CHAPTER 1

Preparation of Human Genomic DNA

Introduction

The goal of this experiment is to isolate and purify a large quantity of high molecular weight human DNA. The source of the DNA will be your cheek cells obtained from a saline mouthwash (a bloodless and non-invasive procedure). You will also learn how to determine DNA concentration and purity. The isolated DNA will be used in several experiments that you will carry out later in the course. The procedure is a "hybrid" between the phenol and chloroform extraction methods, preceded by proteinase K digestion.

This experiment will be performed during two laboratory periods. The first period will include the procedure for cell collection and the initial steps of DNA purification. During the second laboratory period students will finish DNA isolation and determine DNA concentration and purity. Figure 1.1 presents a schematic outline of the experiment.

Background

DNA constitutes a small percent of the cell material and is usually localized in a defined part of the cell. In procaryotic cells DNA is highly condensed and localized in a structure called the nucleoid, which is not separated from the rest of the cell sap by a membrane. In eucaryotic cells the bulk of DNA is localized in the nucleus, which is separated from the rest of the cell sap by a complicated membrane structure. Usually approximately 90 percent of the DNA is localized in the nucleus (chromosomes); the rest can be separated into other organelles such as mitochondria or chloroplasts. In viruses and bacteriophages, DNA is encapsulated by the protein coat and constitutes between 30 and 50 percent of the total mass of the virus. The amount of DNA, as a percent of the total mass of cell material in procaryotes and eucaryotes, is much smaller than that of viruses and is less than 1 percent.

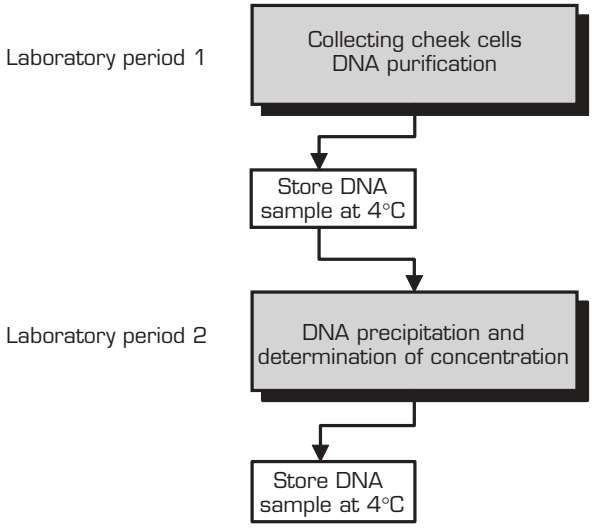


Figure 1.1 Schematic outline of the DNA isolation procedure.

Table 1.1 Composition of living cells

Component	**Percent of total cell weight**	
	***E. coli* cells**	**HeLa (human) cells**
Water	70.0	70.0
Inorganic ions	1.0	1.0
Amino acids	0.4	0.4
Nucleotides	0.4	0.4
Lipids	2.2	2.8
Proteins	15.0	22.3
RNA	6.0	1.7
DNA	1.0	0.85

For example, the approximate composition of rapidly dividing *Escherichia coli* cells and human cells (HeLa) is given in Table 1.1.

The goal of DNA purification is to separate DNA from all of the components of the cell listed in Table 1.1. There is no difficulty in separating DNA from small molecules since the molecular weight of DNA is very large. Thus, the components that constitute "major impurities" and should be removed are protein and RNA.

There are several methods of purification of DNA that exploit differences in the physical properties between DNA and proteins. All methods of purification involve five essential steps.

1. Cell breakage.
2. Removal of protein.
3. Removal of RNA.
4. Concentration of DNA.
5. Determination of the purity and quantity of DNA.

Cell Breakage

Cell breakage is one of the most important steps in the purification of DNA. The usual means of cell opening, such as sonication, grinding, blending, or high pressure, cannot be used in DNA purification. These procedures apply strong forces to open cells that shear DNA into small fragments. The best procedure for opening cells and obtaining intact DNA is through application of chemical (detergents) and/or enzymatic procedures. Detergents can solubilize lipids in cell membranes resulting in gentle cell lysis. In addition, detergents have an inhibitory effect on all cellular DNases and can denature proteins, thereby aiding in the removal of proteins from the solution. The lysis of animal cells is usually performed using anionic detergents such as SDS (sodium deodecyl sulfate) or Sarcosyl (sodium deodecyl sarcosinate).

Removal of Protein

The second step in purification involves removing a major contaminant, namely protein, from the cell lysate. This procedure is called deproteinization. Removal of proteins from the DNA solution depends on differences in the physical properties between nucleic acids and proteins. These differences are differences in solubility, differences in partial specific volume, and differences in sensitivity to digestive enzymes.

Deproteinization using organic solvents

The most frequently used methods for removing proteins explore the solubility differences between proteins and nucleic acids in organic solvents. Nucleic acids are predominantly hydrophilic molecules and are easily soluble in water. Proteins, on the other hand, contain many hydrophobic residues making them partially soluble in organic solvents. There are several methods of deproteinization based on this difference and they vary by the choice of the organic solvent.

The organic solvents commonly used are phenol and chloroform containing 1 percent isoamyl alcohol. The method that uses phenol as the

deproteinizing agent was introduced by Kirby (1957) and is usually referred to as the **Kirby method**. Use of chloroform isoamyl alcohol mixtures was introduced by Marmur (1961) and is named the **Marmur method**. These methods have undergone many modifications and improvements from the time of their first publications so they bare little resemblance to the original descriptions.

