CH 2. PRINCIPLES OF NUCLEIC ACIDS EXTRACTION

I. INTRODUCTION

DNA and RNA isolation are essential techniques in molecular biology since they are the first steps in the analysis of genome structure and gene expression and function. Nearly all molecular biology techniques and approaches start with extraction of nucleic acids. The quantity, quality and integrity of DNA and RNA will directly affect the results of the investigation.

This chapter is devoted to description of the principles of nucleic acid extraction not the protocols which are so diverse. In fact, among the multitude of extraction methods, there is no a perfect one since each method has advantages and disadvantages. Choice of the method depends on which DNA or RNA we want (genomic, plasmid, mitochondrial or chloroplast DNA, total RNA, mRNA, nuclear RNA,…) as well as on the required quantity and quality in terms of degradation and contamination.

An effective extraction method should yield a nucleic acid without major contaminants. Moreover, it should be efficient and nonselective; that is most of the cellular nucleic acids, and all its species, should be isolated and purified with equal efficiency. The method should not physically or chemically alter the purified molecules neither break them. In addition, the extraction method should be relatively fast and simple enough since it is just the beginning of the experiment and not an end in itself.

II. DNA EXTRACTION

DNA extraction is the separation of DNA from all other components and prepare a homogeneous DNA solution. DNA constitutes a small percentage (about 1%) of cell components and is usually enveloped by membranes (except nucleoid of prokaryotes). In eukaryotic cells, the bulk of DNA (~90%) is localized in the nucleus assembled with an equal mass of proteins. In viruses and bacteriophages, DNA is encapsulated by a protein coat and represent 30 to 50% of the total mass.

There is no difficulty in separating DNA from small molecules (amino acids, glucose, metabolites,…) since it has a large molecular weight. Consequently, the main cellular components that may present difficulty to be removed during DNA purification are proteins and RNA.

There are several methods for isolation of DNA which all involve four essential steps: cell breakage, removal of protein and RNA, concentration of DNA and quantitative and qualitative analysis of the sample.

1. Cell lysis

Cell breakage is one of the most important steps in the isolation of DNA and may be achieved chemically, mechanically and enzymatically. The mechanical means of cell breaking, such as sonication, grinding, blending or high pressure cannot be used for DNA preparations since they apply strong forces that shear DNA into small fragments. The best cell lysis procedures to obtain intact DNA are chemical (detergents) and/or enzymatic.

Since the products released by cell lysis could change pH, lysis (chemical and enzymatic) is performed in a buffer (e.g. Tris) in order to keep pH near the neutral value (between 6 to 9 according to constraints of the experiment). A low pH of the lysate causes DNA depurination and causes DNA to pass into phenol phase during deproteinization and high pH (>12) results in DNA denaturation. Moreover, lysis buffer should include DNase inhibitors such as ethylene-diamine-tetra-acetic acid (EDTA), and detergents. EDTA is a Mg^{2+} chelator (bivalent cations chelator in general) and a powerful inhibitor of DNases since most cellular DNases require Mg^{2+} as cofactor in order to be active. In addition, the presence of EDTA in extraction buffers inhibits Mg^{2+} ion-induced aggregation of nucleic acids to each other and to proteins. Moreover, EDTA destabilizes proteins and other cell structures that require calcium ions thereby facilitating cell lysis.

Along with EDTA, addition of detergents is necessary for lysis since they solubilize lipids[1] of membranes and inhibits all cellular DNases. In addition, many detergents cause proteins denaturation, thereby facilitating their removal later on. The lysis of animal cells is usually performed using anionic detergents such as sodium or lithium deodecyl sulfate (SDS or LDS, anionic detergents), sodium deodecyl sarcosinate (Sarcosyl), sodium 4-aminosalicylate, sodium tri-

[1] Lipids are easily removed during the phenol/chloroform extraction.

isopropylnaphthalene sulfonate or hexadecyltrimethyl ammonium bromide (CTAB, cationic detergent).

