PREPARATION AND ANALYSIS OF DNA

2

continued

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continued

CHAPTER 2
Preparation and Analysis of DNA

INTRODUCTION

The application of molecular biology techniques to the analysis of complex genomes depends on the ability to prepare pure, high-molecular-weight DNA. This chapter begins with protocols for purification of genomic DNA from bacteria, plant cells, and mammalian cells. These protocols consist of two parts: a technique to lyse gently the cells and solubilize the DNA, followed by one of several basic enzymatic or chemical methods to remove contaminating proteins, RNA, and other macromolecules. The basic approaches described here are generally applicable to a wide variety of starting materials. A brief collection of general protocols for further purifying and concentrating nucleic acids is also included.

Virtually all protocols in molecular biology require, at some point, fractionation of nucleic acids. Chromatographic techniques are appropriate for some applications—e.g., separation of double- and single-stranded nucleic acids (*UNIT 2.13*)—and may be used for separation of plasmid from genomic DNA as well as separation of genomic DNA from debris in a cell lysate (*UNIT 2.14*). Gel electrophoresis, however, has much greater resolution than alternative methods and is generally the fractionation method of choice. Gel electrophoretic separations can be either analytical or preparative, and can involve fragments with molecular weights ranging from less than 1000 Daltons to more than 10^8. A variety of electrophoretic systems have been developed to accommodate such a large range of applications.

In general, the use of electrophoresis to separate nucleic acids is simpler than its application to resolve proteins. Nucleic acids are uniformly negatively charged and, for double-stranded DNA, reasonably free of complicating structural effects that affect mobility. A variety of important variables affect migration of nucleic acids on gels. These include the conformation of the nucleic acid, the pore size of the gel, the voltage gradient applied, and the salt concentration of the buffer. The most basic of these variables is the pore size of the gel, which dictates the size of the fragments that can be resolved. In practice, this means that larger-pore agarose gels are used to resolve fragments larger than 500 to 1000 bp and smaller pore acrylamide or sieving agarose gels are used for fragments smaller than 1000 bp. Sections II (large fragments) and III (small fragments) of this chapter describe analytical and preparative applications of such gels. Section II also includes a protocol for resolution of very large pieces of DNA on agarose gels using pulsed-field gel electrophoresis (*UNIT 2.5*).

Frequently it is desirable to identify an individual fragment in a complex mixture that has been resolved by gel electrophoresis. This is accomplished by a technique termed Southern blotting, in which the fragments are transferred from the gel to a nylon or nitrocellulose membrane and the fragment of interest is identified by hybridization with a labeled nucleic acid probe. Section IV of this chapter gives a complete review of methods and materials required for immobilization of fractionated DNA (*UNIT 2.9*) and associated hybridization techniques (*UNIT 2.10*). These methods have greatly contributed to the mapping and identification of single and multicopy sequences in complex genomes, and facilitated the initial eukaryotic cloning experiments.

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Other commonly encountered applications of gel electrophoresis include resolution of single-stranded RNA or DNA. Polyacrylamide gels containing high concentrations of urea as a denaturant provide a very powerful system for resolution of short (<500-nucleotide) fragments of single-stranded DNA or RNA. Such gels can resolve fragments differing by only a single nucleotide in length, and are central to all protocols for DNA sequencing. A detailed description of such denaturing polyacrylamide gels is found in *UNIT 7.6*. Such gels are used for other applications requiring resolution of single-stranded fragments, particularly including the techniques for analyzing mRNA structure by S1 analysis (*UNIT 4.6*), ribonuclease protection (*UNIT 4.7*), or primer extension (*UNIT 4.8*). Denaturing polyacrylamide gels are also useful for preparative applications, such as small-scale purification of radioactive single-stranded probes and large-scale purification of synthetic oligonucleotides (*UNIT 2.12*).

Resolution of relatively large single-stranded fragments (>500 nucleotides) can be accomplished using denaturing agarose gels. This is of particular importance to the analysis of mRNA populations by northern blotting and hybridization. A protocol for use of agarose gels containing formaldehyde in resolution of single-stranded RNA is presented in *UNIT 4.9*. The use of denaturing alkaline agarose gels for purification of labeled single-stranded DNA probes is described in *UNIT 4.6*.

Gels and Electric Circuits

Gel electrophoresis units are almost always simple electric circuits and can be understood using two simple equations. Ohm's law, $V = IR$, states that the electric field, V (measured in volts), is proportional to current, I (measured in milliamps), times resistance, R (measured in ohms). When a given amount of voltage is applied to a simple circuit, a constant amount of current flows through all the elements and the decrease in the total applied voltage that occurs across any element is a direct consequence of its resistance. For a segment of a gel apparatus, resistance is inversely proportional to both the cross-sectional area and the ionic strength of the buffer. Usually the gel itself provides nearly all of the resistance in the circuit, and the voltage applied to the gel will be essentially the same as the total voltage applied to the circuit. For a given current, decreasing either the thickness of the gel (and any overlying buffer) or the ionic strength of the buffer will increase resistance and, consequently, increase the voltage gradient across the gel and the electrophoretic mobility of the sample.

A practical upper limit to the voltage is usually set by the ability of the gel apparatus to dissipate heat. A second useful equation, $P = I^2R$, states that the power produced by the system, P (measured in watts), is proportional to the resistance times the square of the current. The power produced is manifested as heat, and any gel apparatus can dissipate only a particular amount of power without increasing the temperature of the gel. Above this point small increases in voltage can cause significant and potentially disastrous increases in temperature of the gel. It is very important to know how much power a particular gel apparatus can easily dissipate and to carefully monitor the temperature of gels run above that level.

Two practical examples illustrate applications of the two equations. The first involves the fact that the resistance of acrylamide gels increases somewhat during a run as ions related to polymerization are electrophoresed out of the gel. If such a gel is run at constant current, the voltage will increase with time and significant increases in power can occur. If an acrylamide gel is being run at high voltage, the power supply should be set to deliver constant power. The second situation is the case where there is a limitation in number of power supplies, but not gel apparati. A direct application of the first equation shows that the fraction of total voltage applied to each of two gels hooked up in series (one after

another) will be proportional to the fraction of total resistance the gel contributes to the circuit. Two identical gels will each get 50% of the total voltage and power indicated on the power supply.

Finally, it should be noted that some electrophoretic systems employ lethally high voltages, and almost all are potentially hazardous. It is very important to use an adequately shielded apparatus, an appropriately grounded and regulated power supply, and most importantly, common sense when carrying out electrophoresis experiments.

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PREPARATION OF GENOMIC DNA

SECTION I

This section begins with a protocol describing basic techniques for purifying and concentrating DNA samples (*UNIT 2.1*), followed by similar protocols for purifying DNA from mammalian tissue (*UNIT 2.2*), plant tissue (*UNIT 2.3*), and bacteria (*UNIT 2.4*). Various properties and useful measurements of DNA are presented in *APPENDIX 1* in Figure A.1B.1 and Tables A.1B.1 and A.1B.2.

IMPORTANT NOTE: The smallest amount of contamination of DNA preparations by recombinant phages or plasmids can be disastrous. Many person-years have been wasted reisolating previously cloned sequences that contaminated preparations of DNA used to create recombinant DNA libraries (see Hall, 1987, for an account of such a mistake) and many researchers have been embarrassed to find that the "extra" genes they found on their Southern blots were actually contaminating plasmid DNA. All materials used for preparation of plasmid or phage DNA should be kept separate from those used for preparation of genomic DNA, and disposable items should be used wherever possible. Particular care should be taken to avoid contamination of commonly used rotors.

