Comprehensive Molecular Diagnostics and Advanced Gene Expression Analysis

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Week 01

Lecture 05 : Nucleic Acid Isolation and Detection Methods

Namaskar. Welcome to the NPTEL lecture series, Comprehensive Molecular Diagnostics and Advanced Gene Expression Analysis. So, we are at the very first week of our lecture series, where we are discussing different essential fundamentals of molecular biology and molecular diagnostics. Today, after the completion of all the basic molecular biology phenomena, which are occurring in our body like replication, transcription, translation that is DNA synthesis from the parent DNA, then mRNA or other RNA synthesis and the protein synthesis from RNA. Today, in this lecture class, we are going to learn about how the nucleic acids DNA, RNA they are isolated from the cells and how they are detected. So, for today's class, these are the broad topics we are going to cover that they are a different methods of DNA isolation, different methods of RNA isolation and then the different detecting techniques for DNA and RNA.

So, what exactly nucleic acid isolation? It is basically extracting the DNA and RNA, which are located within the cell. So, we need to isolate them and have to get the pure form of DNA or RNA, which are free from contamination from other constituents of the cell. Like if we are trying to isolate DNA that should be free from the proteins of the cell, proteinaceous debris from the cell as well as free from RNA as well. So, we need the pure DNA on or RNA and those are used for PCR sequencing for so, the purity is very much critical.

Now, the first isolation of DNA was done by Friedrich Maschner in 1869. The basic principle by which the DNA is I so, DNA or nucleic acid they are isolated is basically we need to get them out from the cell. So, for that we need to lyse the cell or break open the cell. So, the first step is cell lysis, where we are disrupting the cell membrane and releasing the DNA within the cell and how that cell lysis is done. There are different methods starting from grinding or sonication technique or using some lysis buffer, which actually dissolve or destroy the cellular boundaries like membrane or cell walls like that.

So, there is one extraction buffer for that, which actually dissolve the cell membrane

also it helps in removal of the other contaminants. Now, for the membrane lipid removal we use detergent in that extraction buffer also in the cell lysis buffer they are mostly non ionic detergents like salt EDTA they are helpful. Now, EDTA remember EDTA is one very important chelating agent, which chelates the divalent ions. Now, this amongst this divalent cations, magnesium is one such important divalent cation, which is the cofactor for the enzymes like DNases. So, this DNase because due to the application of the EDTA as magnesium is chelated DNase cannot act and thus the it imparts protection over the DNA.

Next step is to precipitate the other contaminants apart from the DNA for that the protein and other contaminates should be removed by precipitation also here proteinase, which breaks down the proteins can be added then mostly we use proteinase in most of the extraction buffers. Then DNA itself is precipitated by alcohol alcohol, which is the chilled alcohol and it forms a pellet at the very bottom of the solution. So, just like here we precipitate the DNA and the pellet we isolate rather collect this pellet for the pure DNA and what remains here are the other contaminants. So, if we isolate the DNA this is the pure DNA, but further purification can be done by centrifugation as well as different column based techniques. Then what we need to do we also need to remove the liquid for that we need for this helps in concentrating the DNA in the isolated solution for that vacuum centrifugation or lyophilization these are the important steps, which removes the remaining liquid.

So, this is the basic principle starting from breaking up the cell then clearing or removing all other contaminants after that for isolation of the DNA itself is precipitated by a ethanol extraction this alcohol based pellet extraction method is some we commonly called ethanol extraction after that that extracted DNA is purified and concentrated. So, this is the basic principle for DNA or nucleic acid isolation. Now, there are different types of extraction method starting those are mostly two types broadly categorized under these two techniques one is chemical based method another is physical method. Now, in chemical based method definitely there are different types of organic or inorganic chemicals are used in organic extraction method the very common one is phenyl chloroform method, which we are going to discuss in details. Then inorganic extraction method there is protein SKA method, salting out method also solid phase extraction method like silica based method.

Then in physical method we have magnetic bead based extraction method also paper based extraction method. So, let us go one by one. So, phenyl chloroform method remember this is a very important very commonly used method and the principle is based on liquid liquid extraction, which follows the differential solubilities in different liquid hydrophilic or hydroscopic water or different types of in different types of liquids different components of the cell is basically solubilize. So, based on this differential

solubilities liquid liquid extraction method is followed in case of nucleic acid isolation. Now, in DNA isolation what happens the aqueous sample that is the isolated DNA.