Use of other non detergent denaturing agents (e.g. guanidium thiocyanate, β-mercaptoethanol which denature protein) in the lysing buffer is common. Ionic strength of lysis solution is usually maintained by the addition of NaCl to preserve DNA structure (hydrogen bonds are broken in a medium with low ionic force).

Breakage of the tough plant cell wall (and bacterial cell wall) requires application of vigorous physical force that can shear large DNA molecules. Therefore, plant cells are first treated with enzymes that make the cell membrane accessible to detergents. For instance, before application of detergents, bacterial cells are treated with lysozyme. As to plant cell walls, they can be removed by treatment with xanthogenates[2] or by enzymes that partially or totally degrade cellulose.

2. Protein removal

Removal of proteins from DNA solution, known as deproteinization, depends on the differences in physical properties between nucleic acids and proteins. These differences are in terms of solubility in organic solvents, of partial specific volume (related to density) and of sensitivity to digestive enzymes.

a. Extraction with organic solvents

Nucleic acids are hydrophilic molecules and are easily soluble in water whereas proteins, contain many hydrophobic residues making them partially soluble in organic solvents such as phenol or chloroform. When an aqueous protein solution is mixed with an equal volume of phenol, the phenol molecules which are extremely hydrophobic, tend to be more soluble in the hydrophobic cores of the protein than in water. As a result, phenol molecules diffuse into the core of the protein, causing the protein to swell and eventually to denature. The denatured protein, with its hydrophobic groups exposed and surrounded by micelles of phenol, are far more soluble in the phenol phase than in the aqueous phase. Therefore, most of the proteins are partitioned into the phenol phase or precipitated on the interphase with water where concentration gets very high. Nucleic acids do not have strong hydrophobic groups and are insoluble in phenol.

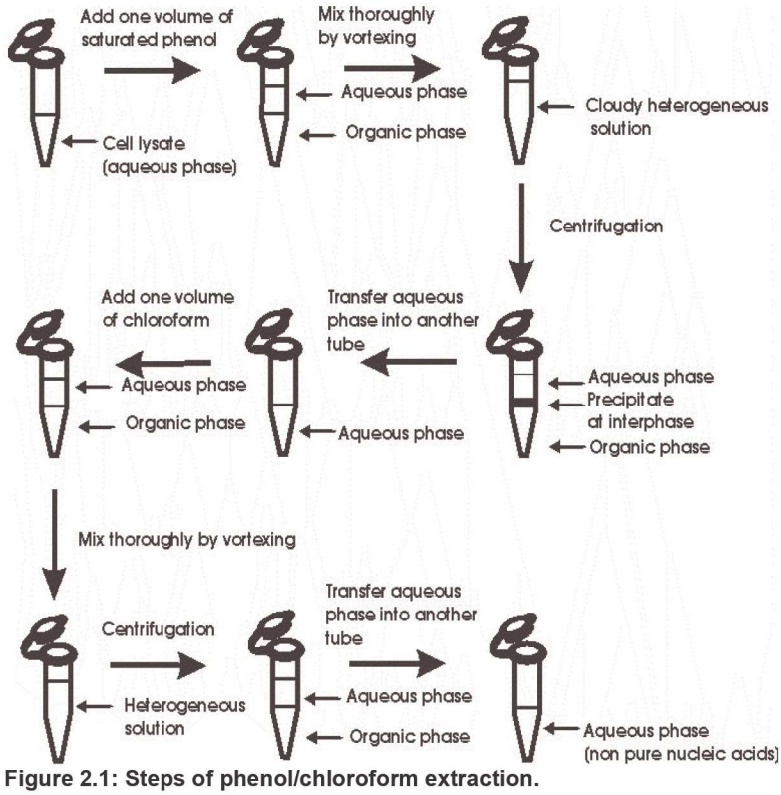

Figure 2.1: Steps of phenol/chloroform extraction.

