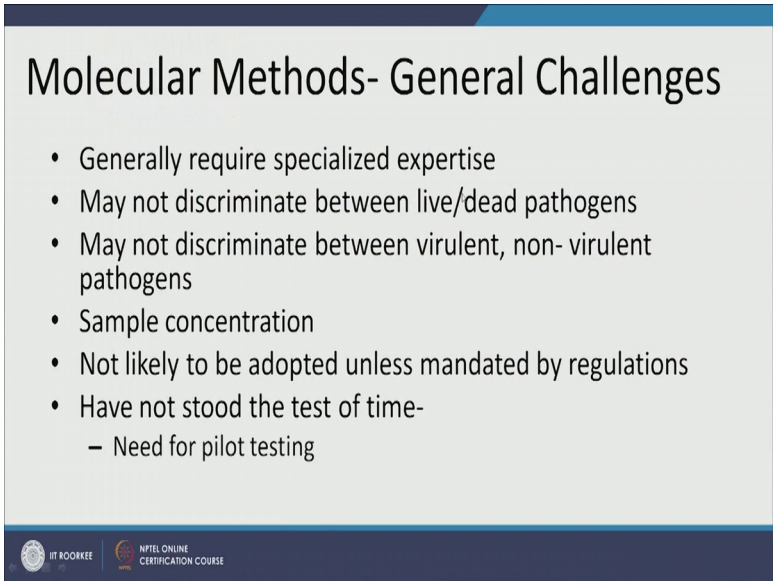

And some of the challenges are they require specialized expertise you need to train people. And use most of them cannot tell between live and dead pathogens in order to do that you will have to do some special tweaking, many of them do not go discriminate between viral and non viral and pathogens. Some pathogens you know they have viral

strains and non viral and strains and the genetic marker might exist in both of them. So, you might know truly and accurately where the pathogen is present or not, but you would not know if that pathogen is violent or not. If it will make you sick or not.

Then there is a step of sample concentration for DNA extraction and here we might lose a lot of our sample our pathogens. And here is the thing most likely our regulators because they have such a good successful history with culturable methods, culture based methods and using fecal indicators that they are most likely regulators will not adopt my molecular methods in near future.

And because the reason is because they have not stood the test of time we still need to do pilot scale testing. So, we have done testing in for example, the study I shared they tested for six waste water reclaiming fascist, the facilities for one year, but we need to do bigger studies.

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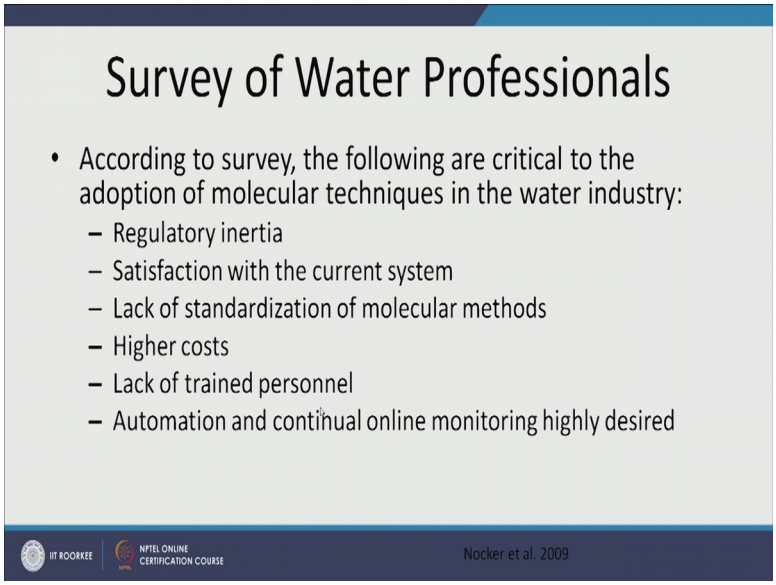

So, let us do a survey of water professionals what do they have to say. They say that to adopt molecular methods in water industry they have to get over their inertia and this is a serious thing now no laughing matter, because they are very satisfied with the current system. The molecular methods have not been standardized it has higher cost, but they do not have the personnel which are trained to you do these analyses. And they would like some kind of automation. So, this is where the lab on chip and gene expert come into picture.