So, sorry the I mean the extraction buffer, which contains everything in fact. So, when the cell is lysed we get everything in the extraction buffer. Now what we do this aqueous sample is mixed with equal volume of phenyl and chloroform mixture. So, after centrifugation what happens there are two distinct phases which are formed. So, if we do the centrifugation we get one aqueous layer, then there is interface of this aqueous layer and the this chloro phenyl chloroform layer.

Now proteins and hydrophobic lipids they are dissolvable or soluble in hydrophilic medium. So, they will remain in the above section here whereas, nucleic acid they accumulate here . So, during the isolation of DNA the cellular extract which is present in aqueous medium. So, this is the basically one aqueous sample it is mixed in equal volume equal volume of phenyl and chloroform. Now after centrifugation we do a centrifugation in that aqueous sample and phenyl chloroform mixture after the centrifugation two distinct phases are formed.

So, if this is our centrifugation tube there are two distinct phases based on the definitely the density. So, this is our aqueous phase and this is the hydrophobic medium or phenyl chloroform medium. Now proteins and hydrophobic lipids they remain in the lower organic phase here protein lipids and because the nucleic acids are soluble in the aqueous medium they remain in the upper layer. So, this is one differential solubility based separation method where we have separated the nucleic acid from other constituents, but remember in the upper column there are contaminants like different types of salt, sugars which are soluble in aqueous medium they are also present. Then what we need to do in we extract or we collect this upper portion and follow ethanol extraction.

In ethanol extraction we isolate the DNA and then we resuspend it in TE buffer TE is the Tries EDTA buffer where trees acts as the buffer and trees salt and EDTA I told you it helps in chelating the magnesium ion also it maintains the proper pH for the medium. So, this is how in phenyl chloroform method DNA is isolated. Coming to protein S K or salting out method protein S K remember protein S K is a protein digesting enzyme. So, it basically destroys the protein and when the sample is digested for 65 degree centigrade 2 hours the proteins are digested followed by centrifugation then we in. So, in centrifugation the proteins are cleared and from the clear fluid or the supernatant we get the DNA by again chilled alcohol precipitation or ethanol extraction.

Now this method is very rapid high yield technique, but the problem is the stability of the protein S K as well as the isolated DNA its quality is not up to the mark. Also salting out method where high concentration of salt sodium chloride are used which is used for

protein precipitation and DNA is present in the supernatant again it has to be isolated by ethanol extraction. Coming to solid phase extraction method and it is basically solid liquid phase extraction where silica it binds with the DNA during purification. Now basically this silica helps in extraction via chaotropic interaction. So, chaotropic interaction of the salts like guanidine hydrochloride it basically disrupts the cell deactivate the nucleases and also this nucleic acid binds with the silica due to chaotropic effect.

Now this life sample in the buffer solution along with ethanol or isopropanol they are transferred over a spin column and the column is centrifuged. What happens in the column there is a silica gel membrane upon which the DNA is bound at optimal pH and salt concentration. Then from that membrane we need to elute the elute out the DNA bound DNA by elution buffer. Again here no precipitation is required for the DNA or other techniques and it is very rapid and highly pure, but problem is this spin column or the silica column they are costly. Then coming to magnetic bead based method where reversible binding between the DNA and the magnetic beads occur because the magnetic beads are positively charged they are coated with positively charged molecules which helps in binding of the DNA which is actually negatively charged.

And that coating is basically antibodies or some functional groups which are specific for the for interacting with the DNA. So, along with charge the specific binding technique is utilized here. Now this bound DNA with the magnetic bead is separated using magnetic field purification is then done with ethanol extraction it is a rapid and simple method, but definitely once again it is a costly technique. Then coming to density gradient centrifugation is a cesium chloride gradient centrifugation. Now during the high speed centrifugation what happens in at the isopnicic point where the density of the DNA and the cesium chlorides gradient became same the DNA actually accumulated there.

So, a band will appear at the isopnicic point. So, if this is the cesium chloride solution suppose here the isopnicic point where the density of the because this is a gradient centrifugation remember the density of cesium chloride is constantly or rather. So, we are coming to density gradient centrifugation where a density gradient is formed by cesium chloride. So, here the solutions density is constantly ascending or descending. So, throughout the tube the density is not same rather it is constantly increasing or decreasing this is the density gradient.

So, at that level where the density of the DNA and the gradient cesium chloride gradient are same here the DNA will accumulate and that we will get a DNA band. Basically this experiment is developed by Meselson and Stoll and after the band appeared after we isolate the DNA cesium chloride has to be removed and that is removed via dialysis or by precipitation for extraction we used ethidium bromide. And finally, after constant or

repeated extraction with organic solvent this ethium bromide is removed from the isolated DNA. Next we are coming to RNA extraction. RNA can be extracted via different techniques starting from organic extraction where organic solvents are used one such common organic solvent is phenol guanidine isothiocyanate or GITC based solution.

