Introduction to Proteogenomics

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Supplementary - 07
Genomic Analysis using Droplet PCR - II

Welcome to MOOC course on Introduction to Proteogenomics. In today's lecture we will continue with Mr. Abijith Dixit who will talk about the Droplet Digital PCR which will be continuation of his previous lecture, where he started discussing about the basis of this technology. He will talk about the type of samples one can use as the input for the instrument, instrument can be used for multiplexing as well even up to 8 plex assays using single flow cells have been reported.

The principle requirement for multiplexing is the significance size difference between the amplicons at least there should be 30 base pair difference. So, let us now welcome Mr. Abijit to have an interactive session on digital PCR technology.

Is everybody clear with that technology how it works? Anybody has any queries? Yeah sure.

Student: How does the sample binds to the droplet?

The sample does not bind to the droplet ma'am.

Student: It enters inside.

Yes. So, these are droplets which are made where your oil is making a cover right and the sample and the reaction which is inside those droplet us. It is an amalgamation micelle in case you know what micelles are, that is how it works. Any other queries?

Student: In the process you are talking about.

Right ma'am.

Student: You can use the simple cells directly or you need to do the extraction.

You would have to do extraction, you would have to do extraction right.

Student: So, you have a separate.

You can have your DNA or RNA from any source, does not matter.

Student: for say If you have to analyze a single cell.

Right, ma'am.

Student: Ok.

Student: Then, how do I generate a marker?

You isolate your single cells by a flow cytometer or any other technology.

Student: Suppose I do not have any way to do the separation.

So, what you can do is even if you have a clump even if you have say a pool of cells right, you are wanting to look at a particular type of amplicon.

Student: I want to look at individual cell.

No, in case of individual cell then you will have to have a cell to extract a nucleic acid out of it, if you have a pool how would the system differentiate between what cells it come the DNA or RNA coming from.

Right.

Student: I know that there can be 2 type of cells.

Right.

Student: I want to separate the cells. So, that each well contains a single cell.

Student: Must be extracted.

Right.

Student: And then I want to store whether there is expression or not.

Right then you so; you wanted to do a separation by this method.

Student: Yeah separation from PCR.

No separation you will have to do by any other method; the PCR can happen here, the PCR and the quantification later part can happen in this. What you are talking of is also a different technology which we have.

Student: sample like single cell line.

Right so, it is a work-flow ma'am where you isolate your single cells by a flow cytometer.

Student: And then.

And then go ahead with this right correct, what you are talking of that technology is also there which is a different technology all together. I mean we can have offline discussion; we have another technology which works for that, a single cell isolation and sequencing yeah.

Student: How many samples can you multiplex at once?

At a time you can have 2 floor because there are 2 fluorophos there are 2 lasers you can have 2, but there are publications where people have done up to 8 plex assays with a single fluorophore. What you simply need to do is keep the size of amplicons variable at least a 50 base pair difference between a two amplicons. So, what it would do is yeah.

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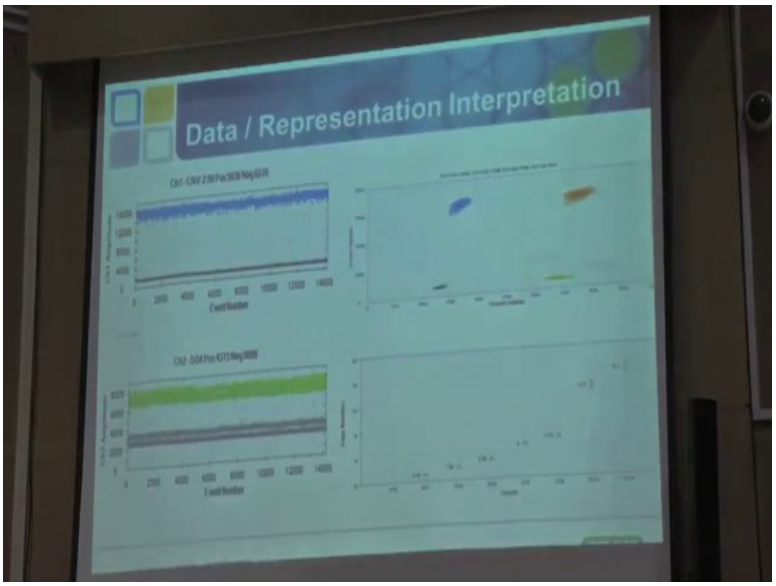

