Comprehensive Molecular Diagnostics and Advanced Gene Expression Analysis

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Lecture 45 : Isothermal Nucleic Acid Amplification

Namaskar. So, we are in the lecture series of comprehensive molecular diagnostics and advanced gene expression analysis. Today we are going to discuss isothermal nucleic acid amplification. Here we are going to cover the different techniques which are used in isothermal nucleic acid amplification, those are looped mediated isothermal amplification, nucleic acid sequence, best amplification, strand displacement amplification, recombinance polymerase amplification, helicase dependent amplification. So, basically what happens in strand displacement DNA synthesis that so, this is the double stranded DNA. So, if a primer is capable of displacing the strand along with the enzyme DNA polymerase, special DNA polymerase which has the ability to displace a strand what we what it will do? It will basically displace this strand and the primer will bind in this region and with the help of DNA polymerase it will be extended. So, that is strand displacement DNA synthesis.

Now in LAMP this strand displacement DNA synthesis technique is basically exploited. Now there are different types of primer which are present in LAMP there are two inner primers and two outer primer. The inner primers are forward inner primer and the backward inner primer similarly there is forward backward outer primer and forward outer primer. Now each contains two distinct sequences corresponding to the sense and antisense sequence of the target DNA.

One is required one inner primer is required for priming in the first stage and the other is required for self priming in the later stage. Now if we see this target DNA you can see there are multiple regions which are denoted with different names and that is basically to help to understand how the different primers are binding in the target DNA region. So, F stands for the forward one forward sequence, B stands for the backward sequence and C stands for the complementary sequence. Now sequence inside both ends of the target region for amplification are designated and F to C and B 2. So, these are if we consider that this is the target region then both ends of the target region one end is denoted as F to C another end is denoted as B 2.

Now these two inner sequences are basically 40 nucleotide apart and the outer sequences to that are basically these two F 3 C and B 3. Then if we consider this is the end of our target DNA inner to that end is F 1 C and B 1 fine. So, again to make you understand this is our target DNA sequence the end of one target is F to C another is B 2 inner sequence which are located inner side of this ends are F 1 and B 1 and the outer sequence is basic to that is F 3 C and B 3. Now the forward primer contains F 1 C a spacer and F 2 which is complementary to F 2 C. So, basically a forward primer can bind with this there is a spacer consider this is the spacer after that there is a sequence region which is complementary to F 2 C.

Similarly the backward primer is complementary to B 1 then there is a spacer once again this region to fulfill this region and after that a sequence which is complementary to B 2. Then there are two outer primers which is complementary to B 3 and another is complementary to F 3 C quite complicated. Let us see how it is actually running in the sample. So, this is our target sequence. So, we have a primer primer which is complementary to F 2 a region which is complementary to F 2 and this F 1 C is basically reverse it has reverse complementary to the F 1 C fine.

So, it is like this. So, if this is our F 2 C and this is our F 1 C. So, what happen F 2 can bind in this way, but F 1 is basically reverse complementary with the sequence fine. Now, this is our forward primer forward primer is attached to the F 2 and here you can see another strand is basically displaced. Now, what happens with the help of the DNA primer is which has the strand displacement ability strand extension is occurring.

So, what happens there is a strand extension. So, this strand is basically extended, but then another forward primer is outer primer that is outer primer is attached in this F 3 region again displacing this strand. This strand is basically replaced or displaced by the outer primer. Again the outer primer is extended using the strand displacement DNA polymerase fine. Now, what will happen? This strand this gray color strand is basically acting as the template strand where backward primer is attached.

The backward primer here binds with the B 2 region whereas, this complementary B 1 C has the reverse complementary like this. Then B 2 using this B 2 primer with the DNA polymerase the strand is extended after that the outer backward primer again is attached here you can see the outer backward primer is attached and displaced this strand. So, this is our target product this is our target product. So, what we wanted if you remember from the if you remember this has been our target sample starting from F 2 to B 2. So, if we see in our amplification we are getting the target which is F 1 to B 1 C.

Now, again if you remember this has reverse complementarity similarly this has reverse complementarity. So, what will happen? There will be a loop formation. So, this F 1

fragment will form a loop and will hybridize with this F 1 C segment forming a loop. Similarly, B 1 C will form another loop and will bind with the B 1 and here we get the dumbbell pattern. Again all the primers will be able to bind here along with that there are loop primers which basically enhances the extension and stability.

So, there are different end products which we get from loop mediated isothermal amplification and because there is tagging of the fluorescent dye we can detect whether there is amplification possible or not based on the hybridization of these different probes. So, this is loop mediated isothermal amplification. Now, the detection can be done in real time can be done via measuring the end products. For end products photometry by measuring the turbidity caused by magnesium pyrophosphate precipitate in the solution can be done. In real time turbidity can be measured by photometry or fluorescence can also be measured by intercalating dyes.

So, quantitative lamp is also possible by measuring the fluorescence. Then in tube detection of lamp DNA amplification can also be done using manganese loaded calcium which starts fluorescence upon complexion complex formation with the manganese by pyrophosphate during in vitro DNA synthesis also hybridization with the complementary gold nanoparticle with the single stranded DNA can also be done for detection. So, this is loop mediated isothermal amplification. Now, another amplification isothermal amplification is nucleic acid sequence based amplification or NASBA where it mimics the retro viral RNA replication. It occurs in two different phases one is a non cycling phase where a target RNA is converted to double stranded DNA by the process reverse transcription after that cycling where this double stranded DNA is actively transcribed into its RNA product.

So, what happens consider this is our RNA target here we are using one primer the primer contains a region which is a promoter region T 7 promoter region where the T 7 polymerase can help in transcription of the product. Now, it is attached in the primers 5 prime end. Now, the primer binds and it synthesize the complementary cDNA strand by reverse transcription after that the sample is treated with RNA's H. So, this RNA DNA from this RNA DNA hybrid only the RNA is our target by RNA's H. So, RNA's H degrades the RNA what we have in our hand is the complementary cDNA which has a T 7 promoter region at its 5 prime end.

Then we have another primer which does not have the T 7 primer T 7 promoter region. So, this is our non T 7 primer now this non T 7 primer forms the complementary strand which is complementary to the cDNA. Again at the end what we are getting is a double stranded DNA. Now, from the double stranded DNA we are getting RNA because there is transcription via T 7 RNA polymerase. Now, remember there is only production of the antisense RNA because remember polymerase can transcribe from 5 prime to 3 prime

ends.

So, the product is antisense DNA, antisense RNA. Now, this antisense RNA is basically once again following the cycle. So, there is again reverse transcription and after reverse transcription from this RNA there is formation of another DNA strand that DNA strand is now amplified using the non T 7 primer. So, there is a constant amplification from the antisense RNA and that can be detected. So, here you can see here we what we are getting after amplification we are getting the antisense RNA which is reverse transcribed using the second primer which is the non T 7 primer.

Then again it is after RNA is treatment we are getting the cDNA strand, cDNA strand is extended using the T 7 primer and this is following the cycle. So, this is how nucleic acid sequence based amplification can detect a specific sequence from its RNA product. Then strand displacement amplification this is a 2 step process where we generate first the target after that that target is amplified exponentially. So, there are 2 sets of 2 pairs of primer one is a bumper primer that is just similar to the standard PCR primer and another is SDA or strand displacement primer. Now, the sand strand displacement primer it contains one restriction site overhang.

So, here is our restriction site overhang. Now, what happens in these double stranded DNA? Remember the basic principle is displacing one strand and amplifying the DNA. Now, what happens first this stand displacement primer binds and amplify the target. So, the stand displacement primer bind and amplify the target and in its prime end we have a restriction site. After the amplification what happens comes the bumper primer.

Now, the bumper primer is basically important to displace these amplified product. So, what happen the bumper primer basically binds a bit upstream just adjacent rather upstream to the SDA primer. Bumper primer binds and extend displacing the SDA primer mediated strand. Now, in this SDA primer mediated strand once again is amplified. So, at the end we are getting a double stranded DNA the target is amplified a double stranded DNA which has the restriction site at its ends.

Now, what happens these restriction sites are identified by the restriction enzyme it induces a nick. Now, here you can see a nick is only introduced in one side of this double stranded DNA because while amplification of the strands we are using thiol modified either of NTP is of the 4 NTPs one is thiol modified. So, the in this modified strand apart from the restriction site the restriction enzyme is not able to digest. So, a nick is only created in its restriction site. Similarly, here also a nick can also be created in the restriction site.

After that because there is a there is creation of nick what will happen the exonuclease

deficient DNA polymerase identifies and by nick translation method it actually extend this part via nick translation method displacing this strand. Again this strand is extended via the primer. So, this is how strand displacement amplification is done via using two different primer one endonuclease one exonuclease deficient DNA polymerase and thiol modified dNTP. Coming to recombinase polymerase amplification recombinase in recombinase polymerase amplification what is used is a recombinase enzyme a single strand binding protein complex and strand displacing DNA polymerase. So, what happen the primers the two oligo primers which are complementary to the to the regions flanking the target sequence they follow the recombination.

How the recombinase enzyme basically forms a nucleoprotein filament where the single strand binding protein they bind with the primer, primer it causes the strand displacement. Now this strand which is displaced is basically stabilized by the single stranded single stranded DNA binding protein. So, this part is stabilized the primer is attached the single strand without the binding. So, this reannealing is constantly displaced in this region due to the binding of single SSB protein and there is constant amplification using the strand displacing DNA polymerase. So, this is how a cyclical amplification of the target region can be done via stabilizing a displaced strand using single strand binding protein and recombinase enzyme and of course, the amplification is done via strand displacing DNA polymerase.

Now helicase dependent amplification, helicase dependent amplification is just a bit difference with the PCR where in polymerase chain reaction we use heat as denaturing agent and for that we need to raise the temperature then during aniline amplification we need to change the temperature. Here in case of helicase dependent amplification we use an enzyme that is helicase, helicase causes the strand denaturation after that there is single strand binding protein which is used to stabilize the single stranded DNA then the amplification using the DNA polymerase is done. Now this helicase is basically a helicase super family 2 protein which is obtained from a thermophilic organism that is thermoanaerobacter 10-consenesis. Now this helicase is a helicase super family 2 protein which is obtained from thermophilic organism that is thermoanaerobacter 10-consenesis and this organism is capable of unwinding the blunt end of nucleic acid at an elevated temperature like 60 to 65 degree centigrade. Now this HD reaction can also be coupled with reverse transcription which causes RNA amplification.

So, amplification of RNA can also be done in isothermal condition using the helicase dependent amplification. So, at the end isothermal nucleic acid amplification is basically a group of molecular biology technique that amplifies the nucleic acid sequence at a constant temperature. So, eliminating the need of thermo cycler amongst them the important one are LAMP or loop mediated isothermal amplification which amplifies the DNA sequences using DNA polymerase and different set of specific primers forward

and backward primers outer primers inner primers and also loop primers. Then NASBA nucleic acid sequence based amplification which amplifies the RNA sequence following three enzymes one is reverse transcriptase then RNase H and finally, producing the transcript via T 7 RNA polymerase. Then strand displacement amplification which amplifies the DNA sequence to strand displacement using reverse transcriptase and DNA polymerase recombinase polymerase amplification RPA it basically follows the recombinase technology where there the single strand DNA binding protein causes the stabilization of the displaced strand followed by amplification using the strand displacing DNA polymerase.

Helicase dependent amplification here the denaturation is done by helicase the single strand is stabilized using single strand DNA binding protein and after that there is amplification via DNA polymerase. So, these are the isothermal nucleic acid amplifications these are the references and. Thank you.