Purification and Concentration of DNA from Aqueous Solutions

UNIT 2.1

This unit presents basic procedures for manipulating solutions of single- or double-stranded DNA through purification and concentration steps. These techniques are useful when proteins or solute molecules need to be removed from aqueous solutions, or when DNA solutions need to be concentrated for reasons of convenience. The basic protocol, using phenol extraction and ethanol precipitation, is appropriate for the purification of DNA from small volumes (<0.4 ml) at concentrations ≤1 mg/ml. Isopropanol may also be used to precipitate DNA, as described in the first alternate protocol. Three support protocols outline methods to buffer the phenol used in extractions, concentrate DNA using butanol, and extract residual organic solvents with ether. An alternative to these methods is nucleic acid purification using glass beads, presented in the second alternate protocol. These protocols may also be used for purifying RNA.

The final two alternate protocols provide modifications to the basic protocol that are used for concentrating RNA and extracting and precipitating DNA from larger volumes and from dilute solutions, and for removing low-molecular-weight oligonucleotides and triphosphates.

PHENOL EXTRACTION AND ETHANOL PRECIPITATION OF DNA

BASIC PROTOCOL

This protocol describes the most commonly used method of purifying and concentrating DNA preparations. The DNA solution is first extracted with a phenol/chloroform/isoamyl alcohol mixture to remove protein contaminants, then precipitated with 100% ethanol. The DNA is pelleted after the precipitation step, washed with 70% ethanol to remove salts and small organic molecules, and resuspended in buffer at a concentration suitable for further experimentation.

Materials

DNA to be purified (≤1 mg/ml) in 0.1 to 0.4 ml volume
25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol (made with *buffered* phenol; first support protocol)
3 M sodium acetate, pH 5.2 (*APPENDIX 2*)
100% ethanol, ice cold

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70% ethanol, room temperature
TE buffer, pH 8.0 (*APPENDIX 2*)
Speedvac evaporator (Savant)

1. Add an equal volume of phenol/chloroform/isoamyl alcohol to the DNA solution to be purified in a 1.5-ml microcentrifuge tube.

   DNA solutions containing monovalent cations ≤0.5 M can be used. Extracting volumes ≤100 µl is difficult; small volumes should be diluted to obtain a volume that is easy to work with.

   High salt concentrations can cause the inversion of the aqueous and organic phases. If this happens, the organic phase can be identified by its yellow color.

2. Vortex vigorously for 10 sec and microcentrifuge 15 sec at room temperature.

   Phases should be well separated. If DNA solution is viscous or contains a large amount of protein, it should be spun longer (1 to 2 min).

3. Carefully remove the top (aqueous) phase containing the DNA using a 200-µl pipettor and transfer to a new tube. If a white precipitate is present at the aqueous/organic interface, repeat steps 1 to 3.

   If starting with a small amount of DNA (<1 µg), recovery can be improved by reextracting the organic phase with 100 µl TE buffer, pH 8.0. This aqueous phase can be pooled with that from the first extraction.

4. Add 1/10 vol of 3 M sodium acetate, pH 5.2, to the solution of DNA. Mix by vortexing briefly or by flicking the tube several times with a finger.

   If the solution contained a high concentration of NaCl or sodium acetate (0.3 to 0.5 M) prior to the phenol extraction step, then no additional salt should be added. It is advisable to make appropriate dilutions to keep NaCl and sodium acetate concentrations below 0.5 M. For high concentrations of DNA (>50 to 100 µg/ml), precipitation is essentially instantaneous at room temperature. If ethanol precipitation is not desirable, residual organic solvents can be removed by ether extraction (second support protocol). In this case, no salt should be added.

5. Add 2 to 2.5 vol (calculated *after* salt addition) of ice-cold 100% ethanol. Mix by vortexing and place in crushed dry ice for 5 min or longer.

   This precipitation step can also be done in a −70°C freezer for 15 min or longer, or in a −20°C freezer for at least 30 min. A slurry of dry ice and ethanol may also be used, but tube labels are less often lost when crushed dry ice is used.

6. Spin 5 min in a fixed-angle microcentrifuge at high speed and remove the supernatant.

   For large pellets the supernatant can simply be poured off. For small pellets (<1 µg), aspirate off the ethanol supernatant with a pipetting device such as a Pasteur pipet or pipettor. This is best accomplished by drawing off liquid from the side of the tube opposite that against which the DNA precipitate was pelleted. Start at the top and move downward as the liquid level drops.

7. Add 1 ml of room-temperature 70% ethanol. Invert the tube several times and microcentrifuge as in step 6.

   If the DNA molecules being precipitated are very small (<200 bases), use 95% ethanol at this step.

8. Remove the supernatant as in step 6. Dry the pellet in a desiccator under vacuum or in a Speedvac evaporator.

The DNA pellet will not stick well to the walls of the tube after the 70% ethanol wash and care must be taken to avoid aspirating the pellet out of the tube.

9. Dissolve the dry pellet in an appropriate volume of water if it is going to be used for further enzymatic manipulations requiring specific buffers. Dissolve in TE buffer, pH 8.0, if it is going to be stored indefinitely.

DNA pellets will not dissolve well in high-salt buffers. To facilitate resuspension, the DNA concentration of the final solution should be kept at <1 mg/ml.

If DNA is resuspended in a volume of TE buffer or water to yield a DNA concentration of <1 mg/ml, small quantities (<25 µg) of precipitated plasmids or restriction fragments should dissolve quickly upon gentle vortexing or flicking of the tube. However, larger quantities of DNA may require vortexing and brief heating (5 min at 65°C) to resuspend. High-molecular-weight genomic DNA may require one to several days to dissolve and should be shaken gently (not vortexed) to avoid shearing, particularly if it is to be used for cosmid cloning or other applications requiring high-molecular-weight DNA. Gentle shaking on a rotating platform or a rocking apparatus is recommended.

PRECIPITATION OF DNA USING ISOPROPANOL

ALTERNATE PROTOCOL

Equal volumes of isopropanol and DNA solution are used in precipitation. Note that the isopropanol volume is half that of the given volume of ethanol in precipitations. This allows precipitation from a large starting volume (e.g., 0.7 ml) in a single microcentrifuge tube. Isopropanol is less volatile than ethanol and takes longer to remove by evaporation. Some salts are less soluble in isopropanol (compared to ethanol) and will be precipitated along with nucleic acids. Extra washings may be necessary to eliminate these contaminating salts.

BUFFERING PHENOL AND PREPARING PHENOL/CHLOROFORM/ISOAMYL ALCOHOL

SUPPORT PROTOCOL

For some purposes, fresh liquefied phenol (88% phenol) can be used without further purification. However, for purification of DNA prior to cloning and other sensitive applications, phenol must be redistilled before use, because oxidation products of phenol can damage and introduce breaks into nucleic acid chains. Redistilled phenol for use in nucleic acid purification is commercially available. Regardless of the source, the phenol must be buffered before use.

CAUTION: Phenol can cause severe burns to skin and damage clothing. Gloves, safety glasses, and a lab coat should be worn whenever working with phenol, and all manipulations should be carried out in a fume hood. A glass receptacle should be available exclusively for disposing of used phenol and chloroform.

