Introduction to Biomicrofluidics
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Lecture - 17
Lab-on-a-chip for Genetic Analysis

Microfluidic platform is of potential to parallelization of that DNA sequencing, PCR, single cell analysis. And at the same time it is largely used for biochemical analysis like; DNA and proteins and other metabolites also. Nowadays, Lab-on-chip is coming to the picture to reduce the time necessary for biochemical analysis. At the same time it can integrate that different discrete steps in one platform by which we can study that several parameters in a short period of time.

So, in all the fields we are utilizing that fluid flow of microfluidics for all the experiments. In this lecture we shall go through that how that PCR means Polymer Chain Reactions for DNA analysis and micro array technology are adopted in microfluidic platform to say that gene analysis particularly for gene expression, then mutations, etcetera. So, first we shall go through that in which way we have designed that the gene analysis.

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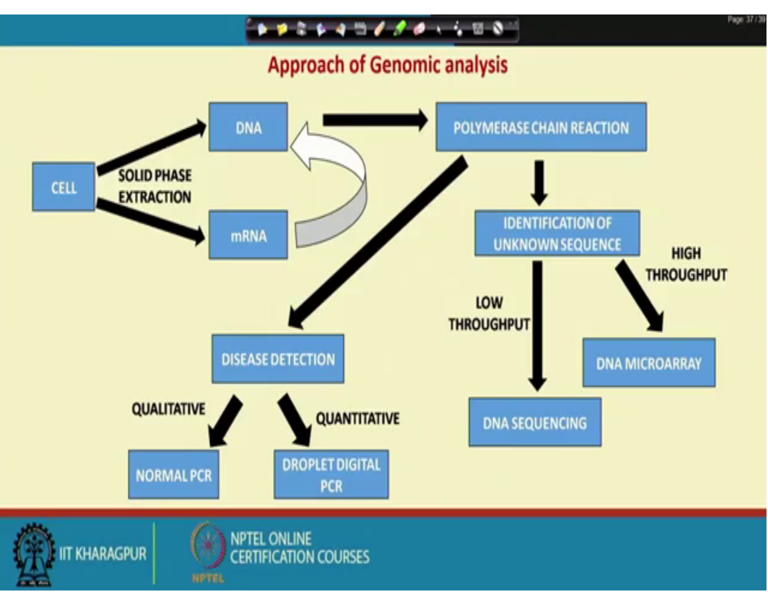

First we shall go through that from cell or starting material is say single cell or may be a cell mass like tissue. From that we have extract nucleic acid either DNA or RNA by solid

phase extraction. Then we shall go for polymerase chain reaction. Why polymerase chain reactions? Because it helps to multiply that DNA to a large quantity by which we can measure.

From polymerase chain reactions we can go for DNA sequencing and DNA microarray. But in that lecture we shall not go for DNA sequencing mainly DNA microarray. And how the polymerase chain reaction will be integrated in that lab on a chip platform by which we can get that readouts, like the how much DNA is there are or what are the DNA expression levels, the cells all those things we shall go through it.

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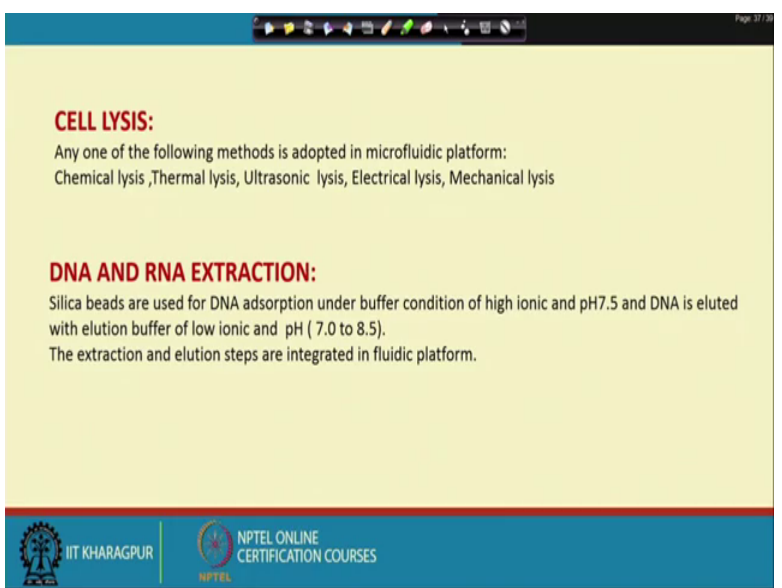

So, how that your starting material is say cells, say in any of the following methods will be adopted for cell lysis, may be thermal lysis, ultrasonic lysis, electrical lysis or mechanical lysis. More or less all this processes are adopted in microfluidic platform. Then after cell lysis DNA and RNA should be extracted. In that extraction process generally that solid phase extraction principle is adopted by which that DNA RNA will be adsorbed in the adsorbed some particle like silica particle at pH 7.5 at high ion extent. And it will be eluted at buffer of low ionic strength around pH 7 to 8.5. After that extraction that DNA will be flowing through the fluidic channel to that PCR platform.

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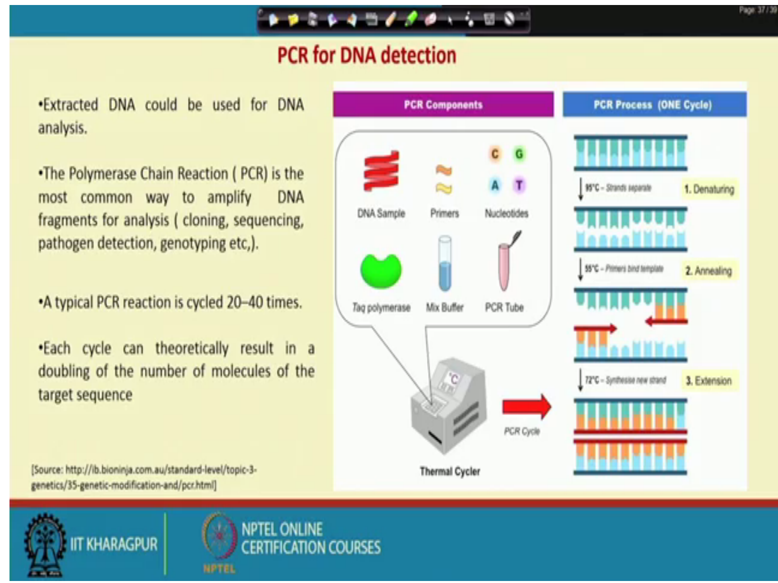

So, what is PCR? PCR is a Polymerase Chain Reactions by which we can amplify the DNA fragments for analysis like say cloning, sequencing, pathogen detection, genotyping etcetera, so basic steps of polymer chain reaction is that. So, you have a DNA template then the polymer chain mix means solution contents that primers. One is forward primer, another is reverse primer then Taq polymerase, then all the nucleotides ATGC, deoxyribonucleotides in a PCR tube around say 20 micro litre volume.

