Comprehensive Molecular Diagnostics and Advanced Gene Expression Analysis

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Lecture 13 : DNA Cloning and Recombinant DNA technology - III

Namaskar and welcome back. We are at our lecture series of Comprehensive Molecular Diagnostics and Advanced Gene Expression Analysis. We were discussing different tools of molecular diagnostics and gene expression analysis and in last two classes we have discussed different methods of recombinant DNA technology. In the previous classes we have discussed the very basic process of cloning, the concept and different steps of cloning, discussed one very important enzyme in this regard that is restriction endonucleosis their functions and different aspects. Then we have discussed different types of vectors which are required for the cloning procedure. Today we are going to discuss DNA library.

So, what are the very basic concept of DNA library, what are the different types like genomic and cDNA library, how they are prepared and from that DNA library how the desired product can be identified. So, you must be remembering this the process of recombinant DNA technology where the new fragment of DNA or a gene can be inserted in different vectors and then it can be cloned and grows in different vector cell. Now these vectors which have the which transfer the recombinant DNA into certain cell the very common one is E. coli.

So, all these E. coli which are transfected or which contain the recombinant DNA they are pool from a library that is our genomic or cDNA library DNA library. Now how this gene library are constructed we are going to discuss. So, DNA extracted from an organism and then digested by the restriction enzyme they form a pool of probes a pool of DNA fragments and their combination is basically the gene library. Now there are two types of gene library one is genomic library which represent the total chromosomal DNA in an organism then cDNA library.

Now cDNA library is basically representing a specific mRNA from a particular cell or tissue at a specific point of time. Remember all the mRNA are not sorry all the gene are not forming mRNA at a specific period of time when required the transcription followed by translation leads to formation of the required product or protein. So, at a specific

frame of time in a cell we are getting those proteins which are currently required in the cellular function. So, those proteins representative mRNA pool is actually represented in the cDNA library. Now which library is required whether it is genomic library or the cDNA library is required is basically depending on the purpose of the recombinant DNA required.

So, recombinant DNA can be required while we need to understand the control of a protein production from a particular gene. So, we need to analyze that gene structure as well as function for that we need genomic library where we are assessing all the total chromosomal DNA. But when we are studying a very specific protein not the genes characteristics or genes product or gene structure analysis rather we are targeting over the end product that is the protein. We need to analyze the protein whether there is any for requirement of a new protein formation or a modified protein formation how this proteins are expressed in a cell what is the pattern of their expression when or the timing of the proteins expression in that case we need cDNA library which can be assessed at a very particular time frame. So, if we compare the genomic and cDNA library it is evident that the genomic library represent the total chromosomal DNA because it represents the DNA in totality it can be isolated from any cell type because the chromosomal content is fixed in all types of cell.

Whereas, when we are targeting a very specific proteins mRNA we need to isolate the sample from a very specific tissue or cell type in a very specific time frame. Now, because we are getting all the chromosomal DNA. So, in genomic library along with the coding region we can get non coding regions we can get promoters, introns everything whereas, in cDNA library remember cDNA if you remember cDNA is formed from mRNA. So, when mRNA is forming the cDNA there is no chance of having introns. So, what we are getting in a cDNA library is only exam.

Then the very very basic procedure how this is formed that is extracted chromosomal DNA after digestion with restriction endonuclease we get the different fragments which is inserted into the vector. Whereas, in case of cDNA library formation we need to extract the mRNA and that is converted to cDNA and that pool is introduced into the vector. So, in case of genomic library the vector contains the chromosomal DNA whereas, in case of cDNA library the vector contains cDNA formed from the extracted mRNA from a cell. Now, if we come to the genomic library preparation. So, this is the very basic step we need to isolate the complete chromosomal DNA from the cell and then we need to digest it with restriction endonuclease.

Now, this restriction endonuclease mediated digestion can be a complete digestion can be a partial digestion. Now, complete digestion is you need to complete the whole digestion process or whole reaction. So, what we will get a large number of very short

fragments. So, this is the product when we are going for complete partial digestion. So, it is completely digested.

In case of partial digestion what happens the reaction is stopped before the completion of the process. So, the enzymes are actually allowed to cleave at different potential restriction site and give rise to differential fragments differential length of fragments. So, you can see if there is partial digestion suppose these DNA is partially digested. So, one pool can be this see this is different the length are different another pool can be this. So, basically this restriction site in this reaction this is the number 1 reaction this is the number 2 reaction this number 2 reaction this restriction site is basically did not get the chance to be digested.

Similarly, here also you can see this restriction site is escape. Similarly, in the third one here you can see this restriction site is escape. So, partial digestion can give rise to different types of fragments of DNA which are having different length because of escape and choice of different restriction sites. Now, the average fragment size basically depend on the relative concentration of the DNA concentration of the restriction enzyme and also the reaction environment the condition of the reaction the duration of the incubation based on this the partial digestion mediated fragments are generated in case of genomic library preparation. Now for cDNA library preparation what we need we need to extract the very specific mRNA from the cell.