Materials

8-hydroxyquinoline
Liquefied phenol
50 mM Tris base (unadjusted pH ~10.5)
50 mM Tris·Cl, pH 8.0
Chloroform
Isoamyl alcohol

1. Add 0.5 g of 8-hydroxyquinoline to a 2-liter glass beaker containing a stir bar.
2. Gently pour in 500 ml of liquefied phenol or melted crystals of redistilled phenol (melted in a water bath at 65°C).

The phenol will turn yellow due to the 8-hydroxyquinoline, which is added as an antioxidant.

3. Add 500 ml of 50 mM Tris base.
4. Cover the beaker with aluminum foil. Stir 10 min at low speed with magnetic stirrer at room temperature.
5. Let phases separate at room temperature. Gently decant the top (aqueous) phase into a suitable waste receptacle. Remove what cannot be decanted with a 25-ml glass pipet and a suction bulb.
6. Add 500 ml of 50 mM Tris·Cl, pH 8.0. Repeat steps 4 to 6 (i.e., two successive equilibrations with 500 ml of 50 mM Tris·Cl, pH 8.0).

   The pH of the phenol phase can be checked with indicator paper and should be 8.0. If it is not, steps 3 to 7 should be repeated until this pH is obtained.
7. Add 250 ml of 50 mM Tris·Cl, pH 8.0, or TE buffer, pH 8.0, and store at 4°C in brown glass bottles or clear glass bottles wrapped in aluminum foil.
8. For use in DNA purification procedure (basic protocol), mix 25 vol phenol (bottom yellow phase of stored solution) with 24 vol chloroform and 1 vol isoamyl alcohol.

   Phenol prepared with 8-hydroxyquinoline as an antioxidant can be stored ≤ 2 months at 4°C.

SUPPORT PROTOCOL

CONCENTRATION OF DNA USING BUTANOL

It is generally inconvenient to handle large volumes or dilute solutions of DNA. Water molecules (but not DNA or solute molecules) can be removed from aqueous solutions by extraction with *sec*-butanol (2-butanol). This procedure is useful for reducing volumes or concentrating dilute solutions before proceeding with the basic protocol.

Additional Materials

sec-butanol
25:24:1 phenol/chloroform/isoamyl alcohol (made with *buffered* phenol; first support protocol)
Polypropylene tube

1. Add an equal volume of *sec*-butanol to the sample and mix well by vortexing or by gentle inversion if the DNA is of high molecular weight. Perform extraction in a polypropylene tube, as butanol will damage polystyrene.
2. Centrifuge 5 min at 1200 × *g* (2500 rpm), room temperature, or in a microcentrifuge for 10 sec.
3. Remove and discard the upper (*sec*-butanol) phase.
4. Repeat steps 1 to 3 until the desired volume of aqueous solution is obtained.
5. Extract the lower, aqueous phase with 25:24:1 phenol/chloroform/isoamyl alcohol and ethanol precipitate as in steps 1 to 9 of the basic protocol, or remove *sec*-butanol by two ether extractions as described in the second support protocol.

   Addition of too much sec-butanol can result in complete loss of the water phase into the sec-butanol layer. If this happens, add ½ vol water back to the sec-butanol, mix well, and spin. The DNA can be recovered in this new, aqueous phase and can be further concentrated with smaller amounts of sec-butanol.

   The salt concentration will increase in direct proportion to the volume decrease. The DNA can be precipitated with ethanol to readjust the buffer conditions.

REMOVAL OF RESIDUAL PHENOL, CHLOROFORM, OR BUTANOL BY ETHER EXTRACTION

SUPPORT PROTOCOL

DNA solutions that have been purified by extraction with phenol and chloroform (first basic protocol) or concentrated with *sec*-butanol (second support protocol) can often be used without ethanol precipitation for enzymatic manipulations or in gel electrophoresis experiments if the organic solvents are removed by extraction with ether. Traces of ether are subsequently removed by evaporation. This procedure is useful only if the solute concentrations in the starting solution are compatible with what is needed in later steps. It is quite useful in purifying high-molecular-weight DNA, as mechanical shearing of large nucleic acid molecules can occur during precipitation with ethanol.

CAUTION: Ether is highly flammable and its vapors can cause drowsiness. All manipulations with ether should be carried out in a well-ventilated fume hood.

Materials

Diethyl ether
TE buffer, pH 8.0 (*APPENDIX 2*)
Polypropylene tube

1. Mix diethyl ether with an equal volume of water or TE buffer, pH 8.0, in a polypropylene tube. Vortex vigorously for 10 sec and let the phases separate.

   Ether is the top phase.

2. Add an equal volume of ether to the DNA sample. Mix well by vortexing or by gentle inversion if the DNA is of high molecular weight.

3. Microcentrifuge 5 sec or let the phases separate by setting the tube upright in a test tube rack.

4. Remove and discard the top (ether) layer. Repeat steps 2 and 3.

5. Remove ether by leaving the sample open under a hood for 15 min (small volumes, <100 µl), or under vacuum for 15 min (larger volumes).

   The DNA solution will be free of organic solvents and will have salt concentrations that are roughly three-fourths of those that were in the aqueous solution before phenol extraction (solute concentrations are lowered in the two phenol/chloroform/isoamyl alcohol extractions steps).

PURIFICATION OF DNA USING GLASS BEADS

ALTERNATE PROTOCOL

The use of a glass beads suspension allows the rapid and efficient purification of DNA from contaminating proteins, RNA, or organic solvents. DNA in solution is adsorbed onto glass beads in the presence of sodium iodide. The DNA–glass beads suspension is washed to remove solution contaminants, and DNA is subsequently eluted into water or a low-salt buffer. Although faster than traditional extraction protocols, this method may result in somewhat reduced yields.

Additional Materials

6 M sodium iodide (NaI) solution
DNA in a 50- to 200-µl volume
Wash solution
TE buffer, pH 8.0 (*APPENDIX 2*)
Glass beads suspension

NOTE: The above materials are also available as commercial kits (e.g., Glas-Pac, National Scientific Supply; GeneClean, Bio101; and Qiaex Gel Extraction Kit, Qiagen).

1. Add 3 vol NaI solution to DNA in a 1.5-ml microcentrifuge tube. Add glass beads suspension as follows: for amounts of DNA <5 µg, use 5 µl glass beads suspension; for amounts of DNA >5 µg, use 5 µl plus an additional 1 µl for each 0.5-µg increment above 5 µg. Incubate 5 min at room temperature.

   For example, if 4 µg of DNA were being purified, 5 µl of the glass beads suspension would be used; if 7 µg of DNA were being purified, 5 µl + 4 µl = 9 µl glass beads suspension would be required.

   Longer incubation times with occasional mixing will improve the binding efficiency, especially with larger volumes.

2. Microcentrifuge DNA/glass beads complex 5 sec. Remove and discard supernatant.

   To enhance yield, save the supernatant and reincubate with another sample of glass beads suspension as in step 1.

3. Wash the DNA/glass beads pellet three times with 500 µl wash solution. Lightly vortex the mixture to resuspend, then microcentrifuge briefly to pellet the beads.

4. Resuspend pellet in TE buffer, pH 8.0, at 0.5 µg/µl. Incubate 2 to 3 min at 45°C to elute DNA from the glass beads.

5. Microcentrifuge 1 min and transfer the DNA-containing supernatant to a fresh tube. Store at 4°C until use.

ALTERNATE PROTOCOLS

PURIFICATION AND CONCENTRATION OF RNA AND DILUTE SOLUTIONS OF DNA

The following adaptations to the basic protocol are used if RNA or dilute solutions of DNA are to be purified.