So, principle is that first you have to denature that DNA to making a single stranded at 95 degree centigrade strands separate then that primers forward and reverse primers will anneal with that your DNA strands. Then after annealing that DNA polymerase will extend that primers to that end of that DNA. So, from 1 DNA you will be getting 2 DNA. Again that same type of cycle is going on in that way around 20 to 30 cycles it will be round in that thermo cycler.

And you will be getting enough DNA by each we can detect some method may be a gel may be a capillary electrophoresis. So, for this method you will need at least say 10 to the power 11 molecules of DNA or more than that by which it can be detected either fluorescent method or by agarose gel or polyacrylamide gel electrophoresis method.

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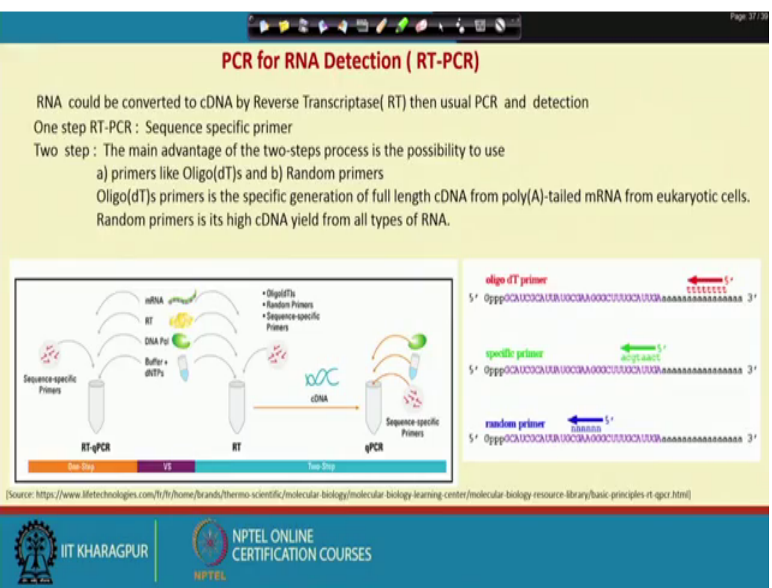

And if you want to go for RNA detection, then we have to isolate that messenger RNA in switch or say we are looking for DNA expression level in a cell or in a tissue. Then you have to isolate that like nucleic acid which contains DNA and RNA. From that you have to isolate that Oligo dT column that messenger RNA, then you can get that messenger RNA.

So, you can do that messenger RNA, it will be converted to DNA by using reverse transcriptase. So, in that process for the if you want to do PCR using that messenger RNA to DNA then PCR, it can be done by two ways. One is first is your single step process, second is your two step process. In single step process in the mixture messenger RNA, reverse transcriptase, DNA polymerase, buffers and dNTP’s.

Then you give sequence specific primer by which in one step that PCR reaction will be going on. And if you go to two step, then first you give Oligo dT by which we will be getting that specific generation of full length cDNA from polyA tailed. Then you give sequence specific or random primer, then that polymer chain reaction will be going on then we will be getting that n of DNA.

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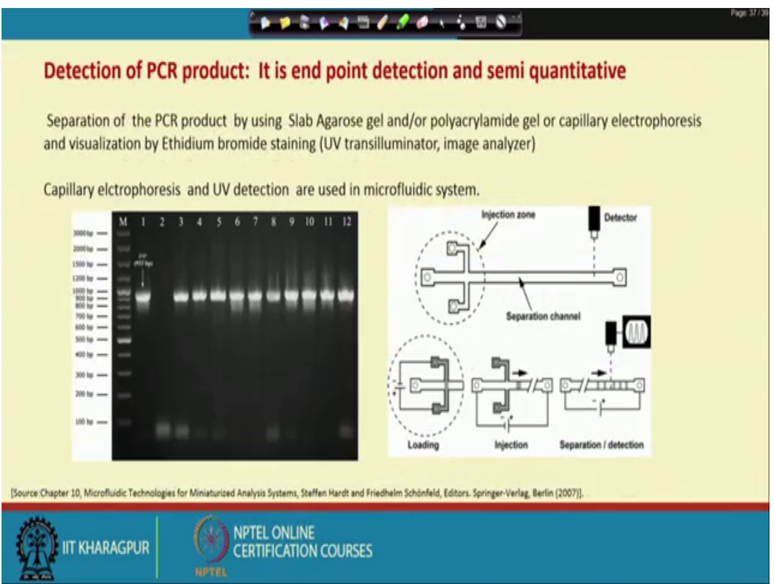

So, how to analyze this PCR product? PCR product could be analyzed by slab gel using agarose gel or polyacrylamide gel; this is demonstrated here. Likes your PCR product is say 900 base sphere, so you give a ladder, then you give that your product will be knowing that what is your base sphere of that PCR product, to know that what is that gene of interest or sequence of interest you are looking for the PCR product.

And this it takes about time around say 15 to 20 minutes. On the other hand, you can do the same type of experiment using capillary electrophoresis and it is very much comfortable to microfluidic platform. It tooks about around say 5 minute and detection by UV method and these are the bands which could be quantified. So, if this PCR, so far we discussed this is a relatively qualitative PCR.

If you want to do quantitative PCR means; how much really was there in your sample or how many DNA's would be expressed in quantitatively then this type of PCR is not good. In that situation we have to give we have to follow, that relative quantitative PCR using that your probe like say TaqMan probe.

