Comprehensive Molecular Diagnostics and Advanced Gene Expression Analysis

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Lecture 44 : Mutation Detection Methods

Namaskar. Welcome back to the NPTEL lecture series of Comprehensive Molecular Diagnostics and Advanced Gene Expression Analysis. In the week of molecular diagnostics in cancer management, we are going to discuss different methods to detect mutation in sample. So, in today's class we are going to discuss different methods which can detect mutation, their principle and a bit of how they are actually done. So, there are list of techniques which can help in detection of mutation. Some are sequencing based, where by sequencing we can detect the mutation.

Some are PCR based technology and some methods neither follow PCR nor follow sequencing, but are very very much important in diagnosis of mutation. Going to one by one, allele specific oligonucleotide hybridization. So, in this method we basically use oligonucleotide probes which are short single stranded DNA probes of 15 to 25 nucleotides. They are complementary to the target DNA sequence that we want to the mutation, the probable mutation that we are assuming that that can be present and those probes are basically they are complementary.

So, they can hybridize to either wild type or the mutant one. So, basically there are two sets of probes one for the reference sample which is the wild type, another is the mutant sample, the probable sample which might contain the mutation. So, after hybridization the excess unbound probe are washed off, then the detection is done by the techniques which are useful in detecting the hybridized oligonucleotide probes that that is they can be autoradiography, they can be fluorescence based technology or enzyme based technology based on that the hybridization can be detected. So, if there is signal which is coming from a mutant oligonucleotide probe, it basically indicates the presence of the mutation and that can be done by comparing the two different sample wild type sample and the target sample. And this is a good way to detect single nucleotide polymorphism or point point mutation, but of course, they are not much suitable for large type large mutation large sequence insertion or large sequence deletion for that this allele specific oligonucleotide hybridization is not much useful.

Coming to ligase chain reaction, so, if you remember this has already been discussed in the variation of PCR. In the ligase chain reaction there are four probes PCR probes with two primers and two enzymes, one thermostable DNA polymerase and another thermostable DNA ligase. Now what happened in this template DNA these four probes are used. So, basically this is our template strand. In this template strand the four probes are attached.

How this is a forward probe and backward probe for one strand and this is another forward probe and backward probe for another strand. So, this is how the four probes are attached to the two both the 5 prime and 3 prime ends of both the DNA template strands they amplify. So, what happens after this hybridization of the probes there is chain elongation via the thermostable DNA polymerase and after that they the strands are ligated via the thermostable DNA ligase. So, this is how the strand is amplified, but the most important thing for detection of mutation is basically the perfect hybridization of these probes. So, if there is any mismatch if there is even a single mismatch at the junction of two probes.

So, the proper ligation will not happen. So, only the duplex which contains a properly perfectly matched primer template they can only survive during the ligation, ligation can occur in those. So, here you can see these this is the wild type DNA here these are the four probes the four colors are the four probes. So, basically after the DNA polymerase mediated chain elongation ligation is possible in wild type whereas, because there is mismatch here these probe are not able to hybridize properly here as well. So, the problem is that they cannot be ligated.

So, there is no ligation. So, this is how ligase chain reaction can help in detection of the mismatch hybridization or the mutation. So, if there is a positive ligation product we can run in agarose gel and can detect whether there is the ultimate formation of the ligation product is present or not. Coming to single strand confirmation polymorphism now by the name it is evident that there is conformational difference of single stranded nucleotide sequence of identical length and that conformational difference is basically induced by the difference in sequences under the experimental condition. So, if the double stranded DNA is denatured what will happen there will be generation of the two strands two denatured strands and if we amplify if this SSCP single strand confirmation polymorphism is combined with definitely PCR.

