Comprehensive Molecular Diagnostics and Advanced Gene Expression Analysis

Prof. Aritri Bir

Dr. B.C. Roy Multi-Speciality Medical Research Centre

Indian Institute of Technology Kharagpur

Lecture 43 : Digital PCR in Cancer detection

Namaskar. Welcome back to the NPTEL lecture series comprehensive molecular diagnostics and advanced gene expression analysis. So, we were at the week of molecular diagnostics in cancer management. Today we are going to discuss the digital PCR in cancer detection. Now in today's class I am going to discuss a bit of ddPCR that is Drop Plate Digital PCR or Digital PCR. What is the exact principle of this Drop Plate Digital PCR, how the work flow is running inside the system and finally, how the analysis is done after quantification.

So, Drop Plate Digital PCR if you remember that you have the basic principle already covered in the variations of PCR in the second week. Then to remind that this Drop Plate Digital PCR technology is basically helping in precise quantification of the target nucleic acid in the sample. And that precise or absolute quantification is done by measuring nucleic acid molecules which are encapsulated in a discrete volumetrically defined water in oil drop plate partition. So, this water oil emulsion drop plate system is basically generating drop plates and those water oil emulsion induced drop plate are basically partitioning each nucleic acid molecules in a compartment.

So, the purpose is in each drop plate there will be nucleic acids those nucleic acids will be amplified. So, here you can see instead of having different tubes here in different drop plates or multiple drop plates there are amplification or PCR reaction. So, here you can see there is increasing of the nucleic acid after PCR amplification. What is the advantage? It needs smaller sample requirement. The basic approach is basically Tackmann similar to the Tackmann probe based assay.

So, the principle here is definitely in helped by drop plate generator which is a combination of microfluidic and proprietary surfact chemistry which basically divide the PCR sample in water in oil drop plates. This drop plate basically contains the nucleic acid which helps in PCR amplification that the template molecules reagents work all the work flow are within this drop plate occurring within this drop plate. So, after PCR amplification what we do? Consider drop plates each drop plate as a chamber. So, in a

chamber we are basically assessing the or the or rather quantifying the amplification. So, instead of the chamber we are considering each drop plate in each drop plate we are analyzing the PCR products via flow cytometry.

Now this DDPCR or Drop Plate Digital PCR based data is basically analyzed to determine the target DNA template concentration in comparison to the or the amplified concentration in comparison to the previous one. So, the workflow is the common one that we need to prepare the sample, sample which must contain the target DNA which we want to amplify along with that we need primers because it is similar to Tackmann probe based assay there should be Tackmann probes with along with all the master mix and a proprietary super mix which is specifically required for drop plate generation. Now here you can see the samples are basically loaded over a cartridge that is drop plate generating cartridge. So, these are this is the cartridge where oil sample droplets have already been marked here. So, in each cartridge we are loading the sample mix or the master mix rather.

Now this drop plate generator cartridge now is placed in a drop plate generator where there as I told you there is a micro fluidic channel based system along with proprietary reagents and they help in generating around 20000 nanometer size drop plates which are of uniform size and volume inside the drop plate generator. So, here you can see on from the input side we introduce the sample and that sample mixes with oil to generate these drop plates. So, this is our drop plate generator. After generation of the drop plate there is PCR process if the PCR amplification occurs within each drop plate the nucleic acid reagents everything are reacting within the drop plates and generating or rather amplifying the target DNA. So, what we do not need is a sorry what we do need is a compatible thermo cycler which can adapt this see this plate basically fine.

After that the amplification is over. So, we are quantifying the end products of this drop plate digital PCR and that is read in a drop plate reader that is analyzed in a drop plate reader. Reader reads each drop plate individually using two color detection system. So, basically multiplexing can be done using different types of fluorescent probe just like we do in TaqMan probe analysis. Now the reading is done in binary fashion whenever there is a PCR positive product we say it is plus or no means 0 means no and that is basically that is basically accessed via fluorescence measurement.

So, the fluorescence in each drop plate is basically measured in two optical channels and after that based on this fluorescence the number of positive and negative drop plates per sample is calculated. Now positive sample considered as at least one copy of target within it target DNA within it and that is definitely giving increased fluorescence in comparison to the negative droplets which does not contain the target DNA. Now based on that a fraction of positive drop plate is actually fitted over the poison distribution.

Poison distribution is basically the probability distribution which determine the absolute copy number of the target molecule in the input reaction mixture and that is represented as copies per drop plate or CPD. CPD is basically not calculating the mass rather it calculating the number of the DNA nucleic acid.

So, it is basically the average number of target nucleic acid in each drop plate after that it can be represented as in microlitre sorry initially it is calculated microlitre after that considering what will be the volume of each drop plate the representation can be done based on copies per drop plate. So, this is how the absolute quantification is done. So, here you can see the drop plate contains this is the sample which is being divided in multiple droplets. The droplets are basically the discrete partition. Now each partition are actually random partition.

Random partition which may contain one molecule of the nucleic acid may contain multiple molecules of nucleic acid may contain 0 number of nucleic acid based on that the reading is done. Now here you can see for the positive reaction there is more fluorescence in comparison to the negative one after that we fit in fit this reading in the Poisson statistics and then the balance is done by one compartment might contain more target molecule creating more fluorescence. So, that is balanced in the Poisson statistics. So, the ultimate is to calculate the fluorescence to measure the fluorescence and represent it in terms of number of nucleic acid per droplet. Now, coming to copy number variation analysis in BDP-CR.

Now what is copy number? Copy number is basically the number of copies of a target locus or target gene sequence in a genome in comparison to the reference one. So, consider there are two genomes or two samples rather one is a reference sample. So, the purpose is to check how many copies of alleles are present in our target sample in comparison to the reference one. So, if there is any variation or any change or any alteration with respect to the reference that is represented as copy number variation. So, copy number variation is basically the variation can be there if there is deletion which reduce the copy number or duplication which increase the copy number deletion or duplication of the target locus with respect to the reference locus.

So, basically it helps in detection of inter individual variability in genome which is much helpful in identifying different types of polymorphism, point mutation which are quite common in not only cancer, but in other genetic pattern genetic alterations which are finally, leading to different types of diseases. So, the purpose is quantification of the target sample locus as well as the reference locus and finally, representing them in terms of ratios. So, this copy number variation can be done by using d d p c r. Now, why I am saying copy number variation, why I am highlighting that because remember in d d p c r we need much less sample and this d d p c r is highly sensitive. So, even if there is a

meager see the point mutation is basically in the whole genome in a single region there is a change in the base polymorphism deletion insertion etcetera.

So, that minute alteration is very much sensitively detected using this d d p c r technology. Then rare mutation detection this is also one as I am talking about rare mutation. So, the detection there is a variation which can be present in very very low frequency in a pool of wild type background. So, if you compare it with the reference genome everything is matching with the in the target sample everything is matching with the reference or wild type sample except there is a minute low frequency change and that is rare mutation detection or R M D. Now, R M D is one very important mode which is much helped by d d p c r.

So, basically these two sequences are very similar except there is one small change due to this rare mutation detection. Now, what is done there are two different types of fluorescent tag with a single set of primers, two competitive probes competitive probes one contains hex another contain fan these are the fluorescent different fluorescent die which are attached to the oligonucleotide probe these are our oligonucleotide probe. Now, they will bind to the target DNA and will detect the presence of mutation. Now, let us see what is the result. So, we basically represent the results in 2D plot.

Now, there can be 4 types of fluorescence which can be detected. So, the number one is double negative droplet. So, there is no that specific oligonucleotide sequence is neither present in wild type nor present in the sample one fine. Then here we are getting wild type only droplets. So, here you can see different types of colors which are basically generated by the differentially tanned fluorescent probes.

So, here the wild type per hex amplitude if we detect it. So, wild type only basically is directed by this green fluorescence remember if you if we go back. So, this is our hex which is for the wild type or control and this is our target mutation which we want to check whether this mutation is present or not. So, basically one oligonucleotide probe is matching with the wild type another oligonucleotide probe is matching with the rare one. Now, when we get only green it indicates that there is only wild type only wild type oligo the oligonucleotide probe is binding with the wild type only giving rise to only wild type sequence.

Now if there is a homozygous mutation what will happen only the mutant or the target which is of blue color that oligonucleotide will bind will give rise to the mutant template that is the blue one. And finally, if there is a heterozygous condition where both the wild type and the mutant oligonucleotide sequence are present they will give rise to one orange sequence. So, it contains the both after that the concentration of this wild type and mutation templates are calculated based on the number of droplets in each cluster

then by calculation we indicate whether that rare molecular changes or rare mutation is present or not which is statistically significant or not. So, this is how different types of rare mutation can be detected by DDPCR. So, what are the actual advantages of DDPCR? Of course, absolute quantification we can quantify not the mass, but the number of the nucleic acid without even running a standard curve fine.

And that is very much important in measuring if you remember in viral DNA viral load microbial quantification for this this absolute quantification is very much helpful. Now because then detection ability is very very sensitive it can identify a low copy number as minute alteration in the change in the DNA sequence. So, rare mutation then rare genetic variations can be easily detected which is much more common in cancer. Then different micro RNA can be absolutely quantified based on their expression level which is also indicating of the presence of cancer, because in cancer or in tumor environment the micro RNA expression is differentially altered. Also the for the NGS sample library preparation validation is very much helpful during to validate the accuracy of the sequencing for single nucleic detection of single nucleotide polymorphism or copy number variation.

For single cell analysis it is also important because in a cell high degree of cell in sorry in a pool of cells the cell to cell variation can be detected with higher sensitivity and also different changes like homogeneous post mitotic progenitor stem cell populations they can also be well identified. After that editing genome editing the validation can be done because in CRISPR crash technology after the during the CRISPR crash technology the homologous or non homologous joining whether that is properly done or not can also be checked by dd PCR. So, the finally, at the end I want to highlight the important part of dd PCR the dd PCR is basically a variation of traditional PCR, but here we can absolutely quantify the nucleic acid where the nucleic acids are encapsulated in tiny droplets. In each droplets there are nucleic acid we consider there are nucleic acid those nucleic acid within those droplet are amplified in presence of reagents and master mix etcetera. Then those amplified PCR products are counted in each droplet via the absolute quantification that is the average number of nucleic acid present per droplet and that absolute quantification is basically giving high precision and high sensitivity.

So, these are the references and. Thank you see you in the next class.