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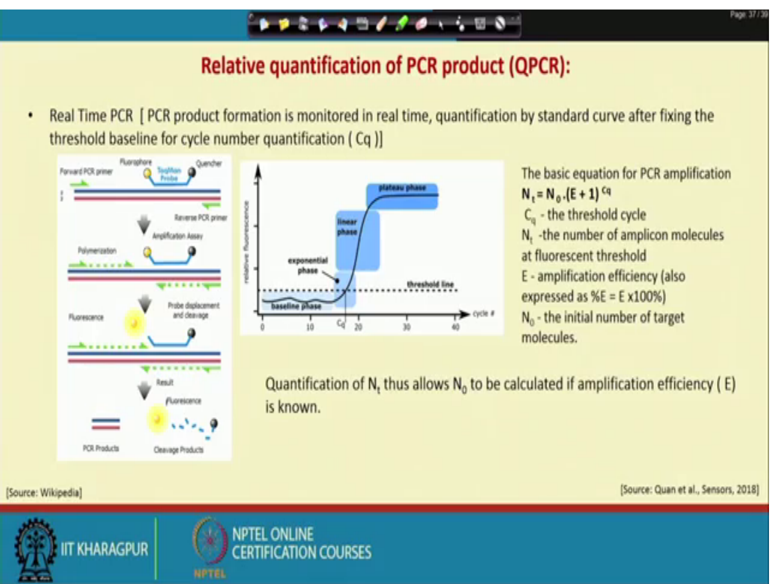

TaqMan probe is nothing but a DNA sequence. You can tell that is a like a primer type of sequence specific to gene of interest which has a two fluorophores , it is a frate sphere, this flurophore this fluorescence is quenched by that this is a quencher. In presence of this is a two flurophores you will be getting no fluorescence. And when that chain is progressing; means cycle after cycle then, that DNA polymerase is progressing.

And when it is coming to that place it has a 5 prime to 3 prime exonuclease activity, it will cut that your fluorophore and fluorescence will be getting. That means, chain progression quantitatively could be measured by measuring that fluorescence and at the same time with the relative fluorescence and that number of cycles going on we can plot. And from this plot we can generate this type of that curve from which we can determine that how much your DNA present in the in your PCR tube at the beginning, that means 0 time.

So, in that curve will be (Refer Time: 10:16) it looks like a general growth curve of bacteria. Say this is a baseline phase around say 10 to 15 cycles, then this is the exponential phase around say 20 15 to 20 cycles then it is a linear phase then plateau phase. And that equation basic equation for that exponential phase N t equal to N 0 into E plus 1 by C q to the power C q. C q is that threshold cycle number at the exponential phase at that, N t is the number of amplicon molecule at fluorescent threshold, E is the

amplicon efficiency and N 0 the initial number. Actually our target is to determine the N g, N 0 basically.

So, if you know N t and efficiency, then C q, then you can determine N 0; that initial number of target molecules. But we cannot get absolute number, always we have to compare with a known gene which should be multiplied using that relative quantitative PCR then we can making a standard curve and you can determine the how much you are DNA of interest is there.

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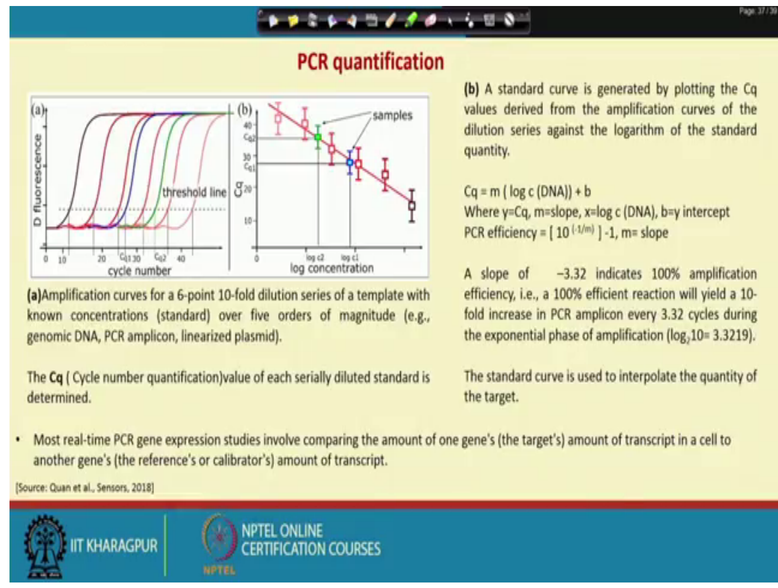

So, this is that say 10 times dilution of that known gene or known DNA sequence. And we are running that quantitative PCR and this is the threshold line. And we are plotting Cq versus log C, means your known concentration of that DNA, then you will be getting this type of plot. And from this you can determine these are 2 points are unknown that determine that what will be your N 0 value means initial DNA concentration.

So, means from that if that efficiency is near about 100 percent the slope will be around minus 3.32. And on principle if it is 100 percent efficient, 1 molecule of DNA will be multiplied to 10 times within 3.32 cycles; means log 10 base 2 equal to 3.32. So, from this standard curve we can know that N0 value of that unknown DNA sequence. Most real time PCR gene expressions studies involve comparing the amount of one gene's that it targets amount transcript in a cell then another genes. So, it is relative means quantitation.

Can we do PCR by which we can quantitate absolutely? In other words, can we without any background or without any compare that standard things, can we get the DNA sequence of a gene? This is question number 1. Second is that say in a sample when that quantaminated DNA you have to determine means; it is a it is concentration is very less normal ways PCR or any other ways you cannot determine. So, how you can go out? Basically, can we determine that we are asking the question, single DNA molecule by PCR?