Purification and Concentration of RNA

The procedure outlined in the basic protocol is identical for purification of RNA, except that 2.5 vol ethanol should be used routinely for the precipitation (step 5). It is essential that all water used directly or in buffers be treated with diethylpyrocarbonate (DEPC) to inactivate RNase (see *UNIT 4.1*, reagents and solutions, for instructions).

Dilute Solutions of DNA

When DNA solutions are dilute (<10 µg/ml) or when <1 µg of DNA is present, the ratio of ethanol to aqueous volume should be increased to 3:1 and the time on dry ice (step 5) extended to 30 min. Microcentrifugation should be carried out for 15 min in a cold room to ensure the recovery of DNA from these solutions.

Nanogram quantities of labeled or unlabeled DNA can be efficiently precipitated by the use of carrier nucleic acid. A convenient method is to add 10 µg of commercially available tRNA from *E. coli*, yeast, or bovine liver to the desired DNA sample. The DNA will be co-precipitated with the tRNA. The carrier tRNA will not interfere with most enzymatic reactions, but will be phosphorylated efficiently by polynucleotide kinase and should not be added if this enzyme will be used in subsequent radiolabeling reactions.

Recovery of small quantities of short DNA fragments and oligonucleotides can be enhanced by adding magnesium chloride to a concentration of <10 mM before adding ethanol (step 4). However, DNA precipitated from solutions containing >10 mM magnesium or phosphate ions is often difficult to redissolve and such solutions should be diluted prior to ethanol precipitation.

DNA in Large Aqueous Volumes (>0.4 to 10 ml)

Larger volumes can be accommodated by simply scaling up the amounts used in the basic protocol or by using butanol concentration as described in the second support protocol. For the phenol extraction (steps 1 through 3), tightly capped 15- or 50-ml polypropylene tubes should be used as polystyrene tubes cannot withstand the phenol/chloroform mixture. Centrifugation steps should be performed for 5 min at speeds not exceeding 1200 × *g* (2500 rpm), room temperature. The ethanol precipitate (step 6) should be centrifuged in thick-walled Corning glass test tubes (15- or 30-ml capacity) for 15 min in fixed-angle rotors at 8000 × *g* (10,000 rpm), 4°C. Glass tubes should be silanized (see *APPENDIX 3*) to facilitate recovery of small amounts of DNA (<10 μg).

REMOVAL OF LOW-MOLECULAR-WEIGHT OLIGONUCLEOTIDES AND TRIPHOSPHATES BY ETHANOL PRECIPITATION

ALTERNATE PROTOCOL

The use of ammonium acetate in place of sodium acetate allows the preferential precipitation of longer DNA molecules. Thus, small single- or double-stranded oligonucleotides (less than ~30 bp) and unincorporated nucleotides used in radiolabeling or other DNA modification reactions can be effectively removed from DNA solutions by two rounds of ethanol precipitation in the presence of ammonium acetate. This approach is not sufficient to completely remove large quantities of linkers as used in cloning procedures (*UNIT 3.16*). If the nucleic acid is to be phosphorylated, this protocol should not be used because T4 polynucleotide kinase is inhibited by ammonium ions.

Additional Materials

4 M ammonium acetate, pH 4.8

1. Add an equal volume of 4 M ammonium acetate, pH 4.8, to the DNA solution. Mix well.
2. Add 2 vol (calculated *after* salt addition) of ice-cold 100% ethanol (67% final). Vortex and set tube in crushed dry ice for 5 min.
3. Microcentrifuge 5 min at high speed, room temperature. Carefully remove supernatant and redissolve pellet in 100 μl TE buffer, pH 8.0.
4. Repeat steps 1 to 3, then proceed to step 5.

   Reprecipitation is required, particularly if the DNA solution from step 1 contained Mg^{++} or other divalent or polyvalent cations that will facilitate the precipitation of the oligonucleotides.

5. Add 1 ml of room-temperature 70% ethanol to the tube and invert several times. Microcentrifuge 5 min at high speed, room temperature.
6. Discard ethanol and dry pellet as in basic protocol (step 9).

   Although the removal of unincorporated nucleoside triphosphates, reaction products, and small oligonucleotides is effective, it is not absolute and the procedure should not be used to purify DNA from these small molecules prior to detailed biochemical or analytical studies.

REAGENTS AND SOLUTIONS

Glass beads suspension

Transfer a volume of ~200 to 300 µl of 200-µm glass beads (National Scientific Supply) into a 1.5-ml microcentrifuge tube; add an equal volume of water. Vortex briefly to suspend just before using.

If glass beads do not come acid-washed, prepare as follows: Wash by soaking 1 hr in concentrated nitric acid. Rinse thoroughly with water. Dry in a baking oven, cool to room temperature, and store at 4°C until needed.

6 M sodium iodide (NaI) solution

Dissolve 0.75 g Na_2SO_3 in 40 ml H_2O. Add 45 g NaI (Sigma) and stir until dissolved (~30 min). Filter through Whatman paper or nitrocellulose and store 3 to 4 months in the dark (in aluminum foil). Discard if precipitate is observed.

Wash solution

20 mM Tris·Cl, pH 7.4
1 mM EDTA
100 mM NaCl

Add an equal volume of 100% ethanol and store 3 to 4 months at 0°C.

COMMENTARY

Background Information

It is often necessary to purify or concentrate a solution of DNA prior to further enzymatic manipulations or analytical studies. The most commonly used method for deproteinizing DNA is extraction with phenol, which efficiently denatures proteins and probably dissolves denatured protein (Kirby, 1957). Chloroform is also a useful protein denaturant with somewhat different properties—it stabilizes the rather unstable boundary between an aqueous phase and a pure phenol layer. The phenol/chloroform mixture reduces the amount of aqueous solution retained in the organic phase (compared to a pure phenol phase), maximizing the yield (Penman, 1966; Palmiter, 1974). Isoamyl alcohol prevents foaming of the mixture upon vortexing and aids in the separation of the organic and aqueous phases (Marmur, 1961). Denatured protein forms a layer at the interface between the aqueous and organic phases and is thus isolated from the bulk of the DNA in the aqueous layer. This procedure is rapid, inexpensive, and easy to perform.

Ethanol precipitation is useful for concentrating DNA solutions and for removing residual phenol and chloroform from the deproteinized aqueous solution. It is also useful for providing DNA that is relatively free of solute molecules when buffer conditions need to be changed. In the presence of relatively high (0.1 to 0.5 M) concentrations of monovalent cations, ethanol induces a structural transition in nucleic acid molecules which causes them to aggregate and precipitate from solution (Eickbush and Moudrianakis, 1978). However, because most salts and small organic molecules are soluble in 70% ethanol, ethanol precipitation and washing of the pellet will effectively desalt DNA. Although sodium chloride, sodium acetate, and ammonium acetate are each capable of inducing precipitation, it is more difficult to remove sodium chloride due to its lower solubility in 70% ethanol.

The glass beads protocol, modified from Vogelstein and Gillespie (1979), provides a simple, nontoxic method for removing DNA from contaminating impurities (*UNIT 2.6*). In the presence of high salt, DNA binds to the small glass particles. The resulting precipitate is washed to remove NaI and impurities from the original sample, and subsequent suspension in water or TE buffer causes dissociation (elution) of the DNA from the glass. Because fewer manipulations are required, this method is faster and easier to perform than the other organic-based extraction methods. However, the yields are somewhat lower, generally ranging from 50% to 75% of the starting material. The procedure seems to work best with DNA fragments larger than 500 bp; smaller-length fragments apparently bind tightly and irreversibly to the glass. DNA fragments >3 to 5 kb may become sheared by the glass, although the suspension provided with the Qiaex gel extraction kit consists of activated silica-gel particles

that allow efficient binding and subsequent elution of DNA fragments ranging from 50 bp to 50 kb in length.