Now, this cluster which you see is from one type of amplicon right, if another amplicon is smaller or bigger than that, that would give a different layer here; you can quantify each of this clusters individually. For example, in 2 D you get it here right. So, you will have another cluster altogether. So, you can have I mean there are lot of publication people have one up to 8 plex assays.

Student: What is the base difference which you mentioned?

Sorry yes ma'am.

Student: Base difference.

For a multiplex.

Student: Yeah

At least a 30 to 50 base pair difference, if you have to go more than 2 plex assays.

Student: Actually now human cells will misguiding for there is not a single cell per say.

Right, it is not for single cell isolation it is after you have a single cell isolation, because that amount of nucleic acid coming out of the single cell is going to be very minimalistic. So, the system is capable enough sensitivity wise to capture even that minuscule amount of nucleic acid.

Student: But for that I need a system that in force isolate my single cell.

A single cell right.

Student: Are we extracting DNA and RNA from the single cell for PCR.

Yes.

Student: We are not we why do all like.

It is not very difficult ma'am, you can do a single cell isolation by flow cytometer very easily.

Student: Yeah.

A flow cytometer can do your single cell isolation very easily and I was mentioning ma'am we have another system also it is another technology altogether, where we can do a single cell right from isolation to base 2 sequence that is a parallel technology different altogether.

Student: As another point when you talk about it is real time taken that is not real time.

It is end point PCR.

Student: It is an end point PCR.

Yes.

Student: It is not a real time.

Yeah no what I meant was this a real time display how it comes, it is a end point PCR yeah. Anything else? So any other queries?

Student: So, is there a limit on amplicon size?

Yes, a ideal limit watch we say is from a 60 base pair to a 350 base pair.

Student: So, if you are going make 2 or 8 multiplexing then you need to think of is amplicon.

Right, right, right.

Student: If the windows quite small it is being actually manipulated.

It is actually not as I told what is recommended is from 60 base pairs to 350 base pairs like is for real time PCR as well, but you have got people who have amplified and specifically quantified up to 2 KB a fragments as well very easily doable. Is what I am talking of for the ideal conditions which come from the literature, which is published when it gets manufactured. What people have done there are more than around 4000 publications now if I am not wrong with this technique and people have done things which even we could not imagine of.

Student: When you are talking about the mutation if there is a single mutation

Right.

Student: Primer set is using a specific this?

Yes.

Student: So, that is when you can do.

Absolutely so, the prime requirement or the best amplification result which you will get with this, does not depend on the quality of your nucleic acid. It depends on the specificity of your primers. If your primers are specific data will get is absolutely specific, the quality of nucleic acid would not hamper your data, yes ma'am.

Student: That means, it cannot be de novo.

No it cannot be, you need to have; you need to have a specific primer.

Yes, you need to have specific primer for your amplification that you are looking for.

Student: Right, we did I did follow the y axis, how does it show the amplification?

Ma'am this is the fluoresce amplitude as each of your droplets is emitting a fluorescence, if there is a amplifiable DNA in the droplet it be fluorescence right. And, when it emits a fluorescence it will be plotted here, if it does not emit it is here.

Student: How does that depend on the size of the fragment?

Right, ma'am the system is optimized in such a way that your specific length of a amplicon, if it emits a fluorescence it will emit a fluorescence in certain range, a system takes care of those aspects as well.