In addition, ionic detergents unfold proteins thereby helping to expose hydrophobic regions of the polypeptide to phenol micelles which aids partitioning of proteins into the phenol phase. Similarly to phenol, chloroform is not miscible with water and its deproteinization action is based on the ability of denatured polypeptide chains to partially enter or be immobilized at the water-chloroform interphase. The resulting high concentration of protein at the interphase causes protein to precipitate.

In most cases phenol extraction is followed by chloroform extraction in order to remove phenol traces of from the aqueous phase. The remaining traces of chloroform are easily removed because it is highly volatile. Note that phenol and chloroform extractions remove all the lipids which are more

[2] Xanthogenates are used by paper industry for their ability to solubilize polyhydric alcohols such as cellulose.

soluble in the organic phase. The technical steps of liquid-liquid phenol-chloroform extraction are depicted in Figure 2.1.

b. Other deproteinization methods

- "complex formation" which is based on the property of some compounds or detergents to form insoluble complexes with proteins or DNA or RNA in a selective manner. The formed water-insoluble complexes are removed by centrifugation.

- DNA binding to silica surface whereas proteins, due to the predominance of hydrophobic characteristics in high salt, will not. The retained DNA is eluted from the glass beads by lowering salt concentration. This simple method is widely used in kits.

- isopycnic (density gradient) sedimentation due to the difference of partial specific volume[3] (proteins : 0.70 to 0.73 cm^3/g; DNA : 0.55 cm^3/g).

- enzymatic treatment due to the specificity of action.

3. Removal of RNA

Removal of RNA from DNA preparations is usually carried out using an enzymatic procedure (RNase treatment) although it can be performed by isopycnic sedimentation and by mild alkaline treatment. Two widely used ribonucleases, RNase A and RNase T1.

RNase A, isolated from bovine pancreas, is an endoribonuclease that cleaves RNA after Cp and Up residues. The reaction generates oligonucleotides ending with 3'P pyrimidine nucleotide. The enzyme is active under an extraordinarily wide range of experimental conditions, such as in the presence of detergent, at high temperatures, in even in the presence of phenol residues etc., but it is very difficult to inactivate. At NaCl concentrations below 0.3 M, the enzyme can cleave both ss and dsRNA, but at high salt concentrations (above 0.3 M) the enzyme digests only ssRNA.

RNase T1, isolated from Aspergillus oryzea, is an endoribonuclease that is very similar to RNase A in its reaction conditions but it cuts after Gp residues, generating oligonucleotides ending in a 3'P guanosine nucleotide. At high NaCl concentration, as with RNase A, the enzyme is active only on ssRNA.

4. Concentrating the nucleic acid sample

a. Selective precipitation of nucleic acids

After removal of proteins and lipids, there are still small organic molecules and mineral salts contaminating the nucleic acid sample. Moreover, the sample is found in a large volume which must be reduced. This step of DNA purification procedures uses alcohol and salts and serves to concentrate DNA from deproteinized solutions and second, it removes small organic molecules such as free nucleotides, amino acids and other low-molecular-weight impurities remaining in the solution after cell breakage.

Two alcohols can be used for nucleic acid precipitation: ethanol and isopropanol. Alcohol precipitation is based on the phenomenon of decreasing solubility of nucleic acids in water. Polar water molecules surround nucleic acids molecules in aqueous solutions. The positively charged dipoles of water interact strongly with the negative charges on phosphate groups (and other polar groups) causing its solubilization. Ethanol which is miscible with water but far less polar, cannot interact with the polar groups of nucleic acids as strongly as water, making it a very poor solvent for nucleic acids causing them to precipitate. Addition of salt (usually sodium or ammonium salts) along with ethanol reduces water activity (in terms of solubility) thereby helping the precipitation of nucleic acids at only 70% ethanol (final concentration). Ethanol-salt precipitation is carried out at -20°C[4] or lower since low temperature, further decreases solubilizing activity of water.

Precipitation of DNA with isopropanol has all the characteristics of precipitation with ethanol. The advantage of isopropanol over ethanol-salt precipitation is that a much lower concentration (50%) of this alcohol is needed to precipitate DNA. However, at this concentration of isopropanol, and at room

[3] Density (g/cm^3) is the reciprocal value of partial specific volume. Density of protein in water is between 1.36 g/cm^3 to 1.42 g/cm^3, whereas density of DNA is about 1.818 g/cm^3.

[4] According to certain authors, precipitation can be performed at room temperature or at 4°C without serious loss of DNA even when DNA concentration in a sample is very low.

temperature, small molecular weight DNA fragments will precipitate poorly. A disadvantage in the use of isopropanol is that it is less volatile than ethanol.

Centrifugation of the sample after precipitation results in a pellet of solid nucleic acids which is washed with 70% ethanol in order to remove the salts[5] that may have precipitated. It is then dried and resuspended in an appropriate buffer.

b. Other concentrating techniques (also removal of small impurities)

- Dialysis which particularly used for isolation of very high molecular weight DNA (200 kb or more) to avoid shearing caused by alcohol precipitation and multiple pipeting.
- Ion-exchange chromatography.

5. Purification of plasmid DNA

The task of plasmid purification substantially differs from that of the preparation of genomic DNA since it should involve removal of proteins but also bacterial chromosomal DNA. Most plasmid DNA purification methods start from the preparation of a crude bacterial lysate and eventually employ the standard protein removal procedures already described above. To achieve separation of plasmid from chromosomal DNA, these methods exploit the structural differences between plasmid and chromosomal DNA. Plasmids are circular supercoiled dsDNA molecules substantially smaller than bacterial chromosomal DNA. There are many methods of plasmid preparation, among them alkaline lysis is widely used.

In the alkaline lysis method, cells are lysed by SDS and DNA denatured by NaOH. Neutralization of the solution results in a fast reannealing of the circular plasmid DNA due to the interconnection of both ssDNA circles. Much more complex bacterial chromosomal DNA cannot reanneal in this short time and forms a large DNA precipitate. Afterward, lowering the temperature results in precipitation of protein-SDS complexes. Subsequently both complexes, DNA and protein, are removed by centrifugation leaving non pure plasmid molecules in the supernatant. Plasmid sample is still contaminated with some proteins and RNA which are removed by the previously described methods.

III. Principles of RNA extraction and purification

Preparation of pure RNA is one of the central techniques in molecular biology and is an essential step in the analysis of patterns and mechanisms of gene expression by northern blotting, nuclease protection assays, RT-PCR, DNA array, cDNA library preparation and *in vitro* translation.

1. Principles of RNA extraction

A typical eukaryotic cell contains about 20 pg of RNA, most of which is localized in the cytoplasm, whereas a prokaryotic cell contains 1000 times less. About 80 to 85% of eukaryotic RNA is rRNA, 15 to 20% correspond to a variety of stable low molecular weight species (e.g. tRNA and snRNA) and about 1 to 3% of the cell's RNA is mRNA that is heterogeneous[6] in terms of size and primary structure. The presence of poly-A tail at their 3'end permits separation and isolation of mRNA from all other RNA classes present in the cell.

Various RNA purification techniques are in use and yield RNA sample of different quantity and quality. Choice of technique depends on the required RNA (quantity, quality and nature). For example, if RNA is going to be used for quantitative RT-PCR, intactness of the purified RNA is not critical, while intact RNA is required for cDNA library preparation or Northern blot analysis. Complete removal of DNA contamination is critical if RNA is to be used in RT-PCR but is not important *in vitro* translation.

The physical and chemical properties of RNA and DNA are very similar, thus, the basic procedures used in RNA purification are similar to those of DNA already described in this chapter. All of the RNA purification methods include the following steps: cell or tissue lysis, effective denaturation of nucleoprotein complexes and removal of proteins, removal of contaminating DNA, concentration of RNA molecules and determination of purity and integrity of isolated RNA.

In contrast to DNA purification, during RNA extraction physical forces during lysis are allowed since RNA are much smaller and much more flexible than DNA molecules. However, precautions should be taken to inactivate internal and external RNases which are omnipresent, very stable and

[5] And isopropanol if it is being used.
[6] Corresponding to the several thousands of expressed protein-encoding genes.

do not require cofactors to function. An irreversible inactivation of endogenous RNases and protection against contamination with exogenous RNase during the isolation procedure are necessary. So, all extraction buffers include powerful RNase inhibitors (e.g. diethyl pyrocarbonate, highly flammable and a suspected carcinogen, RNasin[7]) and all solutions and equipment used are treated to remove exogenous RNases. The most frequent sources of exogenous RNase contamination are manipulator's hands (gloves indispensable), and bacteria and fungi present on dust particles in the air.

The use of strong protein denaturation agents (e.g. guanidium hydrochloride, guanidium isothiocyanate combined with 2-mercapthoethanol[8]) during cell lysis quickly and irreversibly inactivate endogenous RNases and contribute to denaturation of nucleoprotein complexes.

2. Examples of techniques

A widely used method is a single step procedure that breaks cells and fragment DNA into small fragments (<10 kb). Lysis is performed in an acidic buffer (pH 5.5) containing 4M guanidine thiocyanate and high sarcosyl concentration, then phenol-chloroform is added. Under these conditions, RNA remains in the aqueous phase while DNA and proteins are distributed into the organic phase. So, centrifugation yields an aqueous phase containing RNA and other small molecules (e.g. monosaccharides, aa,…). Further purification of the sample and concentration are achieved by alcohol precipitation similarly to DNA.

Another technique, the high-salt lithium chloride method which involves cell breakage in low pH, high salt buffer in the presence of RNase inhibitors. Protein and DNA are removed by acidic phenol/chloroform extraction and RNA is recovered by lithium chloride precipitation. At 4M LiCl, large RNA molecules precipitate while remaining DNA and small molecular weight RNAs are kept in solution. A centrifugation yields an RNA pellet.

Note that DNA contamination may be avoided by performing soft lysis that destroys plasma membrane and keeps the nuclear envelope (eukaryotes) intact so that nuclei are removed by centrifugation. RNA extraction is carried out on the supernatant which corresponds to the cytoplasm. this approach is appropriate if one is not interested in nuclear RNA.

The most powerful technique to separate RNA from DNA after cell lysis in a denaturing medium, is ultracentrifugation on a CsCl gradient which yields two independent fractions since DNA and RNA have different densities regardless of their sizes. All the RNA are in the pellet at the bottom since it much denser than DNA which form bands near the middle of the tube.

Note: Commercially produced RNA isolation kits, using the principles presented here, provide a simple and fast method for preparation of total RNA from bacteria, cultured cells and tissues.

3. Extraction of mRNA

For certain applications, such as construction of cDNA libraries or analysis of low-abundance mRNAs[9], isolation of mRNA instead of total RNA is indispensable. Obviously, analyzing the population of mRNA is more likely to give clear results than analyzing of the total RNA population.

A vast majority of eukaryotic mRNAs are characterized by a 3'-poly-A tail which is ~200 nucleotides in higher eukaryotes and ~50 nucleotides in lower eukaryotes such yeast or green algae. This feature is used to separate mRNA from the remaining non-polyadenylated RNA in cells.

[7] RNasin is isolated from human placenta, it inhibits RNase by non-competitive binding. DEPC causes enzyme inactivation by irreversiblely denaturing proteins

[8] It reduces disulfide bridges.

[9] Gene whose transcription level is very low and its corresponding mRNA is very rare.

Poly-A mRNA are prepared in two steps: first, isolation of total RNA, followed by purification by affinity chromatography to select poly-A mRNA. The total RNA sample is passed through a column[10] filled with very small solid cellulose or latex beads that are coupled to oligo-dT or oligo-U. The poly-A tracks of mRNA are hybridized, at high salt concentration, to oligo-dT oligonucleotides which are covalently linked to the beads (Figure 2.2). Concentration of the buffer allows formation of hydrogen bonds between poly-A tails of mRNA and the oilgo-dT. Therefore, mRNA population is retained in the column and all of the other RNA are excluded (except those containing long stretch of poly A). After extensive washing with the same high salt solution to remove non specifically bound RNA, the retained mRNA are eluted from the solid phase with small volume of water or low-salt buffer (which disrupt hydrogen bonds). This procedure gives a 20 to 50-fold enrichment of mRNA. Depending on to the use of the sample, a second round of purification may be applied to get better enrichment.

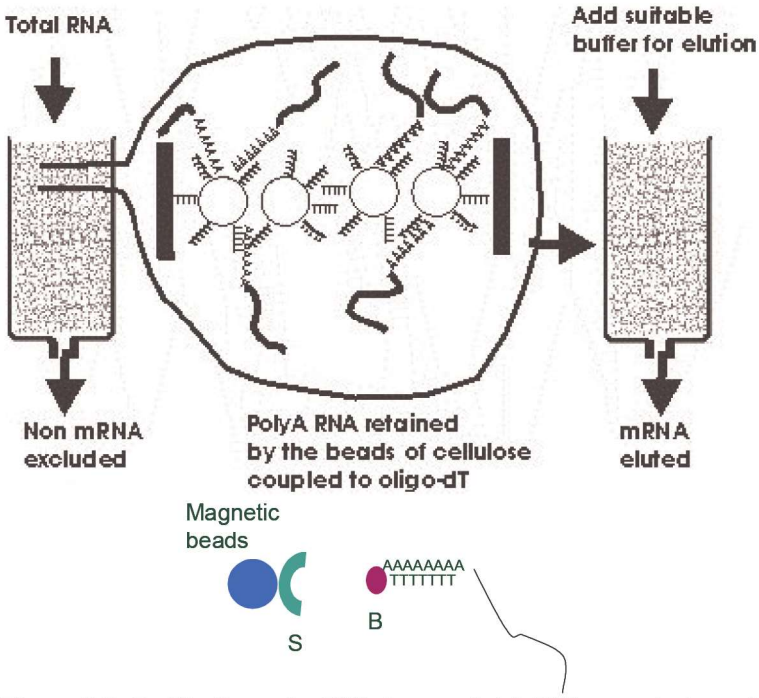

Figure 2.2: Purification of mRNA from a total RNA sample by gel filtration (affinity chromatography, top) on a column of cellulose beads coupled to oligo-dT. In the bottom, magnetic beads indirectly coupled with oligo-dT also serve the same goal.

In another approach, the oligo-dT is directly or indirectly coupled to magnetic beads that permit easy collection of bound mRNA. The tiny magnetic beads are coupled to streptavidin[11]. Biotin which is a ligand of streptavidin is coupled to oligo-dT. Biotin-oligo-dT and streptavidin-beads are added to a pure sample of total RNA in salt condition allowing annealing. In the solution, mRNA anneal with oligo-dT which are linked to biotin which binds to streptavidin coupled to the magnetic beads. A tiny metallic needle is introduced into the solution and takes out the mRNA population in a selective manner. After washing, separation of mRNA from the remaining components is performed by elution similarly to the previous technique. If elution uses large volume of water or buffer, the sample will be diluted. It is concentrated by alcohol precipitation or dialysis (as previously described).

Note that affinity chromatography has many other applications in molecular biology.

IV. Primary analyses of purified nucleic acids

After preparation of any nucleic acid sample, it must be controlled quantitatively and qualitatively before starting further experiments. In other words, one must determine concentration of the sample, its potential contamination with proteins and its intactness (degraded or not) before conducting specific analysis such as northern blotting, Southern blotting, PCR or RT-PCR,...

1. Optical density

UV spectrophotometry is commonly used for the determination of nucleic acid concentration and purity in terms of protein contamination. The resonance structures of pyrimidine and purines bases are responsible for UV absorption. DNA has a maximum and minimum absorbance at 260nm and 234nm (Figure 1.1), respectively. However these are strongly affected by the degree of base ionization and hence pH.

[10] The beads-oligo dT complexes can be added to total RNA sample and collected with hybridized mRNA by centrifugation. After elution, mRNA will be in the supernatant.

[11] A bacterial protein that strongly and specifically binds biotin.

Beer Lambert law correlates light absorption (optical density) with concentration according to the formula: $A_{260} = \varepsilon_{260}$ [C] x l where "A" is OD (absorbance) at 260 nm, "ε" is the extinction coefficient and "l" the path crossed by the light beam (constant = 1 cm). Extinction coefficient for dsDNA is usually taken as 0.02 $\mu g^{-1}xcm^{-1}$ when measured at neutral or slightly basic pH. Thus, an OD (absorbance) of 1 unit at 260nm corresponds to DNA concentration of 50 µg/ml (1/0.02 = 50 µg/ml). The extinction coefficient of ssDNA is 0.027 $\mu g^{-1}xcm^{-1}$ giving a ssDNA concentration of 37 µg/ml for an OD of 1 (1/0.027 = 37 µg/ml).

The linear relationship between absorbance at 260nm and DNA concentration holds in a range between 0.1 to 2 absorbance units. Reliable measurements of DNA concentration can made for solutions of 0.5 to 100 µg/ml using a standard UV spectrophotometer. Samples with an OD > 2 should be diluted before measuring. Measurement of DNA concentration at a lower range (A_{260} <0.2) can be strongly affected by light scattering on dust particles present in the preparation. Measuring the absorbance at 320nm should assess the degree of such contamination. At this wavelength, DNA does not absorb and any absorbance at 320nm is due to light scattering. The absorbance at 320nm should be less than 5% of the A_{260}.

Absorbance measurements at 280nm and determination of the A_{260}/A_{280} ratio is useful for determination of DNA purity in terms of protein contamination. In fact, proteins absorb maximally at 280nm due to the presence of tyrosine, phenylalanine and tryptophan. So, the ratio A_{260}/A_{280} for pure dsDNA was customarily taken to be between 1.8 (weak contamination) to 2 (pure). A ratio < 1.8 informs that the sample is significantly contaminated with proteins.

2. Electrophoresis

Quality of the sample in terms of degradation is determined by electrophoresis on an agarose gel[12]. When a molecule is placed in an electric field it migrates to the appropriate electrode with a velocity that depends on the potential difference between electrodes measured in volts (V, range between 2 to 15 V/cm[13]), the net charge of the molecule, the distance between electrodes (cm), viscosity of the solution (concentration of the gel and the size of its slots) and radius of the molecule (size). smaller molecules are able to penetrate the fine meshwork more easily than larger ones.

Near neutral pH, phosphate groups in the phospho-sugar backbone of DNA and RNA carry large number of negative charges. Therefore, nucleic acid molecules migrate toward the positive electrode (anode) when placed in an electric field during gel electrophoresis. Due to the repetitive nature of the phospho-sugar backbone, the net charge/mass ratio is approximately constant. Consequently, nucleic acid molecules move through the gel according to their sizes and shapes (conformation) but also according to dimension of the gel slots and the applied voltage. Smaller molecules move through the gel matrix more readily than larger molecules, so that molecules of different length migrate as distinct bands that are sorted according to size. Band thickness and intensity are proportional to the amount.

DNA shape (conformation) also influences migration rate. For instance, plasmid DNA can exist in three conformational states: closed circular supercoiled; circular relaxed and linear. Under most conditions, supercoiled DNA migrates faster than linear DNA of the same size since it is more compact. The relative mobility of the three forms is dependent primarily on agarose concentration and, to a lesser extent, on the strength of the current applied and the ionic strength of the buffer[14].

[12] Agarose gels are used for separation and purification of DNA fragments from 0.2 to 50kb in length.
[13] x V/cm, measured as distance between electrodes. Large molecules (>5kb) are run at low voltage for better resolution, small ones are run at high voltage to avoid diffusion.
[14] Circular DNA migrates faster than supercoiled DNA in TAE buffer (Tris-acetate EDTA) and slower than supercoiled DNA in TBE buffer (Tris-borate EDTA).