One alternative for purifying DNA from residual protein is the use of StrataClean Resin (Stratagene), a nontoxic slurry of hydroxylated silica particles. Acidic hydroxy groups on the resin appear to bind proteins in a manner similar to phenolic hydroxyls, and at or near neutral pH display a high affinity for protein and low affinity for DNA. Protein bound to the resin is separated by centrifugation from nucleic acids remaining in solution; two or three extractions with the resin may be required to completely remove protein from a nucleic acid sample. Another product, Phase Lock Gel (available from 5 Prime → 3 Prime; *APPENDIX 5*) that is of an inert silica-based blend of intermediate density improves recoveries in standard organic extractions by reducing loss of sample at the interface. During centrifugation, the normally fuzzy interface is compacted tightly below or within the gel. The gel/interface complex migrates discreetly between the organic and aqueous phases, thus creating a tight partition which allows recovery of virtually all of the aqueous phase.

Critical Parameters

The oxidation products of phenol can damage nucleic acids and only redistilled phenol should be used. For complete deproteinization, extractions should be repeated until no protein precipitate remains at the aqueous/organic interface.

In general, alcohol precipitation of nucleic acids requires the presence of at least 0.1 M monovalent cation in the starting aqueous solution. Precipitation of nucleic acids at low concentrations requires cooling to low temperatures to give good recovery. Precipitation of nucleic acids at high concentrations (>0.25 mg/ml after addition of ethanol) is very rapid at room temperature. Formation of a visible precipitate after adding alcohol and mixing well indicates complete precipitation, and no chilling or further incubation is needed.

In organic extraction, loss of nucleic acid at the interface and into the organic phase is minimized by back-extracting the organic phase. To ensure a reasonable yield from the glass beads method, NaI and wash supernatants should be extracted with the glass beads a second time. The procedure works much more efficiently with DNA fragments ≥500 bp.

Anticipated Results

These procedures should result in virtually complete removal of proteins and quantitative recovery of nucleic acids. However, sequential extractions or precipitations require care and attention to detail to prevent accumulation of small losses at each step. It is particularly important to carefully recover the aqueous phase and reextract the organic phase to ensure full recovery of small amounts of DNA from phenol/chloroform extractions.

The yield of nucleic acids resulting from the glass beads procedure can be similarly improved (to ≤80% recovery) by increasing incubation times by 5 to 10 min (i.e., doubling or tripling the incubation time) and by subjecting supernatants to an additional binding step.

Time Considerations

Approximately 90 min should be allowed for carrying out steps 1 through 12 of the basic protocol on twelve DNA samples in microcentrifuge tubes. Phenol buffering should be started ≥1 hr before equilibrated phenol is needed. Nucleic acids should not be left in the presence of phenol, but can be indefinitely precipitated in alcohol or dried after precipitation. The glass beads protocol can be performed on twelve samples in 15 to 20 min.

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Preparation of Genomic DNA from Mammalian Tissue

BASIC PROTOCOL

Tissue is rapidly frozen and crushed to produce readily digestible pieces. The processed tissue is placed in a solution of proteinase K and sodium dodecyl sulfate and incubated until most of the cellular protein is degraded. The digest is deproteinized by successive phenol/chloroform/isoamyl alcohol extractions, recovered by ethanol precipitation, and dried and resuspended in buffer.

Materials

Liquid nitrogen
Digestion buffer
Ice-cold phosphate-buffered saline (PBS; *APPENDIX 2*)
25:24:1 phenol/chloroform/isoamyl alcohol (*UNIT 2.1*)
7.5 M ammonium acetate
100% and 70% ethanol
TE buffer, pH 8 (*APPENDIX 2*)
0.1% sodium dodecyl sulfate (SDS; optional)
1 mg/ml DNase-free RNase (optional; *UNIT 3.13*)

Cell preparation

Beginning with whole tissue:

1. As soon as possible after excision quickly mince tissue and freeze in liquid nitrogen.

   If working with liver, remove the gallbladder, which contains high levels of degradative enzymes.

2. Starting with between 200 mg and 1 g, grind tissue with a prechilled mortar and pestle, or crush with a hammer to a fine powder (keep the tissue fragments).

3. Suspend the powdered tissue in 1.2 ml digestion buffer per 100 mg tissue. There should be no clumps.

Beginning with tissue culture cells:

1. Spin suspension cultures out of their serum-containing media. Trypsinize adherent cells and collect cells from the flask. Centrifuge 5 min at 500 × *g* and discard supernatant.

2. Resuspend cells with 1 to 10 ml ice-cold PBS. Centrifuge 5 min at 500 × *g* and discard supernatant. Repeat this resuspension and centrifugation step.

3. Resuspend cells in 1 vol digestion buffer. For $<3 \times 10^7$ cells use 0.3 ml digestion buffer. For larger numbers of cells use 1 ml digestion buffer/10^8 cells.

Cell lysis and digestion

4. Incubate the samples with shaking at 50°C for 12 to 18 hr in tightly capped tubes.

   The samples will be viscous. After 12 hr incubation the tissue should be almost indiscernible, a sludge should be apparent from the organ samples, and tissue culture cells should be relatively clear.

Extraction of nucleic acids

5. Thoroughly extract the samples with an equal volume of phenol/chloroform/isoamyl alcohol.

   CAUTION: *Phenol is extremely caustic.*

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6. Centrifuge 10 min at 1700 × *g* in a swinging bucket rotor.

If the phases do not resolve well, add another volume of digestion buffer, omitting proteinase K, and repeat the centrifugation.

If there is a thick layer of white material at the interface between the phases, repeat the organic extraction.

Purification of DNA

7. Transfer the aqueous (top) layer to a new tube and add ½ vol of 7.5 M ammonium acetate and 2 (original) vol of 100% ethanol. The DNA should immediately form a stringy precipitate. Recover DNA by centrifugation at 1700 × *g* for 2 min.

This brief precipitation in the presence of high salt reduces the amount of RNA in the DNA. For long-term storage it is convenient to leave the DNA in the presence of ethanol.

Alternatively, to prevent shearing of high-molecular-weight DNA, omit steps 7 to 9 and remove organic solvents and salt from the DNA by at least two dialysis steps against at least 100 vol TE buffer. Because of the high viscosity of the DNA, it is necessary to dialyze for a total of at least 24 hr.

8. Rinse the pellet with 70% ethanol. Decant ethanol and air dry the pellet.

It is important to rinse well to remove residual salt and phenol.

9. Resuspend DNA in TE buffer until dissolved. DNA may be shaken gently at room temperature or at 65°C for several hours to facilitate solubilization.

If necessary, residual RNA can be removed at this step by adding 0.1% sodium dodecyl sulfate (SDS) and 1 µg/ml DNase-free RNase and incubating 1 hr at 37°C, followed by organic extraction and ethanol precipitation, as above.

10. Store at 4°C; ~1 mg/ml DNA is a convenient working concentration. From 1 g mammalian cells, ~2 mg DNA can be expected.