Student: Is that coming differences?

Yes, because as I told it is like a flow cytometer the.

Student: That is around 50.

Yes at least 30 or 50 to 30 to 50 base pair difference between two amplicons. Anything else? Technologically if we can answer someone, yes ma'am.

Student: can you go back a slide.

The slide before this ma'am yes.

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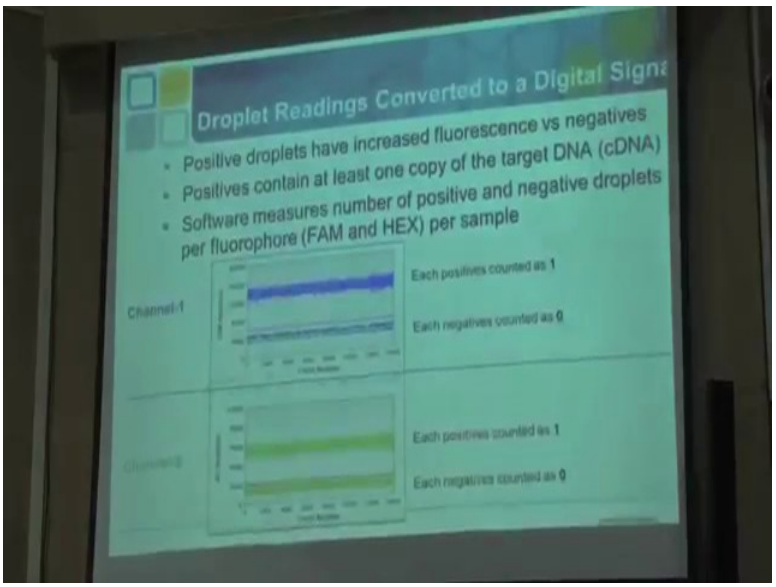

Yeah.

Student: We can do just 8 samples in 1 page know.

1 cartridge.

Student: 1 cartridge.

Right.

Student: Like in.

So

Student: General real time we said it in 95.

96 right so, what?

Student: Here you can do just 8.

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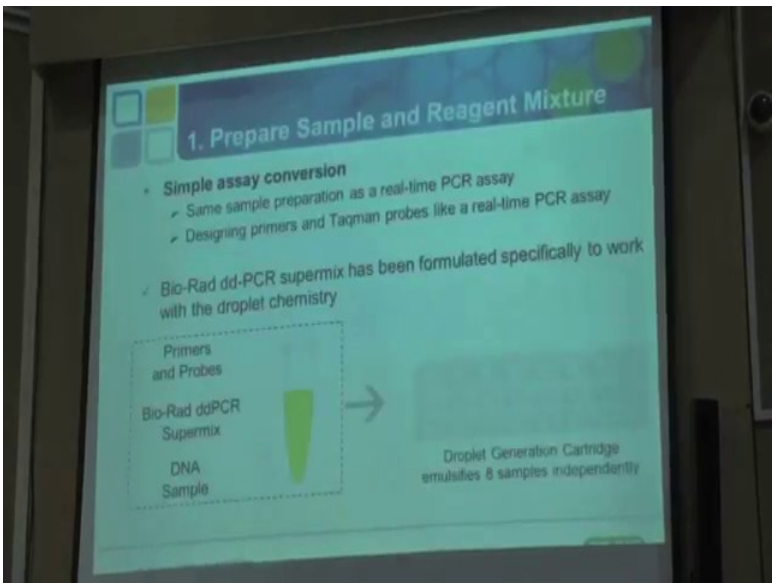

8 at a time for droplet generation.

Student: For droplet generation.

So, these process this we are talking of right, this takes 1 minute.

Student: Ok.

So you transfer your droplets in a PCR plate you generate next.

Student: We have transfer also?

Yes, you are transferring these right you are generating a droplets.

Student: We can.

Which are here in this top row.