Nucleic acids are transparent and require staining to be seen. A common method for visualizing nucleic acid bands on a gel is to incubate the gel in an ethidium bromide (ETB, fluorescent dye) solution[15]. This planar molecule binds to DNA by intercalating between the base pairs. As a result, when the gel is illuminated with UV light (excitation at 305 nm) on a transilluminator, ETB emits orange light. The regions of the gel containing nucleic acids fluoresce much more brightly than the other regions of the gel.

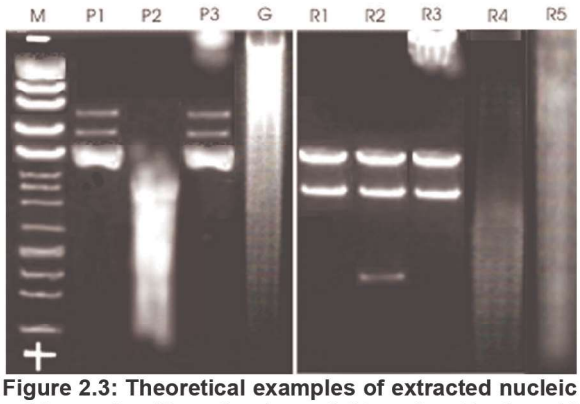

Figure 2.3: Theoretical examples of extracted nucleic acid samples. M: molecular weight marker; P: plasmid sample; R: RNA sample; G: genomic DNA sample. See text for interpretation.

Revelation of nucleic acids separated by gel electrophoresis may be accomplished by the use of radioisotopic markers (the sample is radiolabeled) and autoradiography. Moreover, non-isotopic visualization procedures include chemiluminescence and silver staining. Silver stains are 100-fold more sensitive than ETB for the detection of nucleic acids on gels. Detection of nucleic acids by silver staining depends on the reduction of silver ions to form metallic silver images. This use of a silver reduction reaction to create an image of the nucleic acid distribution in a gel depends on oxidation-reduction potential differences between the sites occupied by the nucleic acid molecules and the remaining sites in the gel.

Theoretical examples of extracted nucleic acid samples are depicted in Figure 2.3 where lane M refers to the molecular weight marker. Lane P1 refers to a perfect plasmid sample with supercoiled, linear and relaxed forms. Supercoiled form is more compact and therefore moves faster than the linear and relaxed forms, which move slowly although they have the same nucleotide content as the supercoiled form. P2 corresponds to a degraded sample of plasmid (or any DNA). P3 is contaminated with genomic DNA which is unable to migrate fast in the gel because of its large size. Therefore, it stays near the origin of migration. Determination of plasmid size is based on the position of the linear form in the gel. Lane G refers to a sample of genomic DNA whose size is large. It consists of a smear rather than a sharp band[16] since there are molecules of different sizes (random breaks occur along the DNA molecules during extraction).

Lane R1 is a perfect sample of total RNA. Only two bands are observed corresponding to the 18s (2.3 kb) and 28s (4.2 kb) ribosomal RNA (the most abundant in the cell). The 28s RNA band stains with approximately twice the intensity of 18s RNA. Occasionally, a diffuse band is visible near the front of migration and corresponds to the 5s (0.12 kb) and 5.8s (0.156 kb) RNA (R2). The other RNA classes are present and sorted according to their sizes but not visible because of their low amount (below sensitivity of ETB staining)[17]. Lane R3 corresponds to an RNA sample which is contaminated by genomic DNA (near the origin of migration). Lane R4 corresponds to degraded RNA sample. Lane R5 corresponds to mRNA sample which forms a smear of slight intensity.

[15] ETB may be added to the sample, to the gel, or to the migration buffer.
[16] Sharp bands are visible only if there are so many molecules of the same size, while a smear consists of a multitude of bands that slightly differ in terms of size.
[17] There must be at least 20 ng of nucleic acid of a particular size in order to be revealed by ETB staining on an agarose gel.