So, triazole is one such reagent where acid guanidine phenol based reagent is used and how it is helpful this triazole reagent basically helps to inhibit the RNAs. Now remember RNA is very vulnerable to RNAs or other enzymes. So, RNAs must be inhibited for RNA extraction and it is very much sensitive to RNAs based distraction. So, this digestion RNAs based digestion has to be inhibited and that is achieved via triazole and also it helps in maintain the integrity of the RNA. This is one very common technique which is used, but the problem is there are chances of contamination.

Again spin column based isolation can be done for RNA via silica membrane, then paramagnetic particle technology is it can be used. Now for these two methods here no organic solvents are required it is a simple efficient and low cost method yields intact RNA with low level of contamination, but the problem is there is contamination of genomic DNA in these techniques. So, these are the different techniques of DNA and RNA isolation. Next we are coming to the quantification or the detection of the nucleic acid. Now using spectrophotometry UV spectrophotometry we can estimate or quantify nucleic acid.

So, if we take absorbance at 260 nanometer if the actually we take reading both at 260 nanometer and 280 nanometer. Now if the ratio is 1.8 or more, but less than 2. So, 1.

8 to 2 it indicates a pure DNA. If the ratio is less than 8 that means, if we are getting more readings from 280 nanometer it indicates that there is contamination with protein. So, actually in 280 we take the absorbance of proteins and in 260 we take the absorbance of nucleic acid. So, when there is presence of protein definitely the absorbance from 280 will be more. So, in that case the ratio will be lower than 1.

8. Now if it more than 2 no it is not the pure thing. So, if it is more than 2 there is contamination with the solvents that is chloroform or phenol. Now we are coming to quantification of this extracted or isolated nucleic acids. So, the quantification can be done by different methods starting from UV spectrophotometric based method or fluorometric based method. Spectrophotometry is one very easy technique where we need to take the reading at 2 different absorbance or wavelength.

Now for 260 nanometer this absorbance is for nucleic acids. Now with that we also take a reading at 280 nanometer and we take the ratio of 260 by 280. Now why such? If the

ratio is more than 8 point 1.8 it indicates the pure DNA.

If it is less than 1.8 that means, there is higher absorb absorbance coming from 280 nanometer for that the ratio is lower than 1.8. So, definitely there is presence of protein or other substances which are giving rise to absorbance at 280 nanometer. What if the ratio is more than 2? Is it the purest one? No. So, there is contamination once again if the ratio is more than 2.

So, it is it indicates that there is presence of solvents isolating solvents like chloroform or phenol when we extract it from phenol chloroform based techniques. Now a reading at 260 or an absorbance at 260 if it is 1 it indicates there is 50 nanogram per ml DNA presence of 50 nanogram per ml DNA. So, consider the absorbance is 5 at it actually not 5 suppose it the reading is sorry suppose the reading is 1.5. So, it indicates that the concentration of DNA in the sample is this fine like that.

Apart from the spectrophotometry or fluorimetric based techniques we can quantify the DNA from agarose joule electrophoresis and UV based detection is done after staining it with ethidium bromide. Now apart from these technique there is one another technique by which we can detect the presence of specific segment or sequence of DNA. So, we are coming to blotting techniques where DNA RNA or proteins are transferred onto a carrier which is a membrane and subsequently detected over this membrane. So, based on that there are 3 types of technique that is southern blotting for DNA detection, northern blotting for RNA detection and western blotting for protein detection. Today I am going to discuss the details of southern blotting only because that these techniques will other 2 techniques will be discussed when we will be discussing the different RNA based detection or diagnostic method or proteomics class.

So, in southern blotting what we detect is the DNA and it is named after Edwin Miller southern. Now because DNA blotting technique is named as over southern keeping the analogy with that RNA extraction RNA detection is named as northern also protein detection is detect named as western. So, the name of the scientist are not northern or western only because Edwin Miller southern has discovered this technique to keep the analogy other names are given. Now, what is done after the gel electrophoresis that electrophoresis sample separated DNA is transferred to a membrane from the gel electrophoresis gel and is detected by probe hybridization method. Now what is probe hybridization method remember this probe hybridization method is will become continuously discussed or the references will be done will be given in different molecular diagnostic method.

So, this probe hybridization technique is very important for detecting a specific segment or sequence of the nucleic acid. Now what exactly it is probe is basically nucleic acid

sequence sequence that is complementary to the sequence of DNA or sometimes RNA which we want to detect. So, it is a complementary nucleic acid strand. So, if suppose we are interested to detect whether in a DNA this sequence is present or not for that we need to design a probe which is complementary to this nucleic acid sequence.

So, for that our probe will be like this. So, if the sequence is present over the DNA the probe will be attached via hydrogen bonding to this sequence and will form the double helix. So, this is basically probe hybridization is basically sequence complementarity based technique. Now the probes are having some basic characteristics like it is 18 to 13 base pair long sequence is a specific sequence complementary to the to our target sequence. Now DNA probes can be single stranded which is synthesized from oligonucleotide molecule or can be double stranded which is isolated from biological sources. Now suppose there is binding of this probe over the sequence how we will know that for that we need to label.

So, this probe is labeled with different types of biomolecule that can be a radioactive biomolecule and non isotopic or non radioactive biomolecule. If it is radioactive biomolecule the commonest one is these isotopes. For non isotope labeling probes are tagged with such moieties that can be used to separate the probe or target hybrid or generate some detectable color or chemiluminescence under appropriate reaction condition. Let us see what are these things. So, this probes if that is radiolabeled or chemiluminescence level they can directly be detected via radio auto radiography or chemiluminescence based technique.

Now if there is indirect detection method what happens the probe is covalently linked with molecules like biotin streptavidin reaction or digoxygenin and its respective anti-biotic. So, what is that? So, consider this is our probe and this is the target sequence. So, if there is binding of the probe so, the loose probe will be in the solution. Now if we are able to bound this over the membrane only those probe attached to the target will be biotin whereas, the single unbound probe will be washed away. After that what we will do if the probe is tagged with biotin the biotin biotin isolated probe will be hybridized to the target sequence and then we will add streptavidin reporter conjugate.

So, streptavidin binds avidly with biotin and this streptavidin abit streptavidin biotin conjugate have high will give rise to some reporting signal that can be chemiluminescence that can be radioactive as well. Similarly, some reporter enzymes like if there is hot extra reddish peroxidase that is HRP or alkaline phosphatase these are the enzyme. Addition of these enzyme generate some chromogenic or chemiluminescence signal because they are made to react in such a way. So, after the unbound probes are washed away what remains in our hand is the bound DNA along with the probe along with the reporter molecule that gives rise to some signal which can

be a color visible color chromogenic substance or can be chemiluminescence signal. So, this is how probe hybridization technique is used to identify specific signal or specific sequence over nucleic acid.

Now, based on this technique southern blotting is done. So, what is done? The very first thing is to isolate the DNA then the DNA is digested with one very specific group of enzyme which is known as restriction endonucleases. So, restriction endonuclease cut the DNA in specific restriction site. So, there are multiple types of restriction endonuclease they have their own specific restriction site. Regarding this we are going to discuss a lot in our next few classes of gene editing etcetera, but for this class what is important that restriction endonuclease has some specific restriction site where it cut off the high molecular weight DNA strands into smaller fragments.

So, if this is our DNA strand that this is our restriction site. So, we are getting different fragments of the DNA. After that this sample which contains all this fragment they are electrophoresed in gel that can be a agarose gel. Then this fragments are separated based on their size in gel electrophoreses after that what we need to do we need to denature the fragments. So, these are converted into single strand remember double strand DNA do not bind with the probe definitely.

So, for that we need to open up the sequences or open up the strands. So, the unwinding or denaturation of the DNA is induced via alkali using sodium hydroxide. After that we need to transfer or blot the DNA fragments from the gel to different membranes membranes like nitrocellulose membrane or nylon membrane and over the membrane it needs to be immobilized. Now this immobilization can be done via baking at 80 degree centigrade for 2 hours that can be done via UV radiation as well. After that we need to apply the probe the complimentary probe which hybridize with it specific target sequence and after that if that is a radioactive probe it is can be detected by autoradiography.

If that is a fluorescent probe by fluorescence based technique it can be detected or chromogenic detection where the color can be generated over the membrane. So, based on this southern blotting technique we can identify a very specific sequence of a DNA whether the sequence is at all present in the DNA can be detected by probe hybridization method if it is DNA then it is southern blotting if it is RNA then it is northern blotting technique. So, what we have learnt from this class that DNA can be isolated via physical or chemical based method the common ones are phenyl chloroform method magnetic bead based techniques RNA can be isolated via GITC based solutions then for the detection of nucleic acid we can use different spectrophotometric fluorimetric technique also probe hybridization technique where we can detect a very specific segment of DNA these are my references. Thank you all and see you in the next class.