REAGENTS AND SOLUTIONS

Digestion buffer

100 mM NaCl
10 mM Tris·Cl, pH 8
25 mM EDTA, pH 8
0.5% sodium dodecyl sulfate
0.1 mg/ml proteinase K

The proteinase K is labile and must be added fresh with each use.

COMMENTARY

Background Information

There are a number of different procedures for the preparation of genomic DNA. They all start with some form of cell lysis, followed by deproteinization and recovery of DNA. The main differences between various approaches lie in the extent of deproteinization and in molecular weight of the DNA produced. The isolation procedure described here is relatively brief and relies on the powerful proteolytic activity of proteinase K combined with the denaturing ability of the ionic detergent SDS. Use of proteinase K for DNA purification was described by Gross-Bellard et al. (1972) and Enrietto et al. (1983). EDTA is included in the digestion buffer to inhibit DNases.

Critical Parameters

To minimize the activity of endogenous nucleases, it is essential to rapidly isolate, mince, and freeze tissue. Tissue culture cells should be cooled and washed quickly. As soon as the tissue is frozen or the tissue culture cells

are added to the lysis buffer, DNA is protected from action of nucleases throughout this protocol. It is important that the tissue be well dispersed and not left in large lumps to permit rapid and efficient access to proteinase K and SDS.

It is crucial to generate very high-molecular-weight DNA for construction of phage (>60 kb) or cosmid (>120 kb) genomic libraries. Two main precautions should be taken to maximize molecular weight: (1) minimize shearing forces by gentle (but thorough) mixing during extraction steps, and (2) after the extraction, remove organic solvents and salt from the DNA by dialysis, rather than by ethanol precipitation.

The absence of both cellular proteins and proteinase K in the final DNA solution is important for susceptibility of the genomic DNA to restriction enzyme action; therefore, care should be exercised in deproteination. To remove protein completely it may be necessary to repeat the proteinase K digestion. In general, highly pure DNA has an A_{260} to A_{280} ratio >1.8, while 50% protein/50% DNA mixtures have OD_{260}/OD_{280} ratios of ~1.5.

Troubleshooting

Failure of the organic phase to separate cleanly from the aqueous phase is generally due to a very high concentration of DNA and/or cellular debris in the aqueous phase. Dilution with more digestion buffer and reextraction can remedy this problem.

Upon addition of the room-temperature ethanol to the extracted DNA solution, the DNA should precipitate in long, stringy fibers. If there is no precipitate or if the precipitate is flocculent, the DNA is either degraded or not purified away from cellular debris. Improper handling of the tissue before digestion or too much tissue in the digestion reaction are possible causes of such problems.

Anticipated Results

Approximately 2 mg DNA should be obtained from 1 g tissue or 10^9 cells. The DNA should be at least 100 kb long and should be digestible with restriction enzymes.

Time Considerations

This protocol involves effort on 2 days: tissue preparation on the first day followed by overnight lysis, and extraction/precipitation on the second day. Actual time spent on the procedure, however, will be less than 1 hr each day. The DNA can be stored indefinitely in the presence of ethanol, or at –20°C.

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Preparation of Genomic DNA from Plant Tissue

This unit describes two methods for preparing genomic DNA from plant tissue. In the first method, plant cells are lysed with ionic detergent, treated with protease, and subsequently purified by cesium chloride (CsCl) density gradient centrifugation. The second method is based upon a series of treatments with the nonionic detergent cetyltrimethylammonium bromide (CTAB) to lyse cells and purify nucleic acid. Nucleic acid is recovered from the final CTAB solution by isopropanol or ethanol precipitation. The first method, although somewhat more lengthy, results in highly purified nucleic acid. The second method requires fewer manipulations, results in very high yields (~10-fold higher per gram fresh tissue depending on species and condition of starting material), and produces DNA that is less pure but nonetheless suitable in quality for use in many molecular biology manipulations.

PREPARATION OF PLANT DNA USING CSCL CENTRIFUGATION

BASIC PROTOCOL

Plant cells are lysed by the detergent *N*-lauroylsarcosine (Sarkosyl), and the lysate is digested with proteinase K. After clearing insoluble debris from the lysate, nucleic acids are precipitated and DNA is purified on a cesium chloride (CsCl) density gradient.

Materials

*For recipes, see **Reagents and Solutions** in this unit (or cross-referenced unit); for common stock solutions, see **APPENDIX 2**; for suppliers, see **APPENDIX 4**.*

Plant tissue, fresh
Liquid nitrogen
Extraction buffer (see recipe)
10% (w/v) *N*-lauroylsarcosine (Sarkosyl)
Isopropanol
TE buffer, pH 8.0
Cesium chloride
10 mg/ml ethidium bromide
CsCl-saturated isopropanol (equilibrate over a CsCl-saturated aqueous phase)
Ethanol
3 M sodium acetate, pH 5.2

250-ml centrifuge bottle
55°C water bath
Beckman JA-14, JA-20 or JA-21, and VTi80 rotors (or equivalents)
5-ml quick-seal ultracentrifuge tubes
15-G needle and 1-ml syringe

Prepare plant tissue

1. Harvest 10 to 50 g fresh plant tissue.

   Plants may be placed in the dark for 1 to 2 days prior to harvest to reduce the starch content in the tissues.

   Younger plants are the preferred source of tissue because they have a lower polysaccharide content.

2. Rinse tissue with deionized water to remove adhering debris and blot dry.

3. Freeze tissue with liquid nitrogen and grind to a fine powder in a mortar and pestle.

   Keep the tissue frozen throughout this procedure by occasionally adding liquid nitrogen.

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Lyse and digest cells

4. Transfer frozen powder to a 250-ml centrifuge bottle and immediately add 5 to 10 ml extraction buffer per gram fresh plant tissue. Stir gently to disperse tissue.

5. Add 10% *N*-lauroylsarcosine to a final concentration of 1%. Incubate 1 to 2 hr at 55°C.

   *It is important to add N-lauroylsarcosine **after** the tissue is resuspended in extraction buffer. If N-lauroylsarcosine is included in extraction buffer, premature lysis of the plant cells will interfere with tissue dispersal and lead to unwanted shearing of DNA.*

   The lysate should be clear, green, and slightly viscous. From this point on solutions should be handled gently to reduce shearing of the DNA—use a wide-bore pipet and do not vortex or mix vigorously.

6. Centrifuge lysate 10 min at 5500 × *g* (6000 rpm in a Beckman JA-14 rotor), 4°C, to pellet debris. Save the supernatant and centrifuge again if necessary to remove undigested debris.

Precipite the DNA

7. Add 0.6 vol isopropanol to the supernatant and gently mix. A nucleic acid precipitate should be visible; if not, incubate 30 min at –20°C.

8. Centrifuge 15 min at 7500 × *g* (8000 rpm in a Beckman JA-14 rotor), 4°C. Discard supernatant.

   Do not let the nucleic acid pellet dry or it will become extremely difficult to dissolve.

Carry out CsCl centrifugation

9. Resuspend pellet in 9 ml TE buffer. If necessary, incubate at 55°C to aid resuspension. Add 9.7 g of solid CsCl and mix gently until dissolved.

   To minimize depurination, limit 55°C incubation to ≤2 hr.

10. Incubate 30 min on ice. Centrifuge 10 min at 7500 × *g* (8000 rpm in a JA-20 rotor), 4°C, and save supernatant.

    This clearing spin removes some of the insoluble debris remaining in the lysate. In addition, a small separate phase may form on the top of the solution after centrifugation; this is due to residual Sarkosyl in the lysate. The Sarkosyl phase can be removed by filtering the supernatant through two layers of cheesecloth. Collect the supernatant but discard the Sarkosyl phase.