Now if we need to study the protein we could have made a library of mRNA, but that is not possible because mRNA is very unstable. So, to prevent the digestion of mRNA what we do we convert the mRNA to cDNA make a pool of that rather make a library of that cDNA and when required we isolate those cDNA then we form mRNA from that and then study the target protein. So, from the mRNA we need to synthesize the cDNA. Now you all know cDNA is synthesized by a very specific RNA dependent DNA polymerase that is reverse transcriptase which is mostly isolated from RNA containing retrovirus. Once this cDNA is formed this cDNA is inserted the spool of cDNA rather is inserted into the vector and then those vectors are cloned to form multiple copies.

Now the while forming cDNA the very first strand of DNA which is complementary to the mRNA template needs to be formed which requires all the 4 DNTPs the enzyme polymerase primer and everything. Now for synthesis of cDNA the first strand we can use different types of primer the primer can be oligo dt primer can be a random hexamer as well. So, you are you can see the first strand of cDNA can be formed by oligo dt or poly dt primer it can be formed by a random hexamer also a very specific primer for that specific mRNA can be used. So, what we get we get one cDNA mRNA hybrid in all the cases. So, one strand of cDNA is formed and the template mRNA is there.

So, they are forming the hybrid. Now following synthesis of the first DNA strand one poly d-c tail is added to the 3 prime end by the enzyme terminal transference using d-ctp. Now why this poly d-c tail is used basically what happens while this poly d-c tail is added to the 3 prime end of the first strand of DNA it also incidentally put a poly d-c tail in on mRNA. These can be exploited when we need to put a second strand when we need to synthesize a second strand let us see how. So, in our hand what we have a mRNA cDNA hybrid.

So, we need to remove the mRNA that mRNA is removed by alkaline hydrolysis. So, mRNA is removed what we have in our hand is single stranded DNA. Now the second strand is basically synthesized over this single stranded DNA. So, here oligo d-g primer can be used which can be best paired with remember the previous poly d-c tail. So, one specific primer that is oligo d-g primer is used by exploiting that poly d-c tail which is added in the single strand synthesis.

So, the final product is basically a double stranded DNA. Now there is another method instead of alkaline hydrolysis what can be used is RNase H. So, basically RNase H creates nick in the mRNA cDNA hybrid over the RNA. So, here you can see this is our mRNA cDNA hybrid. So, RNase induces a nick over the RNA.

Now because of this nick there is one 3 prime hydroxyl group which is utilized by the DNA polymerase as a primer just like replication. So, the second strand is synthesized over the first strand. So, this is how the cDNA library is cDNA is rather prepared. So, this pool of cDNA forms the cDNA library. Now we have a pool of say cDNA or the genomic DNA in the library and there are different types of fragment different types of gene in a library, but we need a very specific sequence or a very specific gene to be isolated from that pool of different types of fragment.

So, we need to screen that gene library in order to isolate our targeted or desired fragment. So, there are multiple methods by which we can isolate those targeted sequence. Colony hybridization technique techniques which exploits PCR based mechanism also there is hybrid select translation mechanism or hybrid arrest translation mechanism. So, what are those? Colony hybridization technique here what is done all those fragment or all those clones are basically allowed to form colony over plate. So, over the basically this these are the agar plates and why we are saying colony because those clones are basically present over different organisms like E.

coli and they are forming the colony. Now this colony is so, we have a plate where we are forming the colony and this plate is replicated over another plate having a nylon membrane over it. Now what happens over this agar plate when this nylon membrane is spread the nutrients is dispersed through the nylon membrane over it. So, the bacteria

can grow over it can form colony after that those colony are lysed. So, here you can see over the nylon membrane we are having the colony.

So, this is our original plate and that original plate is replicated over the nylon membrane. Now this nylon membrane colony are basically lysed and the containing DNA is denatured and liberated over the membrane. Now the membrane is having different type of DNA fragment in different regions. So, if this is the plate this is our colony A colony B colony C or colony D. So, all these colonies are having different types of gene because they are different colony.

Now what we do we need to identify our target desired product by hybridization method. So, we use labeled gene probes which is complementary to our targeted target or desired gene sequence. So, before that what we need to do we need to block the non specific site where the where the probe can bind. So, how this non specific sites are blocked this is blocked by pre hybridization mix. The membrane is incubated with pre hybridization mix, but that pre hybridization mix is it contains different non specific DNA non labeled non specific DNA which blocks the non specific open sites where the probe cannot bind.

Now after this pre hybridization mix we allow to hybridize the label gene probe to the target. So, suppose we are using a probe which can only identify D. So, our targeted labeled probe will only bind in this region. Now the other parts are washed the non bound products are washed off then after that by methods of auto radiography because the probes are already labeled by the method of auto radiography we can detect in which region the gene probe is bound. So, here we will get the positive signal in this region fine.