Student: After generating the droplet we need to transfer those droplets.

To a PCR plate and then put a PCR.

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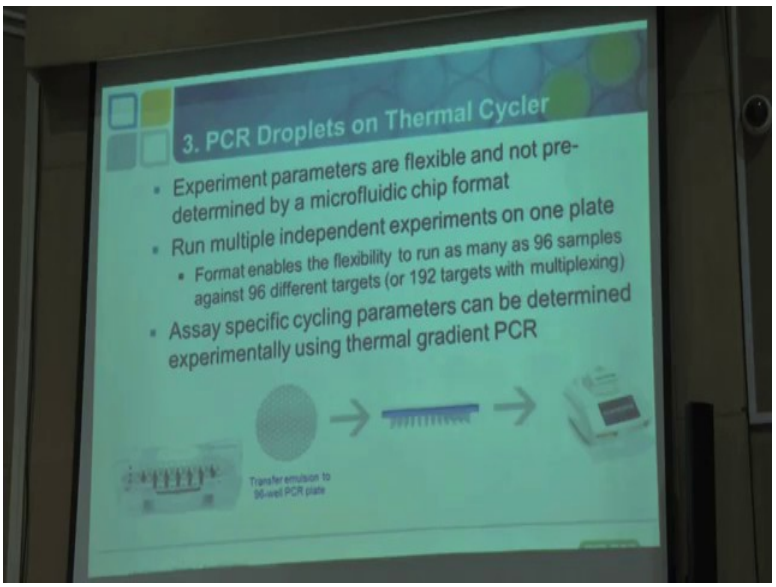

Student: degradation doesn't happen on this.

No they does not happen, does not happen, it is minimal optimized for that, it is minimal optimized for that. The droplets which are formed are very stable.

Student: And in fact, like statistical significance we need to do 3 control 3 replicates suppose we are.

You do not need that in this.

Student: Control.

You do not need that for this technology, because each of your reaction is what is splitting in 20,000 replicates. So, do not need to have technical or biological replicates like you do for real time PCR.

Student: In this like each where it is splitting particular samples size into 20,000 droplets.

Yes.

Student: So.

Sample size no each of your reaction.

Student: Like DNA will be will be dividing to 20,000. So, how can we?

DNA would not get divided, that your DNA is not getting fragmented.

Student: It is not getting fragmented.

Right.

Student: It is getting separate like in the previous video, it was get in a single when they, can you show me the first slide.

First slide for this.

Student: First slide.

This one.

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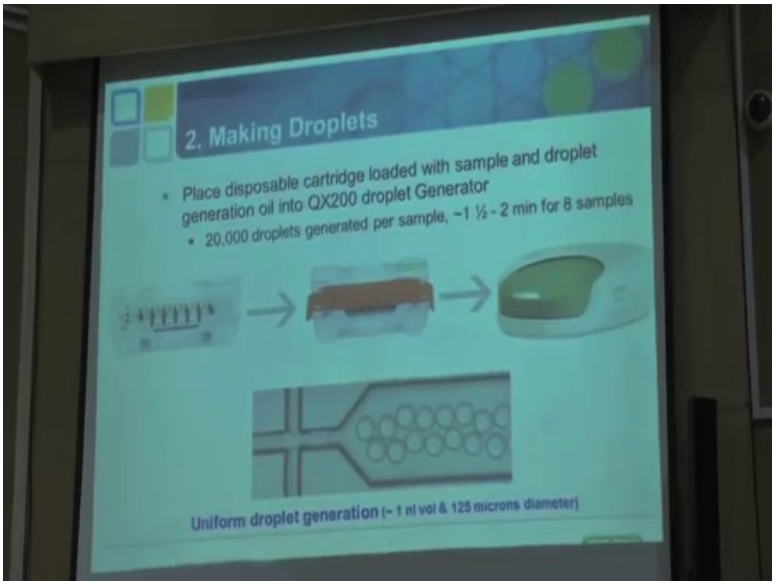

Student: No, which was separated the stem it was a video actually.

It was in the video.

Student: Yes sir.

Yeah wait.

Student: Something else.

I would not be able to forward the video here, because I cannot see anything here, yeah tell me your query ma'am.

Student: Like in that slide when wherever single mutation and to get a single mutation in the gene. We are taking DNA sampling sample in a well and it is taking separated into different-different droplets.

Student: That was the point.

It is not 1 fragment right, they have multiple fragments.

Student: Where will be multiple?

Right.

Student: In that case. If be a suppose 100 suppose 100 strands.

Ok.

Student: Even mutation is in just 1 strand and it is with 100 strands are getting separated into multiple droplets so.

Your DNA is getting; your DNA is getting fragmented, your DNA is not getting fragmented it is getting partitioned.

Student: It is getting partitioned.

Correct.

Student: So, how can we consider it is a 3 replicate sample, like it will be considered as single only.

No what is happening is your, you put your replicates or replicates to eliminate most of the times to eliminate a pipetting errors, if you go for a real time PCR right you should not have more than 0.5 c t value difference.

Student: Yeah.

Right. So, here it does not matter because the data that you get is copies of your DNA per micro liter of your sample.

Student: Her question is different.

Student: But still I doubt.

Right.

Student: The way we know those fragments is not there to be in any of the droplets. So, how would you say that If the same mutation is happened, mutation is happening or rate of the mutation is 0.1 percent.

Right.

Student: If we growing here 20 to 20000 droplet. And then you are saying 33 percent is increasing. Question is that droplet per DNA each there they have it or not have it.

Does not matter sir, not all droplets will anyways not have.

That is ok, we have publications that people have got 0.0001 percent of the mutant as well.

Student: No as a difference.

Right.

Student: This is for statistical significance.

Right so, the data that comes out; the data that comes out comes after implying a Poisson's algorithm, which takes care of your statistical significance.

Student: for the publication.

From a publication specific you are asking, there are more than 4000 publications already with this technology where this direct analysis will be done. Your term of asking is a SD right, the standard deviation that you would get from each 3 replicates that you get, correct. That is required because you might consider that your handling might be different right there will be pipetting errors to take care of those things.

Student: So, in this also we are translating like after formation of those.

Right.

Student: we have to transfer it to the other plate na.

Correct so, but the drop right, but the technology is made in such a way at that transfer droplets none of your DNA molecules are left behind. The technology make sure that all of your reaction mix components are split up and are present in the droplets, none of it is left behind in the cartridge, the system is that rigid.

Student: Your.

It does not leave anything behind in the cartridge.

Student: We are entire droplet is getting transport.

Yes, none of the droplets what we make here for example, in the first step here, these droplets which are made right.

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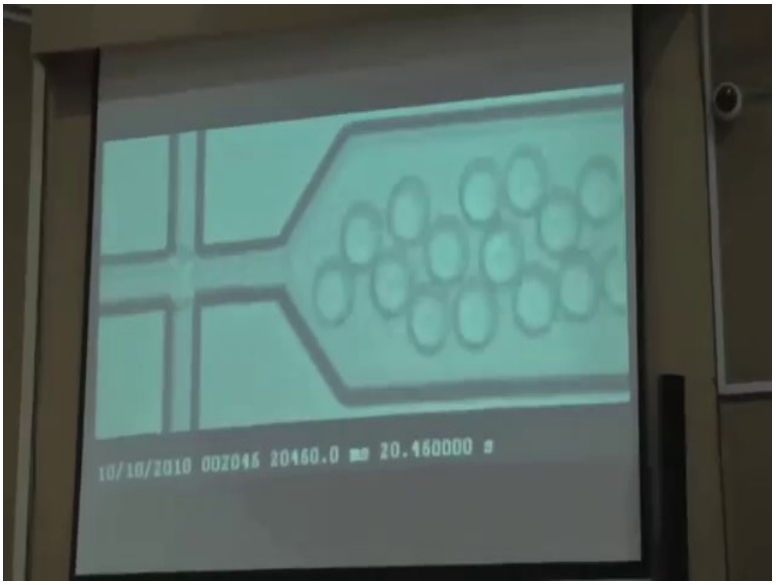

Student: So, the complete what you are getting transferred.