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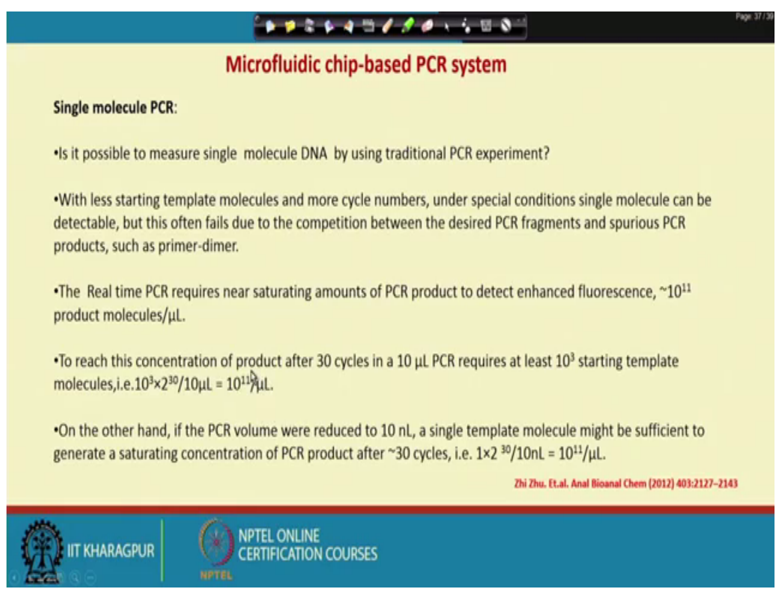

But normal way you cannot do because to generate that signal we need around 10 to the power 11 DNA molecules, by which we can determine that fluorescent intensity. But to get 10 to the power 11 molecules your starting DNA molecules would be around 10 to the power 3. Means, 10 to the power 3 into 30 cycles 2 to the power 30 in 10 micro liters is equivalent to 10 to the power 11 micro liter. So, normal way we cannot do PCR of single DNA molecule.

So, how it could be done? It is possible if we decrease that concentration of a DNA mix in a nanometer scale. Same DNA concentration if we come around that 1 DNA molecule 30 cycles in 10 nano litre then it will be coming 10 to power 11. Means, if we can go about nano litre scale, then only we can get that your PCR's would be done. So, what is the advantage of microfluidic PCR? Why we shall go for microfluidic PCR?

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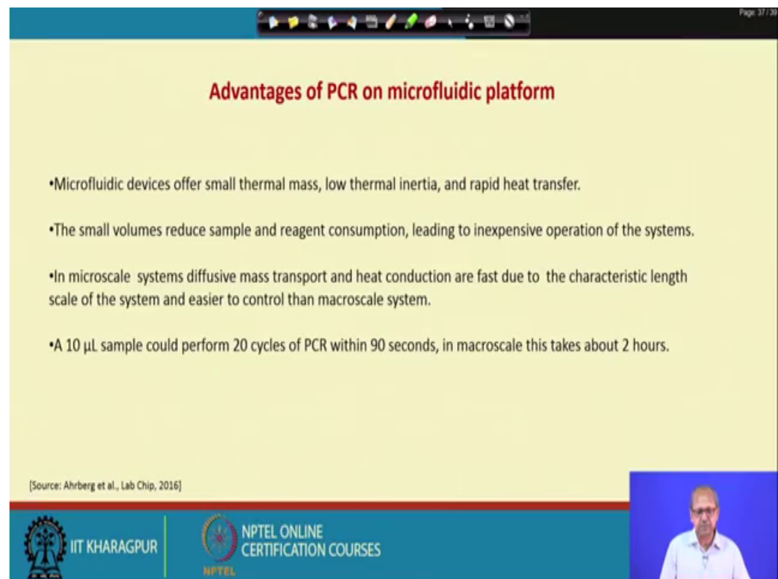

Microfluidic device of our small thermal mass, low thermal inertia, and rapid heat transfer, this is the behavior of any microfluidic system. The small volume reduce sample and reagent consumption leading to inexpensive operation of the systems. The microscale systems diffusive mass transport and heat conduction are fast due to the characteristic length scale.

And a 10 micro litre sample could perform 20 cycles PCR within 90 seconds. Normally, it takes around say 2 hours, so we can decrease that time 2 hours to may be 90 seconds, may be some efficiency some steel is coming, around say 60 seconds.

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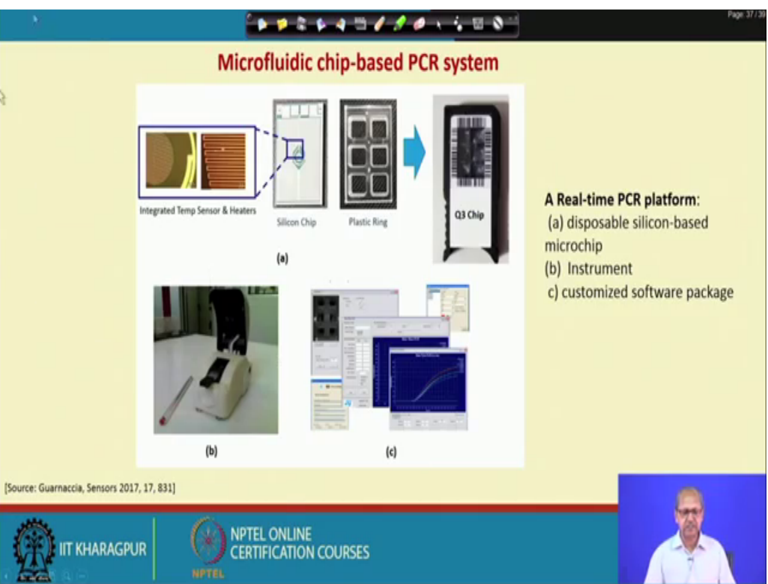

So, this is that just demonstration how that microfluidic chip is developed on the PCR system. This is that thermal cycler things and or that all that things you will be going on. Then we can readout that all that real time PCR by which we can get that relative quantification of that PCR's products.