11. Add 0.5 ml of 10 mg/ml ethidium bromide and incubate 30 min on ice.

    CAUTION: *Ethidium bromide is a mutagen. Be careful and wear gloves.*

12. Centrifuge 10 min at 7500 × *g*, 4°C.

    A large RNA pellet should form. At this point much of the unwanted constituents in the lysate—RNA, protein, and carbohydrates—have been removed.

13. Transfer the supernatant to two 5-ml quick-seal ultracentrifuge tubes and seal tubes.

    Make sure tubes are full, balanced, and well-sealed.

14. Centrifuge 4 hr at 525,000 × *g* (80,000 rpm in a Beckman VTi80 rotor), 20°C, or overnight at 300,000 × *g* (60,000 rpm in VTi80 rotor), 20°C.

Collect and purify DNA

15. Gently remove the tube. Punch a hole in the top (to provide an air inlet) with a large-bore (15-G) collecting needle. Recover the DNA band by inserting needle, attached to a 1-ml syringe, through tube wall directly below the band (see Fig. 1.7.1).

    This operation is identical to that used during plasmid purification, except that only one band should be visible.

    CAUTION: *If UV illumination is used to visualize the DNA, wear UV protective glasses or a face shield. Minimize exposure of gradient to visible light to reducing nicking of DNA caused by ethidium bromide.*

16. Remove the ethidium bromide by repeatedly extracting the collected DNA with CsCl-saturated isopropanol.

17. Add 2 vol water and 6 vol ethanol to the DNA solution and mix. Incubate 1 hr at –20°C.

    DNA may precipitate immediately as a single white mass; it can be collected using a Pasteur pipet with a hook introduced at the tip or by brief centrifugation.

18. Centrifuge 10 min at 7500 × *g*, 4°C.

19. Resuspend pellet in TE buffer and reprecipitate DNA by adding 1/10 vol of 3 M sodium acetate and 2 vol ethanol. Incubate at –20°C if precipitate is not visible and collect DNA by centrifugation.

20. Briefly air dry the final pellet and resuspend in 0.5 to 2 ml TE buffer.

    A DNA concentration of 100 ng/μl is generally convenient for most purposes.

PREPARATION OF PLANT DNA USING CTAB

ALTERNATE PROTOCOL

Alternatively, the nonionic detergent cetyltrimethylammonium bromide (CTAB) is used to liberate and complex with total cellular nucleic acids. This general procedure has been used on a wide array of plant genera and tissue types. Many modifications have been published to optimize yields from particular species. The protocol is relatively simple, fast, and easily scaled from milligram to grams of tissue; it requires no cesium chloride density gradient centrifugation.

Additional Materials

*For recipes, see **Reagents and Solutions** in this unit (or cross-referenced unit); for common stock solutions, see **APPENDIX 2**; for suppliers, see **APPENDIX 4**.*

CTAB extraction solution (see recipe)
CTAB/NaCl solution (*UNIT 2.4*)
CTAB precipitation solution (see recipe)
2% (v/v) 2-mercaptoethanol (2-ME)
High-salt TE buffer (see recipe)
24:1 (v/v) chloroform/octanol or chloroform/isoamyl alcohol
80% ethanol

Pulverizer/homogenizer: mortar and pestle, blender, Polytron (Brinkmann), or coffee grinder
Organic solvent–resistant test tube or beaker
65°C water bath
Beckman JA-20 rotor or equivalent *or* microcentrifuge

Extract nucleic acids

1. Add 2-ME to the required amount of CTAB extraction solution to give a final concentration of 2% (v/v). Heat this solution and CTAB/NaCl solution to 65°C.

   Approximately 4 ml of 2-ME/CTAB extraction solution and 0.4 to 0.5 ml CTAB/NaCl solution are required for each gram of fresh leaf tissue. With lyophilized, dehydrated, or dry tissues such as seeds, 2-ME/CTAB extraction solution should be diluted 1:1 with sterile water. 2-ME should be used in a fume hood.

2. Chill a pulverizer/homogenizer with liquid nitrogen (–196°C) or dry ice (–78°C). Pulverize plant tissue to a fine powder and transfer the frozen tissue to an organic solvent–resistant test tube or beaker.

   Use young tissue and avoid larger stems and veins to achieve the highest DNA yield with minimal polysaccharide contamination.

3. Add warm 2-ME/CTAB extraction solution to the pulverized tissue and mix to wet thoroughly. Incubate 10 to 60 min at 65°C with occasional mixing.

   A 60-min incubation results in larger DNA yields. If maximum yield is not important, 10 min should be adequate. If the tissue contains large amounts of phenolic compounds, 1% (v/v) polyvinylpyrrolidone (mol. wt. = 40,000) may be added to absorb them.

4. Extract the homogenate with an equal volume of 24:1 chloroform/octanol or chloroform/isoamyl alcohol. Mix well by inversion. Centrifuge 5 min at 7500 × *g* (8000 rpm in JA-20; ~10,000 rpm in a microcentrifuge, for smaller samples), 4°C. Recover the top (aqueous) phase.

   Octanol, rather than isoamyl alcohol, is used because it may enhance isolation of nuclei. Slower centrifugation speeds are possible if centrifugation time is increased accordingly; a microcentrifuge may be used for small-scale preparations (≤150 mg starting tissue). After centrifugation, two phases should be evident with tissue debris at the interface.

5. Add 1/10 vol 65°C CTAB/NaCl solution to the recovered aqueous phase and mix well by inversion.

6. Extract with an equal volume of chloroform/octanol. Mix, centrifuge, and recover as in step 4 above.

   The aqueous phase may still be light yellow-brown in color.

Precipitate nucleic acids

7. Add exactly 1 vol CTAB precipitation solution. Mix well by inversion. If precipitate is visible, proceed to step 8. If not, incubate mixture 30 min at 65°C.

8. Centrifuge 5 min at 500 × *g* (2000 rpm in JA-20; ~2700 rpm in microcentrifuge), 4°C.

   Do not increase the speed or time of centrifugation as the pellet may become very difficult to resuspend. If there is no pellet, add more CTAB precipitation solution (up to 1/10 the total volume). Incubate 1 hr to overnight at 37°C. Centrifuge 5 min at 500 × g, 4°C.

9. Remove but do not discard the supernatant and resuspend pellet in high-salt TE buffer (0.5 to 1 ml per gram of starting material). If the pellet is difficult to resuspend, incubate 30 min at 65°C. Repeat until all or most of pellet is dissolved.

   Polysaccharide contamination may make it excessively difficult to resuspend the pellet. Read the A_{260} of the supernatant and discard pellet if nucleic acids are present in the supernatant.

10. Precipitate the nucleic acids by adding 0.6 vol isopropanol. Mix well and centrifuge 15 min at 7500 × *g*, 4°C.

    Ethanol can be used for the precipitation, but isopropanol may yield cleaner pellets.

11. Wash the pellet with 80% ethanol, dry, and resuspend in a minimal volume of TE (0.1 to 0.5 ml per gram of starting material).

    Residual CTAB is soluble and is removed by the 80% ethanol wash. Further purification of the DNA with RNase A and proteinase K may be done using standard methods (UNITS 2.4 & 3.13).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2; for suppliers, see APPENDIX 4.