So, the product after denaturation is actually the product is basically the amplified one which on denaturation gives rise to two different strands the two strands of the double stranded DNA. Now, after denaturation what happens this single strand they undergo a characteristic three dimensional folding and that characteristic three dimensional folding is basically different if there is difference in the sequence. Now if these samples are run

in polyacrylamide gel non denaturing polyacrylamide gel what will happen there will be the difference of different run in this gel by the wild type and the mutant one. So, basically if we compare the migration pattern of two samples now the samples if they are similar they will give rise to similar migration pattern whereas, if there is mutation it will be detected by the migration pattern or the mobility of the two different sample. So, by the change by the difference of the confirmation of the single stranded DNA denatured single stranded DNA we can detect the presence of mutation remember these are the older techniques by which the mutation used to be detected.

Coming to denaturing gradient gel electrophoresis definitely there is gradient gel electrophoresis and there is denaturing. So, what is there the mobility of the partially melted double stranded DNA fragments in the denaturing gradient gel and that is also influenced by the sequence composition. So, if there is a change in the sequence the migration in the denaturing gel in two different samples can be detected. Now because this is one denaturing gradient gel in the gel two denaturing agents are incorporated one is urea another is formamide and that is also a gradient gel. So, basically there is increasing concentration of the gradients along the length of the gel is present.

So, in different regions while the electrophoresis is done in different region the speed or the nature of migration will be different because there is increasing concentration of the gel. Now during electrophoresis what happen this DNA molecule while run through the gel matrix definitely the run depends on the size as well as the sequence composition. So, while running through the gel what happens the nature of migration is affected by the increasing gradient of the gel as well as the sequence because based on the sequence the denaturing product is showing their migratory ability. So, what will happen there will be a different migratory pattern in the reference and while reference means wild type and in the mutated sample. So, the comparison will be shown if we check or compare the different the presence of the mutation will be shown if we compare the migration pattern of the wild type and the target sample.

Then coming to hetero duplex analysis of mutation detection. Now hetero duplex analysis is basically a bit similar with the denaturing gradient gel electrophoresis here the target sample is mixed with the wild type they are denatured and then allowed to hybridize again. Now what will happen there will be either hetero duplex formation or the homo duplex formation. So, here you can see this is our wild type DNA and this is the mutant DNA the target sample which might contain the mutant sequence. Now consider these are our wild types 2 strands and these are our target sample.

If the sequence are similar if there is no mutation what will happen if we hybridize then the this is the wild type duplex consider this the sequence is like this fine. This is the target duplex if there is matching we will get another duplex which contains one strand

from the wild type another from the target samplers similarly other one. If there is no mutation we will get only homo duplex, but if there is mutation we will get hetero duplex as well. So, here you can see because there is mutation there is hetero duplex where the strands in a region is basically not able to hybridize the H hydrogen bond is not formed because of the mutation. So, this is the homo duplex wild type this is the homo duplex of the mutant type, but these are the hetero duplex where one strand from the wild type and another strand from the mutant is present.

Now these hetero duplexes are of basically 2 type if there is small deletion or insertion which creates a stable hetero duplex which is known as bulge type hetero duplex and that can easily be detected in electron microscopy, but single base substitution which forms the bubble type hetero duplex and that single base substitution is not much easy to detect and that can be easily detect by hetero duplex analysis. So, if the sample is run here you can see the wild type homo duplex creates a band while mutant homo duplex can create another band which is not matching with the wild type one, but by molecular weight it is located somewhere else and in the hetero duplex part we will get a strand which is matching with the wild type a strand which is matching with the single strand of the mutant one. So, a pattern will be generated in hetero duplex or if there is a homo duplex mutation there will be a separate homo duplex bond band which is located in a single position. So, this is how mutation screenings are mostly done one very good example is CFTR gene deletion in cystic fibrosis. So, this hetero duplex analysis can also be coupled with the capillary based HDA where the fluorescence capillaries are capillary system are present.

So, basically the primers are labeled with different types of fluorescent dyes and that in the capillary based system multiple types of detection multiple samples can be detected simultaneously. So, basically multiplexing is possible. So, again in the gel you can see. So, if there is no mutation, no mutation what will happen? The reference which contain the wild type mutation wild type band the both bands are of similar sequences similar complementary sequences. So, they will give rise to a band in one single location.