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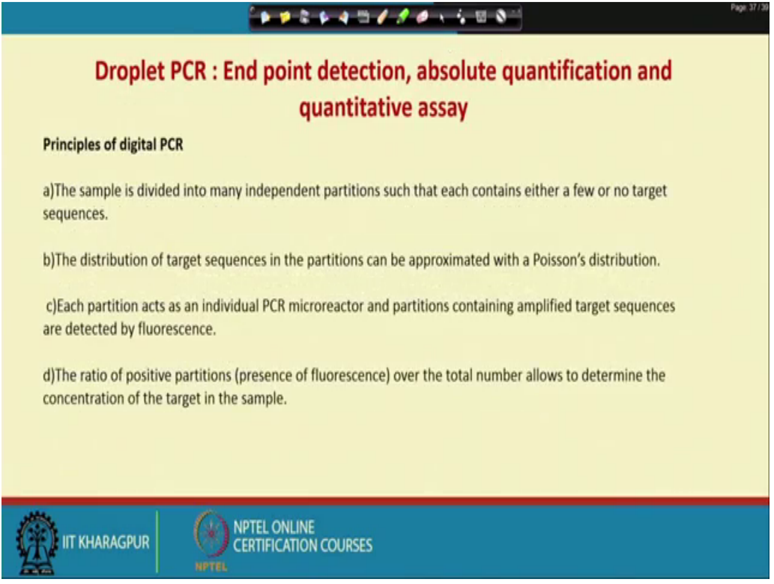

So, in the microfluidic platform as we are interested to quantify single DNA molecule and PCR mix volumes should be the nano litre, then droplet fluidics coming to the picture and here that droplet PCR or digital PCR. So, principle of digital PCR is that the

sample is divided into many independent partitions, such that each contain either a few or a no target. So droplets should be in such a way that droplet volume either it have a one target or no target. The distribution of the target sequence in the partitions can be approximated by the Poisson's distributions.

And each partition acts an individual PCR microreactor and partitions containing amplified target sequences are detected by fluorescence method, normal your by using that confocal microscopy or florescent activated cell sorting instrument. The ratio of positive partitions means, presence of fluorescence over the total number allows the determination of the concentration of the target sample and this is a absolute. You have not to compare with any standard things. And as it is a all over non phenomenon means one droplet will giving a signal which is positive. And another droplet which having nothing or no signal that is v 0 means 0 1. That is why droplet PCR is another name is your digital PCR.

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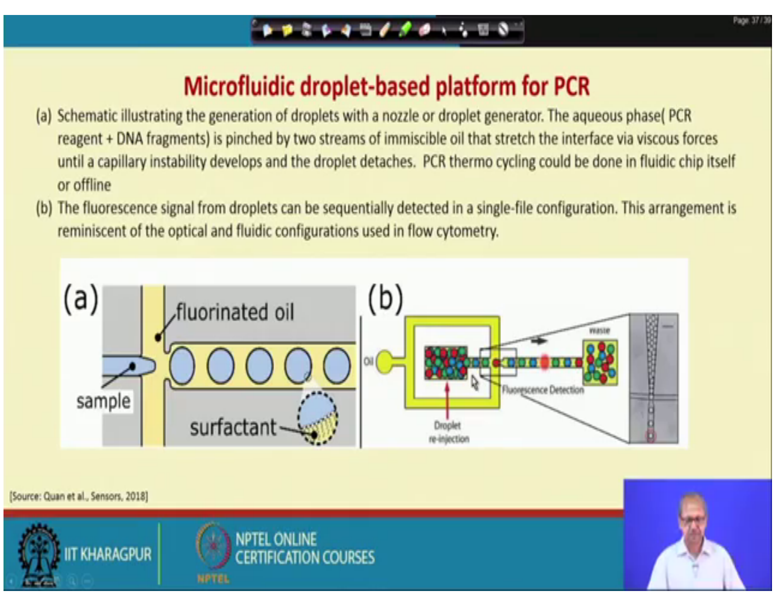

So, that platform there are varieties of platforms to generate that nano litres scale droplets. Here that simple platform is that this is your sample containing that PCR mix and your DNA or any samples. And this is silicon oil, fluorinated silicon oil and this fluorinated silicon oil pinched that sample in a nano litre scale volume by which that single DNA molecule will be trapped along with PCR mix. Then this will be put in a

thermo cycler and you will be getting fluorescence and fluorescence will be monitored by your fluorescence activated cell sorting machine or using a DEP based instrument.

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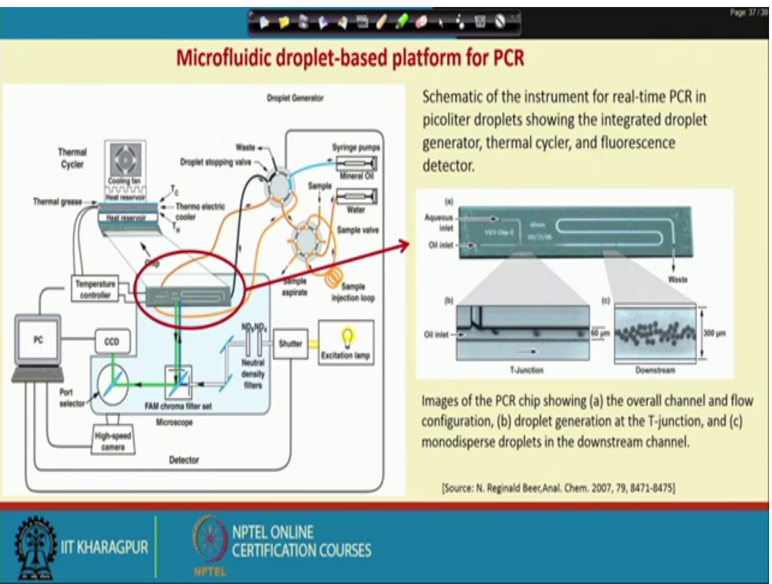

And that whole that setup is integrated in a lab on a chip platform, this is the platform this grown up figure is here that this is the T-junction where that micro droplet's are formed. And thermo cycle then it is monitored by that your CCTV camera going to the computer and you will be getting positive signal out of that you can calculate that absolute number of DNA of interest present that droplets.