CTAB extraction solution

2% (w/v) CTAB
100 mM Tris·Cl, pH 8.0
20 mM EDTA, pH 8.0
1.4 M NaCl
Store at room temperature (stable several years)

CTAB precipitation solution

1% (w/v) CTAB
50 mM Tris·Cl, pH 8.0
10 mM EDTA, pH 8.0
Store at room temperature (stable several years)

Extraction buffer

100 mM Tris·Cl, pH 8.0
100 mM EDTA, pH 8.0
250 mM NaCl
100 µg/ml proteinase K (add fresh before use)
Store indefinitely at room temperature without proteinase K

High-salt TE buffer

10 mM Tris·Cl, pH 8.0
0.1 mM EDTA, pH 8.0
1 M NaCl
Store at room temperature (stable for several years)

COMMENTARY

Background Information

CsCl gradient purification

This protocol is an adaptation of common DNA isolation procedures—cell lysis by detergent, protease treatment, and CsCl gradient purification. Because whole cells are lysed, DNA purified using this protocol will correspond to both the nuclear genome and cytoplasmic (mitochondrial and chloroplast) genomes. Methods for purifying nuclear DNA—free of plastid and mitochondrial DNA contamination—have been described by Watson and Thompson (1986). In addition, a miniprep protocol for isolation of total plant DNA has been described by Dellaporta et al. (1983); the miniprep protocol is similar to the protocol described here, except that it omits the CsCl gradient centrifugation.

CTAB purification

The alternate protocol, cetyltrimethylammonium bromide (CTAB) DNA isolation, was initially used in bacteria (Jones, 1953; *UNIT 2.4*) and later modified to obtain DNA from plants (Murray and Thompson, 1980). CTAB forms an insoluble complex with nucleic acids when the initial NaCl concentration is lowered

to ~0.5 M (Rogers and Bendich, 1985). Polysaccharides, phenolic compounds, and other enzyme-inhibiting contaminants found in plant cells are efficiently removed in the supernatant because most do not precipitate under the conditions described. The nucleic acid–CTAB complex is only soluble in high salt; detergent is removed by raising the NaCl concentration and precipitating the nucleic acids. The residual CTAB is removed by washing the nucleic acid pellet with 80% ethanol; CTAB is more soluble in ethanol and is discarded with the wash solution.

The CTAB method of DNA isolation is widely used on plants because of its versatility. Total genomic DNA has been isolated from many genera of monocotyledons and dicotyledons (Murray and Thompson, 1980; Rogers and Bendich, 1985). Various types of tissue can be used, including whole seedlings, leaves, cotyledons, seeds/grains, endosperm, embryos, tissue culture callus, and pollen. In addition, milligram amounts of tissue can be used when sample size is limiting. The starting material can be lyophilized, dehydrated (even mummified), frozen, or fresh.

The authors' (M.R. and S.R.) laboratory has successfully used this protocol to isolate DNA from *Arabidopsis thaliana, Zea mays, Gossypium hirsutum, Flaveria* sp., *Linum usitatissimum, Petunia hybrida, Glycine* sp., *Nicotiana tabacum*, and *Lycopersicum esculentum*. In addition, each year *The Plant Molecular Biology Reporter* publishes several modifications to this general protocol optimized for species from which nucleic acid extraction is difficult.

Critical Parameters

The aim of any genomic DNA preparation technique is to isolate high-molecular-weight DNA of sufficient purity. Two factors affect the size of the DNA isolated: shear forces and nuclease activity. As noted in the protocols, lysates should be treated gently to minimize shear forces. Plant cells are rich in nucleases. To reduce nuclease activity, the tissue should be frozen quickly and thawed only in the presence of an extraction buffer that contains detergent and a high concentration of EDTA.

Plant DNA isolated using the basic protocol should be in the range of 50 kb in length, which is quite acceptable for most applications. *Arabidopsis* DNA isolated using this protocol can be digested with restriction enzymes and ligated efficiently into cloning vectors. However, in some cases it may be necessary to modify the steps in order to reduce contamination by polysaccharides, phenolics, and other compounds that interfere with DNA isolation. Polysaccharides pose the most common problem affecting plant DNA purity. These carbohydrates are difficult to separate from the DNA itself, and they inhibit many enzymes commonly used in cloning procedures. The recommended procedure for polysaccharide removal is chloroform extraction of lysates in the presence of 1% CTAB and 0.7 M NaCl, as described by Murray and Thompson (1980) and in the alternate protocol. If the DNA pellet obtained using the alternate protocol is excessively difficult to resuspend, it may be due to the presence of polysaccharides that were not removed during CTAB precipitation. The DNA should be soluble in TE buffer; passage of the solution over an anion-exchange column should remove much of the contamination (Fang et al., 1992; *UNIT2.14*). If phenolic compounds are a problem, 1% polyvinylpyrollidone (mol. wt. = 40,000; Sigma) can be included during tissue homogenization to absorb them. Chloroform/octanol is preferred over chloroform/isoamyl alcohol for organic extractions because it has been reported to isolate nuclei more efficiently (Watson and Thompson, 1986). Finally, RNase A and proteinase K digestion followed by phenol extraction can be performed if further purification is required.

Anticipated Results

CsCl gradient purification. Yields should be in the range of 10 to 40 μg DNA (50-kb length) per gram of fresh plant tissue. Isolated DNA should digest well with restriction enzymes and be ligated efficiently into cloning vectors.

CTAB purification. Yields should be in the range of 100 to 500 μg DNA per gram of fresh plant tissue. The greatest yields will always be obtained using the youngest, freshest tissue available. DNA ≥50 kb can be obtained if care is taken not to shear it (by using wide bore pipettes and gentle mixing) and if nucleases are avoided (by keeping tissue frozen or lyophilized and thawing or rehydrating only in the presence of CTAB extraction solution). In addition to DNA, RNA is also efficiently liberated and purified by this method and can be separated from the DNA if desired (e.g., see *UNIT4.3* and Taylor and Powell, 1982).

Time Considerations

CsCl gradient purification. Approximately 4 to 6 hr are required to work through the protocol to the point where the lysate is loaded

onto the CsCl gradient. The gradient can be centrifuged overnight at 300,000 × *g* or 4 hr at 525,000 × *g*. Approximately 3 to 4 hr are required to process the banded DNA.

CTAB purification. This procedure should take between 2 and 6 hr depending on the quantity of starting material, desired purity, and yield.

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Preparation of Genomic DNA from Bacteria

BASIC PROTOCOL

MINIPREP OF BACTERIAL GENOMIC DNA

Bacteria from a saturated liquid culture are lysed and proteins removed by digestion with proteinase K. Cell wall debris, polysaccharides, and remaining proteins are removed by selective precipitation with CTAB, and high-molecular-weight DNA is recovered from the resulting supernatant by isopropanol precipitation.

Materials

TE buffer (*APPENDIX 2*)
10% sodium dodecyl sulfate (SDS)
20 mg/ml proteinase K (stored in small single-use aliquots at –20°C)
5 M NaCl
CTAB/NaCl solution
24:1 chloroform/isoamyl alcohol
25:24:1 phenol/chloroform/isoamyl alcohol (*UNIT 2.1*)
Isopropanol
70% ethanol

1. Inoculate a 5-ml liquid culture with the bacterial strain of interest. Grow in conditions appropriate for that strain (i.e., appropriate medium, drug selection, temperature) until the culture is saturated. This may take several hours to several days, depending on the growth rate.

2. Spin 1.5 ml of the culture in a microcentrifuge for 2 min, or until a compact pellet forms. