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THE PRINCIPLES OF ISOLATION, PURIFICATION AND ANALYSIS OF NUCLEIC ACIDS

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Introduction

Advanced Biotechnological research is largely depended on the genome analysis and recombinant DNA technology. Good quality nucleic acid is an essential prerequisite for consistent results in most of the down stream applications in the genome analysis and recombinant DNA technology. The general principle underlying the isolation of nucleic acids is common with few modifications depending on the type of nucleic acids being isolated. The type of the nucleic acid intending to isolate is to be made free from the other biological macromolecules and cell debris. This is achieved by properly lysing the cell wall or cell membrane as the case may be and by selectively denaturing the other macromolecules like proteins. Nucleic acids thus recovered in its native form are to be purified by removing the very closely associated molecules. The finely purified molecule is precipitated by alcohol and suspended in sterile buffer or distilled water. Finally, the qualitative integrity of the isolated nucleic acids is to be checked by agarose gel electrophoresis and ethidium bromide staining before proceeding with the further downstream applications. Quantitative estimation of nucleic acids are carried out by spectrophotometric and fluorimetric methods.

The types of nucleic acids usually isolated on a routine basis are:

1. Total genomic DNA
2. Total RNA
3. Plasmid DNA & Mitochondrial DNA

A) Total Genomic DNA

Breaking of the bacterial and plant cell walls as well as solubilising the cell membrane of animal tissue are to be carefully carried out under optimum conditions. Even the rapid stirring of solution can break high molecular weight DNA into shorter fragments. Vigorous shaking will cause nicks and even cut open the covalently closed circular structures of plasmid and mitochondrial DNA. If physical disruption is necessary as is the case with certain types of tissues, it should be kept to the minimum, and should involve cutting or squashing of cells, rather than the use of shear forces. Ultra sonic sounds are used to disrupt the tough cell wall of certain bacteria. Care has to be taken to prevent degradation of DNA by deoxyribonucleases. These enzymes are found in most cells, and may also be present in dust, which could contaminate laboratory glasswares. Hence all the glass wares, plastic wares and the homogenizing buffer are to be made sterile by autoclaving. Using

EDTA in buffers, which will chelate the Mg++ ions needed for DNase activity, can inhibit this enzyme activity. Cell disruption and most of the subsequent steps should be performed at 4°C.The cell wall could be lysed enzymatically as well. The enzyme Lysozyme usually lyses the bacterial cell wall. The cell membranes on the other hand are solubilised by including suitable detergent in the homogenizing buffer. Upon lysis the nucleic acids will be released into the cytoplasm and now the target molecule, DNA, is to be made free from RNA and other associated proteins. The RNA molecules can be selectively denatured by enzymatic treatment with RNase. Prior to its use, the RNase is to be heat treated to inactivate any DNase contaminants. RNase is relatively stable to heat as a result of its disulphide bonds, which ensure rapid renaturation of the molecule on cooling. The other major contaminant, protein, is removed by enzymatic treatment with proteinase K followed by shaking with water-saturated phenol or with phenol-chloroform mixture, either of which will denature proteins but not nucleic acids. Centrifugation of the emulsion formed by this mixing produces a lower, organic phase, separated from the upper, aqueous phase by an interface of denatured protein. It is advisable to use cut micro tips while proceeding through these steps. The aqueous solution is recovered and deproteinised repeatedly until no material is seen at the interface. Finally the deproteinised DNA preparation is mixed with two volumes of absolute ethanol, and allowed to precipitate out of solution in a freezer. After centrifugation, the DNA pellet is redissolved in a buffer containing EDTA for protection against DNases, and this solution can be stored at 4°C for at least a month. DNA solutions can be stored frozen, but repeated freezing and thawing tends to damage long molecules by shearing and hence the DNA preparations in frequent use are normally stored at 4°C.

B) Plasmid & Mitochondrial DNA

The principle of isolation of plasmid and mitochondrial DNA is based on the structural characteristics. Plasmids are double stranded, Covalently Closed Circular (CCC) or super coiled structures. Similarly mt. DNA is also having the same structural characteristics and hence almost the same isolation procedure can be adapted. Bacterial cell wall is to be broken by enzymatic treatment (lysozyme) in a suitable buffer with a suitable metallic chelator like EDTA before initiating the isolation process. The tissue for the mtDNA isolation is to be thoroughly homogenized under ice-cold conditions.

The classical method is to isolate the plasmid and mitochondrial DNA by Caesium chloride density gradient ultra centrifugation in the presence of ethidium bromide. Ethidium bromide causes unwinding of DNA as it binds to it, simultaneously producing a decrease in its buoyant density. Since the super coiled plasmid and mtDNA can unwind to only a very limited extent, it will not bind as much ethidium bromide as with the linear and open circle forms of DNA in the presence of saturating levels of ethidium bromide. Because of this density difference, plasmid and mt DNA can be separated from other DNA by ultra centrifugation.

Another method, which is relatively fast, is based on alkaline lysis. In this method the property of super coiled DNA to remain intact at pH between 12 and 12.5 is exploited for the isolation. At this pH selective denaturation of linear DNA will occur where as the super coiled DNA will remain intact. Further reduction of the pH to acidic condition will enhance the formation of a complex network of proteins and linear DNA and the resultant supernatant after centrifugation will contain the intact plasmid or mtDNA. This can be purified and precipitated as in DNA isolation procedures. For mitochondrial DNA, this method works well with fresh tissues with minimum nicks.

Mitochondrial DNA can also be isolated by differential centrifugation technique. This involves the selective isolation of the mitochondria, which is further lysed with suitable detergents to release the mtDNA. This will be further purified and precipitated by conventional means.

C) RNA

RNA molecules are relatively short, and there fore less affected by shearing. RNA is, however, very vulnerable to digestion by RNases which are present abundantly even on fingers. These enzymes are stable and generally require no co-factors. Hence gloves should be worn, and a strong detergent should be included in the isolation medium to denature any RNases immediately. The solutions used are to be treated with nuclease inhibitors like Diethyl pyrocarbonate (DEPC). Care should be taken while using DEPC, as it is a suspected carcinogen. Glasswares should be baked at 300°C for 4 to 5 hours, as autoclaving alone may not be sufficient to fully inactivate RNases. The plastic ware can be rinsed with chloroform. Tissue homogenization is to be carried out under ice-cold conditions with all the precautions detailed above. As in the case of DNA, RNA is to be made free from DNA and proteins. Proteins are denatured by proteinase K treatment followed by phenol chloroform extraction. This is followed by the ethanol precipitation of RNA in the presence of sodium acetate or sodium chloride. The over night precipitated pellet is washed with 70% ethanol to remove the salts and finally dissolved in DEPC treated water. Contaminating DNA can be removed by treatment with RNases free DNase. The RNase can be inactivated by RNAsin or vanadylribonucleoside complex.

Commercially available kits

Several readymade kits are available commercially and many laboratories are depending on such products for the isolation of nucleic acids. In most of these kits, the nucleic acids are either trapped by ultra filtration membranes or allowed to bind with certain resins, which have affinity towards nucleic acids. The advantage with these kits is that the process is very fast and devoid of using corrosive organic chemicals like phenol. The main disadvantage is that they are quite expensive and hence unaffordable to many laboratories. Hence it is advisable to use alternative non organic protocols, for DNA isolation, based on the use of high concentration of salts for removing proteins in place of phenol, which are

easy to perform in the laboratories especially while isolating from liquid connective tissues like blood, haemolypmph etc. Meanwhile, the commercial kits are effectively used for the isolation of total RNA and mRNA, as the manual isolation is a sensitive process with increased chances of degradation.

Quantitative Estimation of nucleic acids

DNA and RNA can be spectrophotometrically estimated by taking optical density (OD) at 260nm, 1O.D corresponds to 50 micro gram of DNA and 40 micro gram of RNA. Purity of the DNA can also checked spectrophotometrically by taking O.D at 260 &280nms. The ratio of 260 and 280 will result a value of 1.8 with pure nucleic acid preparations.

Suggested readings

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