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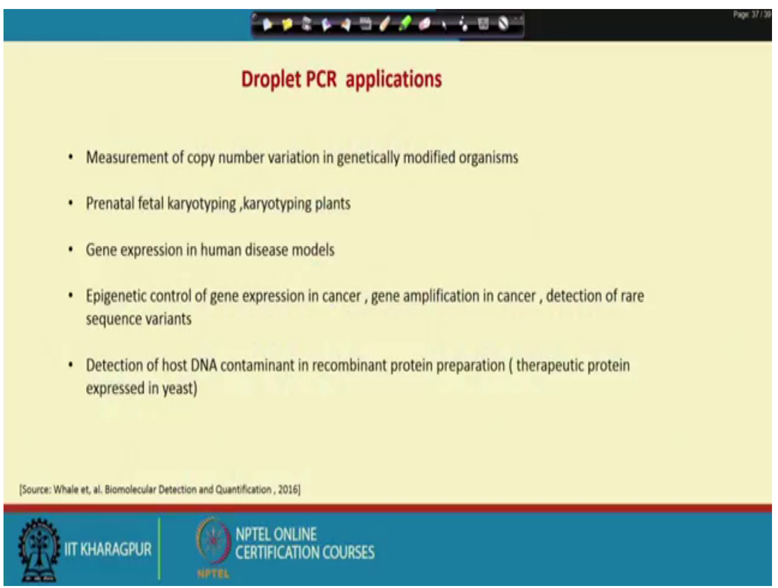

So, what are the application of droplet PCR or digital PCR? Measurement of copy number variation in genetically modified organisms. Prenatal fetal karyotyping or karyotyping of plants, gene expression in human disease models, epigenetic control of gene expression in cancer, gene amplification in cancer, detection of rare sequence variants, detection of host DNA contaminant in recombinant protein preparation; like say, therapeutic protein generally you are expressing in Ucolai or yeast. If that protein preparation contents say few pico down level or pamto gram level DNA that cannot be detected by normal voice. So, droplet PCR is the answer for that and it is a based on the principle of micro predicts.

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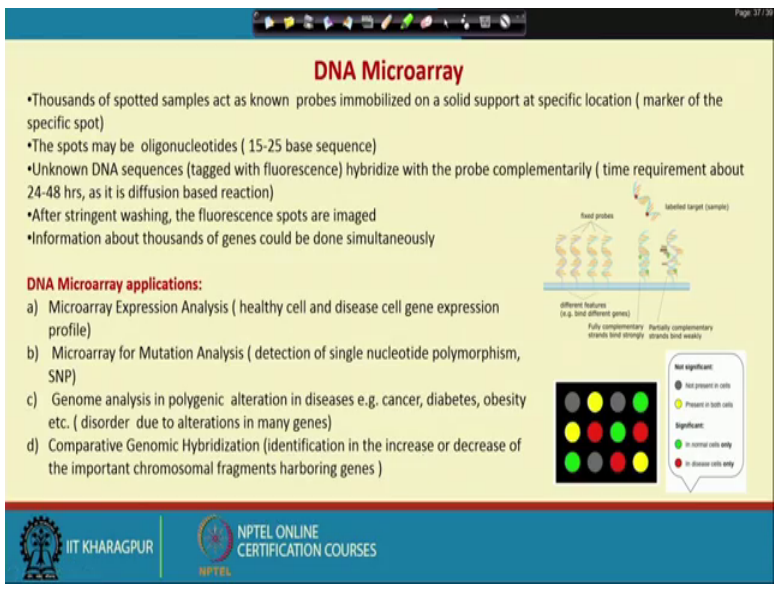

So, another platform is a DNA microarray. So, DNA microarray is a platform where we can a say that thousand of amplicons or say DNA sequence. And it is very much necessary when you are going for a DNA expression analysis or some single nucleotide polymorphism or so on so forth lot of cases we can use that. So, DNA microarray basic principle is that this thousand of spots samples are acted as a known probes immobilized as a solid support. Means, it is a combination of both solid phase and liquid phase reaction basically.

And that as it is a these spots are located in the particular space or particular position that position we know that which probe is where that is very important when you are giving large scale of microarray. Now, this spots may be oligonucleotides of length 15 to 25

base sequence and unknown DNA sequence tagged with a fluorescence hybridize with the probe complimentarily and it tooks around 24 to 48 hours, it is a diffusion based reactions. After that hybridization that stringent washing step is followed it takes lot of time to remove that means, non specific bindings which are not hybridized properly. Even it is strength of DNA microarray is that if it is one nucleotide is not matching then that will be washed out during stringent washing.

So, we can get information about thousands of gene by this DNA microarray this is a very highly powerful setup. And it main applications are microarray expression analysis, healthy persons with (Refer Time: 21:32) disease cells like say this is array example where that we are comparing that normal cells is a green and disease cells is red. When that sequence present in both the cases you will be getting yellow and then you will be getting nothing when nothing is there. In that way you can generate the microarray spots. And it could be used for comparative genomic hybridization identification in increase or decrease of important chromosomal fragments etcetera.

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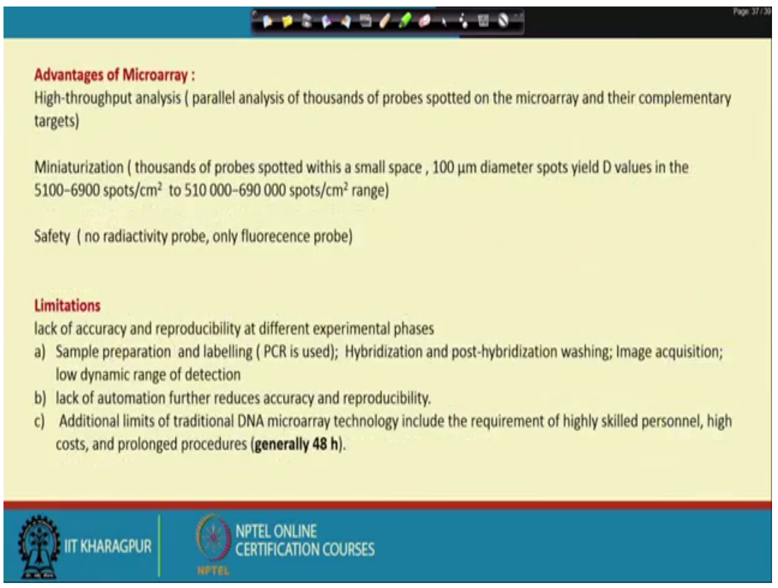

So, what are the advantages of microarray? It is high throughput analysis, parallel analysis of thousands of probes spotted on the microarray and their complementary targets. And now that miniaturization is in the scale you can spot around 5,000 to 510000 spots per square centimeter. Almost we are reaching that maximum level of spotting

different technologies are used nowadays. Hence in with respect of safety concern as there is no radioactivity used only fluorescence probes are used.