If the target sample contains no mutation. So, the both the strands will be same and they will give rise to a similar pattern of band which is matching with the reference one. So, there is no mutation. If there is mutation in the target DNA and that mutation is of homo duplex type means both the strands contain the mutation. So, in that case what will happen because the strands both the strands are similar they will give rise to one pattern of band only not the two bands are migrated to two different location rather both the bands are migrated to one single location only.

So, one band will be generated, but that band is not matching with the reference one, but if it the target contains mutation and that is of hetero duplex pattern what will happen?

Two different bands will be generated. Now, two different bands one will be matching with the wild type, one will be matching with the mutant and they will give rise to a hetero duplex pattern. So, this is how the mutation can be detected by hetero duplex analysis. Coming to HRM or high resolution melting analysis where resolution of the melting pattern of two different DNA sample can be compared. So, again the there is PCR amplification followed by melting in higher temperature and that melting is basically analyzed in real time by measuring the fluorescence.

So, fluorescence how that can be measured definitely there should be intercalating dyes which contains the fluorescent dye with the dyes which can bind with the double stranded DNA and when bound with the double stranded DNA they gives they give rise to the fluorescence. So, whenever there is melting there will be loss of fluorescence. So, initially at the very beginning because there is double stranded bond there is fluorescence slowly when this melting proceeds there will be fall of the fluorescence. Now what if we analyze this melting curve what will happen? There is a pattern of the wild type. If there is homozygous allelic variation the pattern of the melting curve will be same, but there will be a temperature shift.

So, here you can see the pattern is similar with the wild type, but the temperature shift is there, but if there is heterozygous sort of heterozygous duplex hetero duplex. So, what will happen there will be a different types of melting curves. So, initially the wild type melting curves was like this then the homo duplex type of mutation is like this. So, only there is change in the change in the temperature. So, shifting of temperature, but the hetero duplex is like this.

So, the pattern is basically changed in case of the hetero duplex type of mutation and that is due to base pair mismatch because the hetero duplex is destabilized and also the melting profile difference between the 2 genotypes are amplified in such a way that the difference can easily be located. So, that is high resolution melting analysis. Next coming to another technique by which we can detect mutation that is restriction fragment length polymorphism. So, here the basic principle is restriction digestion. So, the DNA sample is digested by different restriction enzymes assuming that in a DNA there are specific restriction points and if we digest this DNA with a specific restriction enzyme, they will give a pattern of pattern of restriction digest or restriction fragment and that can be detected by southern blotting technique after running it in the agarose gel.

Now, if there is single nucleotide polymorphism which is giving rise to inclusion of a new recognition site. So, suppose there is mutation which insert one new restriction site. So, the fragments will be different. So, we will get one additional band. Similarly, if a restriction site is deleted due to mutation in comparison to these if there is a deletion of suppose this part is deleted.

So, if there is a deletion of the restriction site what will happen? The number of band will be decreased. So, this is how by comparing the pattern of the restriction digest in two different sample the single nucleotide polymorphism can be detected. Now, this single restriction fragment length polymorphism can be combined with the PCR where PCR and following PCR amplification the restriction fragment length polymorphism process is followed. After that the sequence currently what is majorly done is sequencing based technique. So, the sequencing of the targeted region is done to identify any variation in the any variation in the genome or transcriptome and compared with the reference one.

So, even identification of the single nucleotide polymorphism can be done via checking whether there is any variation with the reference genome is present or not. Detection or insertion can be detected structural variation like the duplication, deletion, inversion, translocation those can also be detected. Copy number variation can be analyzed detection of rare variants can be done also sequencing of RNA can help in identifying different types of point mutations, splice site mutation, gene fusion etcetera. And of course, genome wide screening is very much helped via following this sequencing based techniques to detect if there is any variation is present or not. So, these are the techniques I have discussed allele specific oligonucleotide hybridization, ligase chain reaction, single strand, confirmation polymorphism, denaturing gradient gel electrophoresis, hetero duplex analysis, then high resolution melting analysis, RFLP, PCR restriction fragment length polymorphism and also a bit of the modern technique sequencing based technique how they can help in detection of different types of mutation.

So, these are my references. Thank you.