Yes.

Yes, nothing is left behind in your cartridge, all of it is getting transferred nothing like is there back in the cartridge.

Student: So, can we reuse the cartridge?

The cartridge are not, you cannot reuse the cartridge.

Student: We cannot reuse.

No, no; we cannot reuse the cartridge yeah.

Student: We do not use the real time monitoring. So, it is a end time.

It is a end point PCR, sir.

Student: So, how do you differentiate over our real time PCR?

So, that is what I have mentioned, there was a entire slide on that right here.

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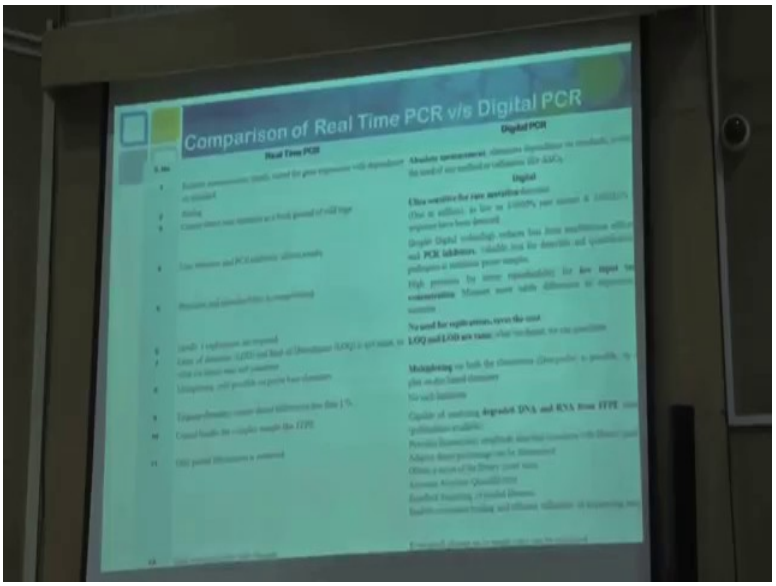

It quantitates it, the data that you get is it will give you how much ever DNA or RNA was present in your sample. It gives you copies of your amplified DNA in each sample in copies per microlitre, it is not a you do not have to do any minor calculation like you do in real time PCR, you have a CT value then you do a false method delta CT, delta delta CT, do not have to do anything. The system quantitates and gives you the precise value, yes ma'am yeah.

Student: What is there any limitation on the amount of DNA that we put in the starting reaction with? That was the I am talking about the templates.

The minimum amount DNA you mean right, again we have got lots of data where people have worked on femtograms of DNA as well. Even a single copies spike you have company called as Horizon which gives you copy number controls; it gives you 1 copy, 2 copy. So, you can spike your sample and check the copy. So, system is being validated to do that.

Student: That template can be of any size?

Any the template can be of any size, but your amplicon should not be very long, ideally it should not be above a KB.

Student: But how does to make sure that very long template we are using?

Right so, in case you are using genomic DNA. Right, we recommend doing a restriction enzyme ligation, if you are using a genomic DNA which you feel is going to be very long.

Student: We isolate DNA.

Correct.

If it is a cDNA, then does not matter based on the genomic DNA alright we recommend doing a restriction ligation. So, that your template is available for the primers to bind and elongate.

Student: It has to go a most wells.

It will go in either of the droplets, it can be 1 droplet also, it can be 10 also, it can be 100 also, it can be 1000 also, does not matter.