So, DNA microarray is very means comfortable or it can tell us safety with respect of that hazards concern. But what are the limitations? Limitations are that lack of accuracy and reproducibility at different experimental phases. What are the experimental phases? Sample preparation and labeling, hybridization and post hybridization washing is very important and image acquisition and low dynamic range of detection means. Low dynamic range means your detection limit concentration upper and lower limit is not very high within a limited zone of concentration you can do it.

And further more it is depends on the skill of the person who is doing the experiment. And lack of automation further reduces the accuracy of reproducibility. And it needs highly skilled personnel, high cost and prolonged procedures around say 48 hours. Where the time is constant, can we reduce that time 48 hours to around say few hours in the scale of hours? So, that again we can utilize that microfluidic principle by which that molecules will be coming together, not by diffusion definitely by diffusion, but diffusion length scale will be decreased due to the micro confinement.

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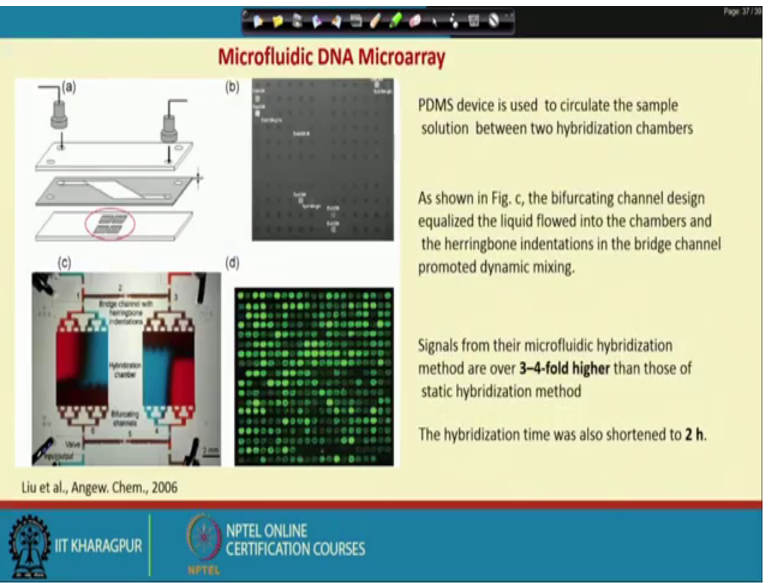

So, here that microfluidic DNA microarray; basic principle is that you have a microarray chip where that microarrays are spotted. And your samples would be passed through that chip with a shortened speed and with a some additional some attributes like say

oscillating flaps and etcetera by which that surface concentration of that DNA molecules which will be probed, means which will be accessed there will be concentration will be increased.

And it tooks around say 2 hours and 3 to 4 fold higher intensity will be getting with respect of that normal PCR. See here is demonstration that herringbone indentation used to for mixing that your means microarray. And that what DNA single stranded DNA should be accessed and you will be getting the spot then you analyze the spots.

Say this time frame to 2 hour could be decreased in the scale of minute scale by using oscillating flaps. And this type of oscillating flaps is discussed by Professor Chakrovarthy in his previous classes by which that DNA hybridization time will be reduced to in the scale of say 40 minutes to 60 minutes.

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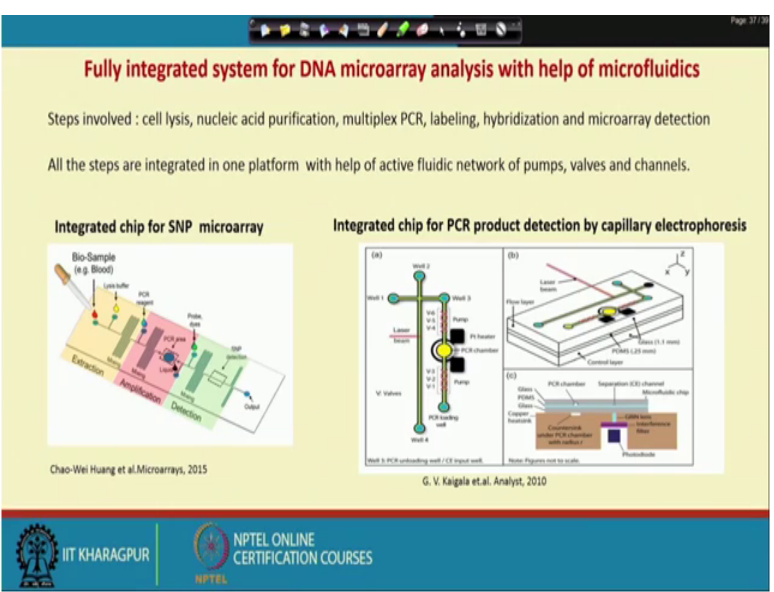

Now, we are coming that lab on a chip platform starting from that sample preparation to readouts, like say; may be your microarray readouts or if you look for that PCR products in a in one platform. Say this an example where we are looking for single nucleotide polymorphism in a samples. So, here you are giving that bio sample like say blood, so lyses buffer, then mix then PCR agent, this is a mixing chamber and this is the PCR area where that thermo cycling is going on and your DNA is amplified. Then probe dais, then again mixing, then it is a your microarray.

So, whole this platforms extraction amplification detection in one platform that is that lab on a chip which can detect a sample within a say 15 to 20 minutes starting from incorporation of the samples to get that read, this is microarray based system. Same type of system can be adopted another lab on a chip platform where that your PCR mix here means PCR sample. Then it is passed through a one capillary electrophoresis and using that capillary electrophoresis you can do that PCR product means qualitative detection of the spots. And that can be adopted for DNA sequencing also using Sanger's Method.