The application of phenol in the Kirby method is based on the following principle. Phenol is crystalline at room temperature, but in the presence of 20 percent water it forms an aqueous suspension containing phenol micelles surrounded by water molecules. Protein molecules generally contain many hydrophobic residues, which are concentrated in the center of the molecule. When an aqueous protein solution is mixed with an equal volume of phenol, some phenol molecules are dissolved in the aqueous phase (approximately 20 percent water and 80 percent phenol). Yet the phenol molecules are extremely hydrophobic. Consequently, they 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 unfold or denature. The denatured protein, with its hydrophobic groups exposed and surrounded by micelles of phenol, is far more soluble in the phenol phase than in the aqueous phase. As a result proteins are partitioned into the phenol phase leaving the nucleic acids in the aqueous phase. Nucleic acids do not have hydrophobic groups at all and are insoluble in the phenol phase.

Application of the phenol method does require mixing the phenol phase with the water phase. This introduces some shearing of DNA molecules. Since only relatively small amounts of protein can dissolve in a given volume of phenol, repeated extraction of the aqueous phase with phenol is required in order to remove all the protein. Because the phenol phase at saturation contains 20 percent water every phenol extraction will remove 20 percent of the DNA into the phenol phase. Even more DNA is lost by entrapment in the interphase layer of precipitated proteins or when the pH of phenol drops below pH 8.0.

Another drawback of the Kirby method is that the oxidation products of phenol can react chemically with DNA (and RNA) molecules. In addition, phenol is highly toxic and requires special disposal procedures.

In order to minimize these effects, several modifications have been introduced.

1. The use of ionic detergents. These detergents, by unfolding the protein, help to expose hydrophobic regions of the polypeptide chains to phenol micelles, thereby aiding partitioning of proteins into the phenol phase.

2. Enzymatic removal of proteins before phenol extraction. This reduces the number of extractions needed, thus limiting the loss and shearing of DNA.

3. Addition of 8HQ (8-hydroxyquinoline) to the phenol. This increases the solubility of phenol in water. In the presence of this compound phenol remains liquefied at room temperature with only 5 percent water. In addition,

8HQ is easily oxidized and, therefore, it plays the role of an anti-oxidant, protecting phenol against oxidation. Since the reduced form of 8HQ is yellow and the oxidized form is colorless, the presence or absence of yellow color is an excellent visual indicator of the oxidation state of phenol.

4. Removal of oxidation products from phenol and prevention of oxidation upon storage or during phenol extraction. Because water-saturated phenol undergoes oxidation rather easily, particularly in the presence of buffers such as Tris, phenol used for DNA purification is twice distilled, equilibrated with water, and stored in the presence of 0.1 percent 8HQ.

5. Adjusting the pH of water-saturated phenol solution to above pH 8 by equilibration of the liquefied phenol with a strong buffer or sodium borate.

DNA obtained by the Kirby method is usually of high molecular weight, but contains approximately 0.5 percent protein impurities that can be removed by another method.

The application of a chloroform : isoamyl alcohol (CIA) mixture in the Marmur deproteinization method is based on a characteristic of this organic solvent that differs from phenol. The chloroform is not miscible with water and, therefore, even numerous extractions do not result in DNA loss into the organic phase. The deproteinization action of chloroform 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. Since the deproteinization action of chloroform occurs at the chloroform–water interphase, efficient deproteinization depends on the formation of a large interphase area. To achieve this, one has to form an emulsion of water and chloroform. Since chloroform does not mix with water this can only be done by vigorous shaking. An emulsifier, isoamyl alcohol, is added to chloroform to help form the emulsion and to increase the water–chloroform surface area.

The Marmur method is very efficient in the recovery of DNA, but it requires repeated time-consuming extractions when large amounts of protein are present. In addition, chloroform extractions require rather vigorous mixing that contributes to hydrodynamic shearing of large DNA molecules. Using this method, it is possible to obtain very pure DNA, but of limited size (20,000–50,000 bp). The method is useful for the preparation of DNA from viruses with small genomes or when DNA of low molecular weight is sufficient for experiments (e.g. a polymerase chain reaction or PCR).

A substantial improvement in the method can be accomplished by limiting the number of extractions. This saves time and limits DNA shearing. This can be done by enzymatically removing most of the protein before extraction. Another modification frequently used is combining phenol and chloroform extraction into one step.

The efficient use of the Kirby and Marmur methods of deproteinization of DNA requires prior enzymatic digestion of protein. These methods can only be used without this preliminary step when small amounts of protein contaminate DNA solutions.

Deproteinization using enzymes

Proteins can be removed from DNA preparations using a protease that can digest all proteins, i.e. a general-purpose protease. Two such enzymes are in use, proteinase K and pronase. Both enzymes are very stable, general specificity proteases that are secreted by fungi. Commercial preparations of these enzymes are inexpensive and devoid of DNase contamination, making them safe to use in the purification of nucleic acids. These proteases are active in the presence of low concentrations of anionic detergent, high concentrations of salts, and EDTA and exhibit broad pH (6.0–10.0) and temperature (50–67°C) optima. They can digest intact (globular) and denatured (polypeptide chain) proteins and do not require any co-factors for their activities. Proteinase K and pronase are usually used in DNA purification procedures at final concentrations of 0.1–0.8 mg ml^{-1}. The difference between these two enzymes lies in their activities towards self; pronase is a self-digesting enzyme, whereas proteinase K is not. The fact that proteinase K is not a self-digesting enzyme makes it a more convenient enzyme to use than pronase, because it is unnecessary to continually add it during the prolonged course of the reaction.

The major drawback in using these enzymes is that enzymatic treatment can only remove 80–90 percent of the proteins present. This is because protein digestion is an enzymatic reaction that is dependent on substrate and enzyme concentrations. In practice, the deproteinization rate depends only on the protein (substrate) concentration, because it is not practical to add a large amount of enzyme to accelerate the reaction at low substrate concentration. Therefore, as the reaction proceeds the concentration of substrate decreases progressively, thereby slowing the reaction rate and, indeed, enzymatic reactions will go to completion only given infinite time. At high substrate concentrations and sufficient concentration of enzyme, the reaction proceeds at a maximal rate until 80–90 percent of the substrate has been removed. The reaction rate then becomes too slow to be practical for the removal of remaining protein in a reasonable time.

The characteristics of enzymatic removal of proteins make enzymatic deproteinization an ideal and indispensable first step in nucleic acid purification. This treatment is used when a large amount of protein is present, i.e. right after cell lysis. The remaining proteins can be removed with a single extraction using organic solvent.

Removal of RNA

The removal of RNA from DNA preparations is usually carried out using an enzymatic procedure. Consequently this procedure does not remove all

RNA and, therefore, yields DNA preparations with a very small amount of RNA contamination. Two ribonucleases that can be easily and cheaply prepared free of DNase contamination are used, namely ribonuclease A and ribonuclease T1.

Ribonuclease A (RNase A) is an endoribonuclease that cleaves RNA after C and U residues. The reaction generates 2′:3′-cyclic phosphate which is hydrolyzed to 3′-nucleoside phosphate producing oligonucleotides ending with 3′-phosphorylated pyrimidine nucleotide.

Ribonuclease T1 (RNase T1) is an endoribonuclease that is very similar to RNase A in its reaction conditions and stability. The enzyme cleaves double-stranded and single-stranded RNA after G residues, generating oligonucleotides ending in a 3′-phosphorylated guanosine nucleotide.

Because of the RNA cleaving specificity of these enzymes, it is recommended that they be used **together** for complete RNA removal from DNA samples. The use of only one of these enzymes can result in contamination of DNA preparations with a large amount of oligonucleotides that will make the spectrophotometric measurement of DNA concentration practically impossible.

Concentrating the DNA

Precipitating with alcohol is usually performed for concentration of DNA from the aqueous phase of the deproteinization step. Two alcohols are used for DNA precipitation: ethanol and isopropanol.

Alcohol precipitation is based on the phenomenon of decreasing the solubility of nucleic acids in water. Polar water molecules surround the DNA molecules in aqueous solutions. The positively charged dipoles of water interact strongly with the negative charges on the phosphodiester groups of DNA. This interaction promotes the solubility of DNA in water. Ethanol is completely miscible with water, yet it is far less polar than water. Ethanol molecules cannot interact with the polar groups of nucleic acids as strongly as water, making ethanol a very poor solvent for nucleic acids.

Replacement of 95 percent of the water molecules in a DNA solution will cause the DNA to precipitate. Making a DNA solution of 95 percent ethanol is not practical because it requires the addition of a large volume of 100 percent ethanol. To precipitate DNA at a lower ethanol concentration, the activity of water molecules must be decreased. This can be accomplished by the addition of salts to DNA solutions. Moreover, the presence of salts will change the degree of charge neutralization of the DNA phosphates, eliciting extensive changes in the hydrodynamic properties of the DNA molecules (Eickbush and Moudrianakis, 1978). These changes, simultaneous with water elimination, will cause the separation of the DNA

phase, i.e. precipitation, at the moment of complete neutralization of DNA molecules.

DNA precipitation is customarily carried out with 70 percent ethanol (final concentration) in the presence of the appropriate concentration of sodium or ammonium salts. The use of each of these salts has its advantages and disadvantages. The major advantage of using sodium chloride, in addition to convenience and low cost, is that SDS remains soluble in ethanol in the presence of 0.2 *M* NaCl. The use of sodium chloride is therefore recommended if a high concentration of SDS has been used for lysing the cells. The disadvantage of sodium chloride is its limited solubility in 70 percent ethanol making it difficult to completely remove from the DNA samples. This is particularly true when the precipitated DNA is collected by centrifugation. A high sodium chloride concentration in DNA preparations can interfere with the activity of many enzymes. When sodium chloride is used, the DNA should be spooled rather than centrifuged in order to collect precipitated DNA, making sodium chloride particularly useful in large-scale, high molecular weight DNA preparations.

Sodium acetate is more soluble in ethanol than sodium chloride and, therefore, is less likely to precipitate with the DNA sample. Its higher solubility in 70 percent ethanol makes it easier to remove from a DNA preparation by repeated 70 percent ethanol washes. Sodium acetate is the most frequently used salt in DNA precipitation.

Ammonium acetate is highly soluble in ethanol and easy to remove from precipitated DNA due to the volatility of both ammonium and acetate ions. The use of ammonium acetate instead of sodium acetate is also recommended for removing nucleotide triphosphates or small single- or double-stranded oligonucleotides (less then 30 bp), since these molecules are less likely to precipitate at high ethanol concentrations. In addition, precipitation of DNA with ammonium acetate has proven to be more efficient for the removal of heavy metals, detergents, and some unknown impurities that are potent inhibitors of restriction endonucleases and other enzymes used for DNA manipulation (Crouse and Amorese, 1987; Perbal, 1988).

Usually ethanol precipitation is carried out at temperatures of –20°C or lower. It is reasoned that low temperature and the presence of salts further lower the activity of water molecules, thereby facilitating more efficient DNA precipitation. However, a careful analysis of the efficiency of DNA precipitation at various temperatures and DNA concentrations demonstrated that this step could be performed at room temperature without serious loss of DNA, even when the concentration of DNA in a sample is very low (Zeugin and Hartley, 1985; Crouse and Amorese, 1987).

The best recoveries of DNA (DNA concentrations in the range 5–5,000 ng ml^{-1}) occur at room or 4°C temperatures and the worst when the precipitation is carried out at –70°C. The recovery of DNA at very low concentration (5 ng ml^{-1}) is not substantially different at the various temperatures

and is largely dependent on time. Thus, in the procedures described in this manual DNA precipitation is always performed at room temperature.

Determination of the Purity and Quantity of DNA

The last step of any DNA isolation procedure is evaluation of the results. For DNA this evaluation involves determination of DNA concentration and evaluation of the purity of the DNA.

Ultraviolet (UV) spectrophotometry is used for the determination of DNA concentration. The DNA has maximum and minimum absorbances at 260 and 234 nm, respectively. However, these are strongly affected by the degree of base ionization and, hence, pH of the measuring medium (Beaven et al., 1955; Wilfinger et al., 1997).

The relationship between DNA absorbance at 260 nm (A_{260}) and DNA concentration (N) is described by the following equation:

$$N = A_{260}/\varepsilon_{260} \tag{1.1}$$

where ε_{260} is the DNA extinction coefficient. This coefficient for double-stranded DNA is 0.02 $\mu g^{-1}\,cm^{-1}$ when measured at neutral or slightly basic pH. Thus, an absorbance of 1.0 at 260 nm gives a DNA concentration of 50 $\mu g\,ml^{-1}$ (1/0.02 = 50 $mg\,ml^{-1}$). The value of the absorption coefficient (ε_{260}) for double-stranded DNA varies slightly depending on the percent of GC. As a result the concentration of DNA solutions having an absorbance of 1.0 is not always 50 $\mu g\,ml^{-1}$. This slight variation is usually disregarded. The absorption coefficient of single-stranded DNA is 0.027 $\mu g^{-1}\,cm^{-1}$ giving an ssDNA concentration of 37 $\mu g\,ml^{-1}$ for an absorbance of 1.0 (1/0.027 = 37 $\mu g\,ml^{-1}$).

The linear relationship between absorbance at 260 nm and DNA concentration holds in a range between 0.1 and 2.0 absorbance units. Reliable measurements of DNA concentration can be made for solutions of 0.5–100 $\mu g\,ml^{-1}$ using a standard UV spectrophotometer. Before measurement, samples with an absorbance equal to or greater than 2.0 should be diluted. The measurement of DNA concentration at a lower range (A_{260} lower than 0.2) can be strongly affected by light scattering on dust particles present in the preparation. Measuring the absorbance at 320 nm (Schleif and Wensink, 1981) will assess the degree of such contamination. At this wavelength, DNA does not absorb and any absorbance at 320 nm is due to light scattering. To measure DNA concentration properly the absorbance of a DNA sample at 320 nm should be less than 5 percent of the absorbance at 260 nm.

Absorbance measurements at wavelengths other than 260 nm are used for determination of the degree of protein contamination of the DNA sample. Proteins absorb maximally at 280 nm due to the presence of tyrosine, phenylalanine, and tryptophan and absorption at this wavelength is used for the detection of protein in DNA samples. This is done by determination of the $A_{260}:A_{280}$ ratio. This ratio for pure double-stranded DNA is not 1.8–1.9, as previously thought, but is 2.0 (Glasel, 1995; Huberman, 1995; Manchester, 1995; Held, 1997). The ratio between 1.8 and 1.9 corresponds to 60 and 40 percent protein contamination, respectively (Glasel, 1995). If the absorbance ratio of 260 nm : 280 nm is lower than 2.0, the DNA concentration can be calculated using following formula (Surzycki, 2000):

$$N(\mu g\ ml^{-1}) = 70\ A_{260} - 40\ A_{280} \tag{1.2}$$

where A_{260} and A_{280} are the absorbances of a DNA sample at 260 and 280 nm, respectively.

A better indicator of protein contamination in DNA samples is the ratio of $A_{260}:A_{234}$. DNA has an absorbance minimum at 234 nm and protein absorbance is high due to the absorption maximum for peptide bonds at 205 nm (Scopes, 1974; Stoscheck, 1990). Since the ratio of the DNA extinction coefficient at 234 nm (ε_{234}) to the protein extinction coefficient at the same wavelength is 1.5–1.8, the $A_{260}:A_{234}$ ratio is a very sensitive indicator of protein contamination. For pure nucleic acids, this ratio is between 1.8 and 2.0. The DNA concentration can be calculated from the absorbances at 260 and 234 nm using the following equation (Surzycki, 2000):

$$N(\mu g\ ml^{-1}) = 52.6\ A_{260} - 5.24\ A_{234} \tag{1.3}$$

where A_{260} and A_{234} are the absorbances of a DNA sample at 260 and 234 nm, respectively.

FIRST LABORATORY PERIOD

In this laboratory period you will collect your cheek cells and begin DNA purification.

Safety precautions

Each student should work only with his or her own cells. Any student who does not wish to isolate DNA from his or her own cells should be provided with human genomic DNA certified to be free of human immunodeficiency virus DNA. This DNA is commercially available from a number of companies (e.g. Promega Co. and Sigma Co.).

Special safety procedures are necessary when working with phenol or CIA solutions. Because of the relatively low vapor pressure of phenol, occupational systemic poisoning usually results from skin contact with phenol rather than from inhaling the vapors. Phenol is rapidly absorbed by and highly corrosive to the skin. It initially produces a white softened area, followed by severe burns. Because of the local anesthetic properties of the phenol, skin burns may not be felt until there has been serious damage. Gloves should be worn at all the times when working with this chemical. Because some brands of gloves are soluble or permeable to phenol, they should be tested before use. If phenol is spilled on the skin, flush off immediately with a large amount of water and treat with a 70 percent aqueous solution of PEG (polyethylene glycol) 4000 (http://users.ox.ac.uk/~phar0036/biomedsafety/labsafety/chemicalsafety/phenol.html). **Do not use ethanol.** Used phenol should be collected in a tightly closed, glass receptacle and stored in a chemical hood to await proper disposal.

The CIA reagent should also be handled with care. Mixing chloroform with other solvents can involve a serious hazard. Adding chloroform to a solution containing strong base or chlorinated hydrocarbons could result in an explosion. Prepare CIA in a fume hood because isoamyl alcohol vapors are poisonous. Store the CIA mixture in a hood in a tightly closed, dark glass bottle. Used CIA can be collected in the same bottle as phenol and discarded together.

Technical tips

Two of the most common obstacles in obtaining a high yield of high molecular weight DNA are hydrodynamic shearing and DNA degradation by non-specific DNases.

To avoid hydrodynamic shearing, DNA in solution should always be pipetted slowly with wide-bore pipettes (approximately 3–4 mm orifice

diameter). A wide-bore pipette can be prepared by cutting off the tip of a 10 ml plastic disposable pipette. Alternatively, a pipette aid can be inserted on the tip end of a sterilized 10 ml glass pipette using the other end as the "intake" end. The end of the pipette should always be immersed in the liquid when pipetting DNA. The DNA solution should never be allowed to run down the side of a tube nor should it be vigorously shaken or vortexed.

In order to avoid DNase degradation, all solutions should contain DNase inhibitors. Two kinds of DNase inhibitors are in use, EDTA and detergents. EDTA is a Mg^{2+} ion chelator and a powerful inhibitor of DNases since most cellular DNases require Mg^{2+} ion as a co-factor for their activity. In addition, the presence of EDTA in extraction buffers inhibits Mg^{2+} ion-induced aggregation of nucleic acids. A concentration of 50–100 mM EDTA is usually sufficient for inhibiting DNases present in human cells. In fact, a high concentration of EDTA (above 100 mM) in buffers is not recommended because it leads to a substantial decrease in yields. Detergents commonly used in DNA purification are SDS or lithium deodecyl sulfate and Sarcosyl.

DNA samples should be stored under conditions that limit their degradation. Even at ideal storage conditions, one should expect approximately one phosphodiester bond break per 200 kb per year. For long-term storage, the pH of the buffer should be above 8.5 in order to minimize deamidation and contain at least 0.15 M NaCl and 10 mM EDTA.

During DNA preparation and storage the following conditions will contribute to fast degradation of DNA.

DNase contamination

The most frequent source of this contamination is human skin. In spite of the low stability of most DNases, even short exposure to a very low concentration of these enzymes will result in substantial sample degradation. In order to avoid this contamination, it is necessary to avoid direct or indirect contact between samples and fingers by wearing gloves and using sterilized solutions and tubes. Since DNA is easily absorbed onto glass surfaces only sterilized plastic tubes should be used for storage.

Presence of heavy metals

Heavy metals promote the breakage of phosphodiester bonds. Long-term DNA storage buffer should contain 10 mM or more of EDTA, which is a heavy metal chelator. If EDTA is present, DNA can be stored as a precipitate in 70 percent ethanol. This storage condition is preferred if the sample is stored at 5°C because it prevents bacterial contamination. A 1–2 mM EDTA concentration is sufficient for short-term storage of DNA and more convenient for everyday work.

Presence of ethidium bromide

The presence of ethidium bromide causes photo-oxidation of DNA with visible light in the presence of molecular oxygen. Since it is difficult to remove all ethidium bromide from DNA samples treated with this reagent, such samples should always be stored in the dark. Moreover, due to the ubiquitous presence of ethidium bromide in molecular biology laboratories, DNA samples can be easily contaminated with it. For this reason all DNA samples should be stored in the dark.

Temperature

The best temperature for short-term storage of high molecular weight DNA is between 4 and 6°C. At this temperature the DNA sample can be removed and returned to storage without cycles of freezing and thawing, which cause DNA breakage. For very long-term storage (five years or more) DNA should be stored at a temperature of –70°C or below, providing the sample is not subjected to any freeze–thaw cycles. Long- or short-term storage of high molecular weight DNA at –20°C is not recommended. This temperature can cause extensive single- and double-strand breakage of DNA because, at this temperature, molecular bound water is not frozen.

Protocol

Collecting human cheek cells

1. Pour 10 ml of PBS into a 15 ml conical centrifuge tube. Transfer the solution into a paper cup. Pour all the solution into your mouth and swish vigorously for 30–40 seconds. Expel the PBS wash back into the paper cup.
2. Transfer the solution from the paper cup into a 25 ml Corex centrifuge tube and place it on ice.
3. Repeat step 1 one more time with fresh PBS. Expel the mouthwash back into the paper cup and transfer the solution into the same 25 ml Corex tube.
4. Collect the cells by centrifugation at 5,000 r.p.m. for 10 minutes at 4°C.
5. Pour as much supernatant as possible back into the paper cup. Be careful not to disturb the cell pellet. Discard the supernatant from the paper cup into the sink. Invert the Corex centrifuge tube with cells on a paper towel to remove the remaining PBS.

DNA purification

1. Add 1 ml of lysis buffer prewarmed to 65°C to the cells and gently resuspend them by pipetting up and down.

2. Add 70 µl of proteinase K (20 mg ml^{-1}) and mix by inverting the tube several times.

3. Add 80 µl of 20 percent Sarcosyl and mix well by gently inverting the tube. Clearing of the "milky" cell solution and increased viscosity indicates lysis of the cells.

4. Incubate the mixture for 60 minutes in a 65°C water bath.

5. Add 800 µl of dilution buffer and mix carefully. Hold the tube between your thumb and index finger and very quickly invert several times. Do not allow the lysate to run slowly down the side of the tube. This step lowers the sodium chloride concentration allowing single- and double-stranded activities of both RNases.

6. Transfer the tube to a 37°C water bath and add 25 µl of RNase A and 2 ml of RNase T1. Cap the tube and mix by quickly inverting several times. Incubate for 30 minutes at 37°C.

7. Add 1 ml of phenol–8HQ solution and 1 ml of CIA solution to the tube. Close the tube with a Teflon-lined cap and mix using the procedure described in step 5. The solution should turn "milky" when properly mixed. This should not take more than two to three inversions.

8. Remove the cap and place the tube into a centrifuge. Centrifuge at 10,000 r.p.m. for 5 minutes at 4°C to separate the water and phenol phases. DNA will be in the top aqueous layer.

9. Collect the aqueous phase using a P1000 Pipetman set to 1,000 µl and equipped with a wide-bore blue tip. Prepare a wide-bore blue tip by cutting off 5–6 mm from the end of the tip with a razor blade. The total volume of the aqueous phase should be approximately 2 ml. Avoid collecting the white **powdery looking** precipitate at the interphase. However, do collect as much of the viscose, bluish-white layer from the interphase as possible. This layer contains concentrated nucleic acids, not proteins. Record the volume of aqueous phase and transfer it into a fresh 25 ml Corex tube. To do this place the cut-off end of the blue tip at the bottom of the tube and slowly deliver the solution.

10. Add 0.5 volume (1.0 ml) of 7.5 M ammonium acetate to the DNA solution and mix by inverting the tube.

11. Add approximately 6 ml of 95 percent ethanol to the tube containing DNA solution. The volume of added ethanol should be two times the total volume, i.e. DNA plus ammonium acetate (~3.0 ml). Carefully overlay the ethanol onto the viscose DNA solution. Since ethanol is less dense than the DNA solution, it will be the upper layer.

12. Precipitate DNA by gently inverting the tube several times. The DNA should appear as a cotton-like precipitate.

13. Insert the end of a glass hook into the precipitated DNA and swirl the hook in a circular motion to spool out the DNA. The DNA precipitate will adhere to the hook. **Note:** if at this step DNA does not form a clump and

instead it forms several smaller fragments, do not try to collect them on a glass hook. Go to step 17 instead.

14. Transfer the hook with DNA into a 20 ml tube filled with 5 ml of cold 70 percent ethanol. Wash the DNA by gently swirling the glass hook. Pour out the 70 percent ethanol and repeat the wash two more times.

15. Transfer the hook to a microfuge tube and add 300 µl of TE buffer. Rehydrate the DNA slowly by moving the glass hook back and forth. To speed up the rehydratation of DNA, incubate the solution in a 65°C water bath for 10–15 minutes moving the tube gently every 2–3 minutes.

16. Store the tube in a 4°C refrigerator until the next laboratory period

17. Collect precipitated DNA by centrifugation for 5 minutes at 10,000 r.p.m. Discard the supernatant by gently pouring off the ethanol. DNA will appear at the bottom of the tubes as a white precipitate.

18. Add 5 ml of cold 70 percent ethanol to the tube and wash the pellet by carefully rolling the tube at a 45° angle in the palm of your hand. Take care not to dislodge the DNA pellet from the bottom of the tube during this procedure. Never vortex the tube. Discard the 70 percent ethanol and drain the tube well by inverting it over a paper towel for a few minutes. Repeat the wash one more time.

19. Add 100 µl of TE buffer to the tube and rehydrate the DNA pellet by gently pipetting up and down using a P200 Pipetman equipped with a yellow tip with a cut-off end. Transfer the DNA solution into a 1.5 ml microfuge tube.

20. Add another 100 µl of TE buffer to the centrifuge tube and wash the tube by gently pipetting up and down as described in step 19. Add the solution to the microfuge tube with DNA. Repeat this washing one more time. The total volume of the DNA should be 300 µl. Store the tube at 4°C as described in step 16.

SECOND LABORATORY PERIOD

In this laboratory period we will continue DNA purification. First, DNA will be concentrated by precipitation with ethanol in the presence of ammonium acetate. We will use the ammonium acetate procedure because this salt has proven to be the most efficient for the removal of the heavy metals, detergents, and impurities that are potent inhibitors of restriction endonucleases and other enzymes used for DNA manipulation. Second, we will determine DNA concentration and purity using a UV spectrophotometer.

Safety precautions

The same safety precautions as for the first laboratory period apply.

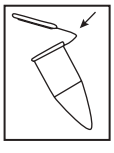
Technical tips

The same technical tips as for the first laboratory period apply.

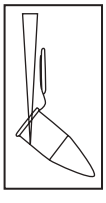
Protocol

Precipitating DNA

1. Retrieve the tube from the refrigerator and add 150 µl of 7.5 M ammonium acetate. Mix by inverting the tube several times.
2. Add 950 µl of 95 percent ethanol and mix by inverting the tube two to four times.



3. Place the tube in a centrifuge, orienting the attached end of the lid away from the center of rotation (see the icon in the margin). Centrifuge the tube at maximum speed for 5 minutes at room temperature.
4. Remove the tubes from the centrifuge. Pour off ethanol into an Erlenmeyer flask by holding the tube by the open lid and gently inverting the end. Touch the lip of the tube to the rim of the flask and drain the ethanol. You do not need to remove all of the ethanol from the tube. Return the tubes to the centrifuge in the same orientations as before. **Note:** when pouring off ethanol do not invert the tube more than once because this could disturb the pellet.



5. Wash the pellet with 700 µl of cold 70 percent ethanol. Holding the P1000 Pipetman vertically (see the icon in the margin) slowly deliver the ethanol to the side of the tube opposite the pellet. **Do not start the centrifuge:** in this step the centrifuge rotor is used as a "tube holder" that keeps the tube at an angle conve-

nient for ethanol washing. Withdraw the tube from the centrifuge by holding the tube by the lid. Remove ethanol as before (step 4). Place the tube back into the centrifuge and wash with 70 percent ethanol one more time.

6. After the last ethanol wash, collect the ethanol remaining on the sides of the tube by centrifugation. Place the tubes back into the centrifuge with the side of the tube containing the pellet facing away from the center of rotation and centrifuge for 2–3 seconds. For this centrifugation, you do not need to close the lids of the tubes. Remove collected ethanol from the bottom of the tube using a P200 Pipetman equipped with capillary tip. **Note:** this procedure makes it possible to quickly wash the pellet without centrifugation and vortexing. Vortexing and centrifuging the pellet is time-consuming and leads to substantial loss of material and shearing of the DNA. Never dry the DNA pellet in a vacuum. This will make rehydration of the DNA very difficult if not impossible

7. Add 35 µl of TE buffer to each tube and resuspend the pelleted DNA. Use a yellow tip (P200 Pipetman) with a cut-off end for this procedure. Gently pipette the buffer up and down directing the stream of the buffer towards the pellet. If the pellet does not dissolve in several minutes, place the tube in a 60–65°C water bath and incubate for 10–20 minutes mixing occasionally.

Determination of DNA concentration and purity

1. Determine the concentration of DNA by measuring absorbance at 260 nm. Initially use a 1 : 20 dilution of the DNA. The absorbance reading should be in the range 0.1–1.5 OD_{260}. Special care must be taken to dilute the viscose solution of DNA when micropipettors are used. Most micropipettes will not measure the volume of a very viscose solution correctly. To prepare a 1 : 20 dilution of DNA, add 100 µl of PBS to a microfuge tube. Prepare a wide-bore, yellow tip by cutting off 5–6 mm from the end of the tip with a razor blade. Withdraw 5 µl of PBS from the tube and mark the level of the liquid with a marking pen. Discard PBS from the tip and draw DNA solution to the 5 µl mark. Transfer DNA to the tube containing PBS. Pipette up and down several times to remove the viscose DNA solution from the inside of the pipette tip. **Note:** DNA concentration should never be measured in water or TE buffer.

2. Determine the absorbance at 260 nm and calculate the DNA concentration using the equation

$$\text{DNA}(\mu\text{g ml}^{-1}) = \text{OD}_{260} \times 50 \times \text{Dilution factor} \qquad (1.4)$$

3. Determine the purity of DNA by measuring the absorbances at 280 and 234 nm. Calculate 260 nm : 280 nm and 260 nm : 234 nm ratios. Calculate the amount of DNA using equations (1.2) and (1.3).

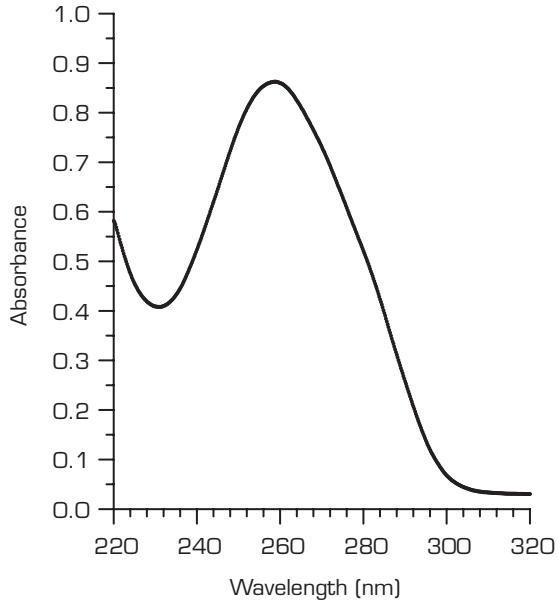


Figure 1.2 Absorption spectrum of DNA purified from human cheek cells. The DNA was diluted 20 times in PBS and scanned using a UV spectrophotometer. The 260 nm : 280 nm absorbance ratio was 1.7 and the 260 nm : 234 nm absorbance ratio was 1.8.

4. Label the tube with your name and group number and indicate the DNA concentration in **micrograms per microliter. Store the tube in a 4°C refrigerator.**

Expected results

A typical spectrum of the DNA purified from human cheek cells is shown in Fig. 1.2. The concentration of DNA isolated from two mouthwashes should be between 0.5 and 1.0 $\mu g \mu l^{-1}$. The total amount of DNA should be 17–35 μg. The DNA is high molecular weight and does not contain RNA.

A low 260 nm : 280 nm and/or 260 nm : 234 nm ratio indicates protein contamination and more often than not is caused by low activity of proteinase K or inadequate mixing of phenol and aqueous phases. The low activity of proteinase K is indicated by the presence of a large amount of "foamy" material at the interphase after the first phenol extraction, whereas inadequately mixed phenol and aqueous phases do not have a uniformly "milky" appearance.

The presence of low molecular weight DNA in the preparation frequently results from DNA mechanical shearing when pipettes with narrow openings are used or by allowing the DNA solution to run down the side of the tube. Inverting tubes too slowly during organic extraction will also result in substantial shearing of DNA molecules.

The presence of low molecular weight DNA in the preparation can also result from insufficient inhibition of DNase activity. This usually results from a too low concentration of EDTA in the lysis buffer.

A low yield of DNA can result from inadequate lysis of the cells or a too high concentration of EDTA in the lysis buffer. Insufficient cell lysis will be noticeable after the addition of Sarcosyl. Adequate lysis of the cells at this step results in a drastic increase in viscosity of the solution.

References

Beaven, G.H., Holiday, E.R., and Johnson, E.A. (1955) Optical properties of nucleic acids and their components. In *The Nucleic Acids*, Vol. 1, E. Chargaff and J.N. Davidson (eds), pp. 493–553. Academic Press, New York.

Crouse, J. and Amorese, D. (1987) Ethanol precipitation: ammonium acetate as an alternative to sodium acetate. *BRL Focus*, **9**(2), 3–5.

Eickbush, T.H. and Moudrianakis, E.N. (1978) The compaction of DNA helices into either continuous supercoils or folded-fiber rods and toroids. *Cell*, **13**, 295–306.

Glasel, J.A. (1995) Validity of nucleic acid purities monitored by 260 nm/280 nm absorbance ratios. *BioTechniques*, **18**, 62–3.

Held, P. (1997) The importance of 240 nm absorbance measurement. The A_{260}/A_{280} ratio just isn't enough anymore. *Biomed. Products*, **7**, 123.

Huberman, J.A. (1995) Importance of measuring nucleic acid absorbance at 240 nm as well as at 260 and 280 nm. *BioTechniques*, **18**, 636.

Kirby, K. (1957) A new method for the isolation of deoxyribonucleic acids; evidence on the nature of bonds between deoxyribonucleic acid and proteins. *Biochem. J.*, **66**, 495–504.

Manchester, K.L. (1995) Value of A_{260}/A_{280} ratios for measurement of purity of nucleic acids. *BioTechniques*, **19**, 208–10.

Marmur, J. (1961) A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J. Mol. Biol.*, **3**, 208–18.

Perbal, B.V. (1988) *A Practical Guide to Molecular Cloning*, 2nd edn. John Wiley & Sons, New York, Chichester, Brisbane, Toronto, and Singapore.

Schleif, R.F. and Wensink, P.C. (1981) *Practical Methods in Molecular Biology*. Springer-Verlag, New York.

Scopes, R.K. (1974) Measurement of protein by spectrophotometry at 205 nm. *Anal. Biochem.*, **59**, 277–82.

Stoscheck, C.M. (1990) Quantitation of protein. *Methods Enzymol.*, **182**, 50–68.

Surzycki, S.J. (2000) *Basic Techniques in Molecular Biology*. Springer-Verlag, Berlin, Heidelberg, and New York.

Wilfinger, W.W., Mackey, K., and Chomczynski, P. (1997) Effect of pH and ionic strength of the spectrophotometric assessment of nucleic acid purity. *BioTechniques*, **22**, 474–80.

Zeugin, J.A. and Hartley, J.L. (1985) Ethanol precipitation of DNA. *BRL Focus*, **7**(4), 1–2.