So, in the next class my dear students we are almost done with all detection techniques for microbes in water. In next class, we will talk about biological drinking water treatment. So, I want to give a little preview into what you are going to talk um. Typically, in a drinking water, though it is usually pretty clean and ideally does not have any pathogen to killers, but usually they are very rich in microbes. I mean compared to what the general impression is that drinking water is.

So, clean does not have any bacteria or virus, but they in general do have micros present. But please note not all microbes are bad for you most of them are actually benign they do not hurt you some of them are essential you need them to survive and be healthy only few of them are dangerous for you only few are pathogens.

So, please we do not have to be anti microbes then most of them are really good for us or benign for us, but in drinking water we have a rich complex we can have rich diversity of microorganisms that are present. And then a lot of funny things interesting things happen in drinking water distribution system, where biofilms can grow where pathogens can intrude if pipe leak somewhere of these corroded portions of the distribution system. And as such the the drinking water can be rich with nutrients because then there is leakage, there is a loss of integrity nutrients and pathogens everything can come in.

The other thing is that water treatment plants do not remove natural organic matter because it is very complex like humic acids. So, there is some carbon source always present in drinking water which it was not removed in our water treatment. The other is that there are certain ions that can be utilized from metabolism by microbes such as there could be sulfites, there could be manganese, they could be ferrous. So, we can have iron oxidizing bacteria, there could be ammonia, so we can have ammonia oxidizing bacteria they can have nitrates. So, we can nitreate reducing bacteria.

So, nitrogen cycle can go on a very rich community can grow and use these microbes and usually what microbes do is in this oligotrophic environment they make biofilms and they trap these nutrients. So, they make their very nutrient rich niche, and where they flourish. And as such it is very important for us to get rid of even these trace nutrients, and the way to do is to come back to biology the way that we know.

So, what is going to happen in drinking water distribution system where biofilms grow and microbes flourish, we can do it in our water treatment plant. There we can have

microbial biofilms that will remove these trace nutrients that will get rid of things that we can get rid of which are still there in water after the conventional treatment.

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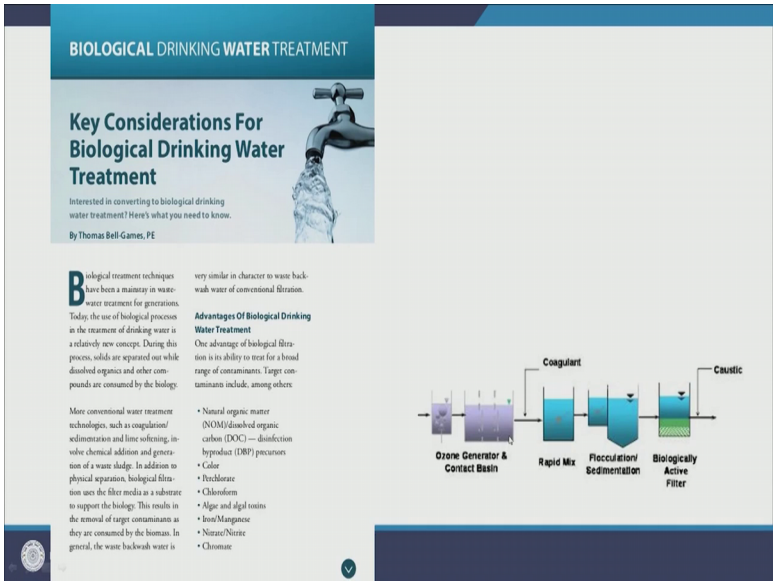

So, on the right here we have a small schematic. So, let us look here so usually you know we have coagulant flocculation we have general initial oxidation to break down complex organic. So, that they can easily coagulate flocculate and easily degrade. In the last step, we have biologically active filter. So, this filter is biologically active it has perhaps microbial mats in it and they will degrade all the remaining organics they will get rid of all the nitrate, ammonium, sulfide even the oxidized iron, so that no one will do it in the pipes and in the pipes will have lesser nutrients available for microbes to utilize.

So, there are lot of advantages with biological drinking water treatment plant and some of them are listed here they get rid of these target contaminants natural organic matter disinfection by products color or precursors of dbps color perchlorate chloroform algae algal toxins, iron, manganese, nitrate nitrite and chromate all of this can be removed using biological water treatment. So, dear students this is all for today. In the next class, we will talk about biological drinking water treatment and that will put an end to our drinking water per week all right.

Thank you very much, see you in next lecture.