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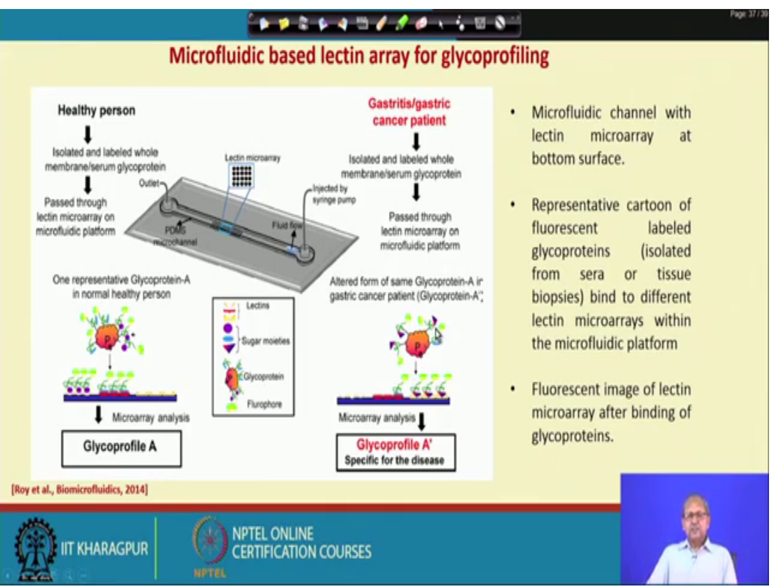

So, using as we have discussed that microarray technology for DNA, that is the common principle of any other type of array technology. So, in our lab we have developed protein array; particularly lectin array for detecting that glycoprotein's. In some disease cases that glyco selection pattern of proteins are changed. Say if we can access that what is that glycoprofiling of that sample using lectins array, we have to have idea of what that status of the samples. So, we have developed that lectin microarray, principle is same here that lectin microarray where we have spotted 17 types of lectins of different sugar binding pockets and each of the spots containing 12 spots around say 122 micro meter diameter. So, principle is that healthy persons are getting the samples and from that sample we are isolated that glycoprotein's from that membranes. Total protein we are isolating rather than glycoproteins and from that patients also doing the same things. And we are using that lectin microarray to detect that glycoprofiling to detect that glycoprofiling.

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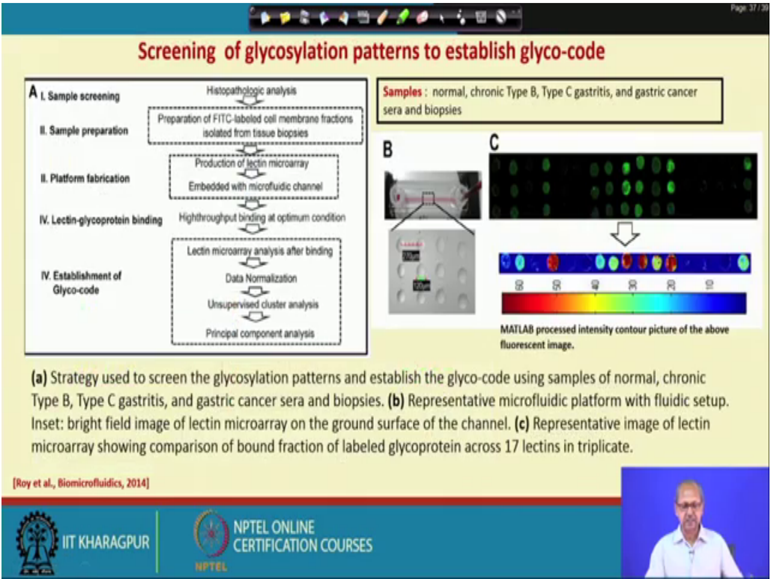

So here is that array one of the spots amplified for this 4 spots of each spot is about distance 270 micro meter and 1 spot diameter is 120 micrometer. Then protein mix labeled with FITC fluorescein isothiocyanate and passed through that microfluidic channel around say 4 microliter per minute around say 4 minute and after that you are giving just one washing. And then generate that fluorescein isothiocyanate that FITC level spots. Then we will analyze the spots using MATLAB to determine the intensity.

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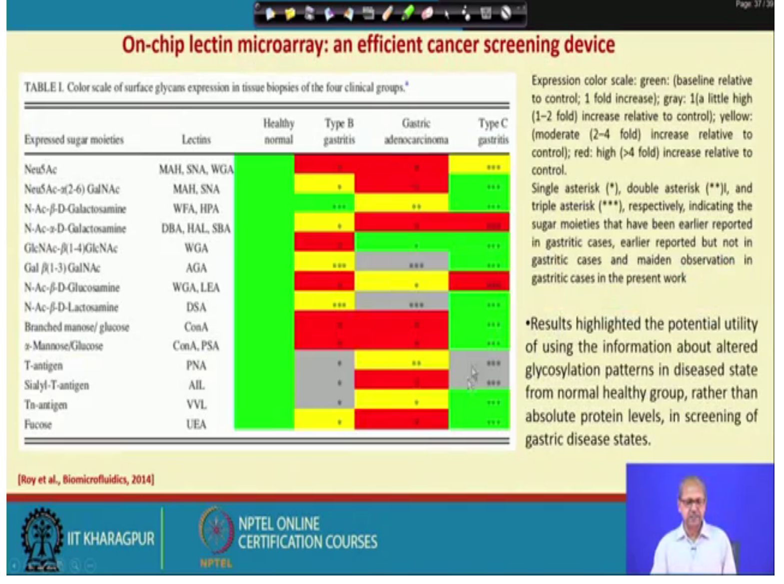

So, what you are getting this type of results, this is healthy persons, this is Type B gastritis, this is gastritis adenocarcinoma and Type C. And you see that these are the red indicates that is highly increasing value. Then yellow indicates that moderate value and then green indicates little high. With these color coding we can have a glycoprofiling of the 3 types of patients disease status with respect to healthy control that what is there means glycosylation status of the protein, these are the all 17 lectins of different means your sugar binding activity. So, in that way we can utilize that microfluidic principle to reduce that time of experiment, say 5 minutes to 10 minutes to get that result. Now, question arises at what situation that microfluidic technology will be used particularly country like us. I think that in that 3 situation we can utilize this microfluidic technology for biochemical analysis.

First point is there when that sample volume is very less or concentration of the sample is less. Like say amniotic fluid, or say cerebrospinal fluid, or say neonatal sample; where you have to analyze very few microlitre or nano litre of samples. Then if you want to parallel experiments in a short period, say; if you want to DNA sequence or you want to amplify that that PCR products or artificial things lot of samples in a very short period of times. And lastly if you want to have a rare sequence if you want to detect within a very short period.

Say particular say disease detection in a remote places where that not enough facilities are there, where you can use this type of strips like things or lab on a chip type of things. Where you can detect 2 or 3 type of disease together means multiplexing that can could be detected. So, these are the situations where we can utilize this type of microfluidic platform ok.