So, in the nylon membrane in this region we have our targeted colony or targeted sequence. So, what we will do we will compare this pattern to our master plate. So, if this is D suppose if this is D. So, our master plate is basically here. So, if we isolate colonies from this region we will get our targeted probe.

So, this is how colony hybridization by based on colony hybridization technique we can identify a specific target or specific gene sequence from the library. Now pluck hybridization and is another similar method where we can identify the desired gene in bacteriophage vectors only thing why it is called pluck instead of colony, colony formed by bacteria. So, here bacteriophage are forming plucks. So, that is why the process is same, but instead of colony pluck is formed by the bacteriophage vectors. So, this is how by hybridization method we can identify the target gene in the gene library.

That gene sequence can be identified by different PCR based method where we can used

primers, primers which can identify vector that is the easier one. Primers which can identify that specific DNA fragment that recombinant DNA, but for that we need to have a very clear idea of what that specific DNA sequence is. Now after the PCR based amplification of the vector suppose we are using the primers against the vector. So, if we suppose if we have amplified both of them a vector which does not contain the specific DNA sequence along with the vector which contains the specific DNA sequence what will be the effect? The see this is the non recombinant vector where the target DNA is absent here the target DNA is present. So, of course, the molecular weight will be higher here.

So, if we run it over one agarose gel electrophoresis the higher segment will be our targeted sequence. Here in case of PCR based method what is the advantage? Definitely it is a very rapid method it can be done in hours where the hybridization technique needs 2 to 3 days. Also the size of the clone insert can be assessed instead of the sequence. In case of hybridization we need to identify the sequence whereas, in case of PCR based screening we can identify the size of the cloned insert. Next we have another method hybrid select translation or hybrid arrest translation.

So, let us see what is there? So, hybrid select translation or hybrid arrest translation these methods are basically used when there is a mixed cDNA library. So, there are multiple types of cDNA in a library from that we need to identify our specific targeted cDNA. So, what is done? First the plasmid vector is extracted from each colony then each preparation each colony is denatured on denaturation what will happen? The DNA will come out from the cell and those are immobilized over the nylon membrane. So, the DNA segment DNHR DNA segment is bound over nylon membrane. Now these nylon membrane are soaked in total cellular mRNA.

What will happen? Each membrane will bind to one specific species of mRNA because the nylon membrane though they all the different colonies are there remember suppose this is the different plasmid colony which are denatured. Now in different nylon membrane one very specific sequence will be present one very specific cDNA will be present. Now even if that is treated with the total cellular mRNA it will bind to it is specific complementary sequence all other sequences will be unbound and those unbound mRNA will be washed off from the membrane. So, suppose amongst these 4 nylon membrane only this membrane is bound by the mRNA. So, what will happen? We can elute this bound mRNA then we can use the direct in vitro translation.

Now in direct in vitro translation what happens that eluted mRNA is allowed to go for translation by which the final targeted protein is formed. Now we have now what we will do we will isolate that target protein via either immunoprecipitation or electrophoresis then from that protein we will identify the mRNA which is coding the protein then if we

do a back calculation. So, we have identified the protein we have identified it is mRNA then we will identify from which membrane we have isolated it. So, that is the segment that membrane or that colony rather is containing our targeted sequence. So, that is hybrid select translation where one protein is selectively expressed whereas, in case of hybrid arrest translation what happened the positivity is shown by absence of a particular translation product.

For that what is done the total mRNA is hybridized with excess cDNA. Now what happened mRNA cannot be translated if it is hybridized to other molecule. So, what will happen the target protein will be absent or non translated in the when it is targeted for in vitro translation. So, this absence of the target protein will direct in the pool of the expressed protein when we will find that this protein is absent. So, the corroborative mRNA and their corroborative cDNA can be identified.

So, this is the hybrid select or hybrid arrest translation method which is utilized for identification of the target sequence in the gene library. So, all these are method for screening of our desired sequence in a gene library. So, for coming to the summary we have discussed the genomic library and cDNA library. Genomic library is collection of the total chromosomal DNA whereas, cDNA library is basically representing the mRNA of a particular cell at a specific point of time. We have discussed how this genetic genomic library and cDNA library are formed.

Genomic library is formed by the complete and partial digestion of restriction endonuclease which can give rise to fragments of DNA having different length. Whereas, cDNA can be synthesized by forming cDNA via reverse transcriptase enzyme. Both of this pool of genomic DNA or cDNA are inserted in the vector they are cloned and form the library. And via the method of colony hybridization technique PCR based screening or hybrid select arrest translation we can identify that specific desired sequence on requirement from the genomic or cDNA library.

So, this is all about recombinant DNA technology. These are my references see you in the next class. Thank you.