Student: The

Because it reads each of them individually; so, this thing what you see here yeah even if you have this 1 droplet positive and everything else is negative. You can be sure that the 1 droplet is because there is some DNA which is gone there, which is amplified and hence there is a fluorescence.

Student: How many DNA is getting fractionated partitioned?

Right, ma’am.

Student: So, what are the chances that a particular droplet I mean can have more than 1?

Yes.

Student: So, what are the maximum number that have being qualified?

Above 5 generally above 10 lakh copies if it is there, then you start because what will happen is all of it will be positive then you will have no negative droplets. All of it will be positive. So, you might not get a very precise amount of DNA, that is above 10 lakh copies if you have, that abundant amount of DNA in 1 sample now if you have.

Student: Per sample you know I am.

Per sample, per sample.

Student: Per droplet.

Per droplet, because 20,000 into 5 becomes that number so, per what happens is, the system while it gives you the data, it takes into account your forward scatter and side scatter, like it does in a flow cytometer. So, even if there are up to 5 copies in 1 droplet it quantifies them as 5 or 4 or 3 or 2 or 1, it does not give you as 1 copy.

Student: So, maximum is 5.

Maximum is 5 per droplet that amounts to more than 10 lakh copies in your sample. Anything else? Yeah.

Student: is there a way to know how specific your primer binding is? so what happened to your primer not designed specific might bind somewhere else.

Right so, in that case yeah you would get multiple clusters here, you would not get a single cluster. If it is amplifying it is binding somewhere else right, the size of the amplicon will be variable, it cannot be of same size right. So, it will grow a different cluster here, you would not find such clean clusters, you will have another clear cluster which can be of a smaller or a bigger size, it is very sensitive for that.

Student: Where this basically amplicon size where we can say for sure it will amplify?

Right so, may be in case you know one of your standard even if you have one standard for the first time if you are putting a new reaction, you have one sample where you know it is going to work for sure right. You just put that for once and see which florescence does that come to for once and you know that value next time. So, if you are getting something variable; that means, there is something non-specific also which is amplifying along with your specific thing.

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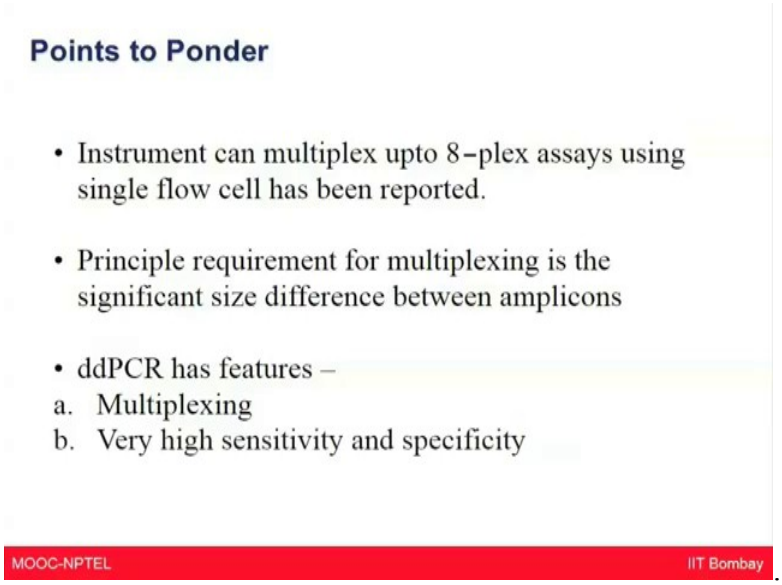

In conclusions, I hope today you have learned how ddPCR can be used for multiplexing. It has very high sensitivity and specificity, ddPCR make sure that the sample is not lost during the droplet formation, hence increases the efficiency of the process. In the next supplementary lecture, we will talk more about various proteomics applications.

Thank you.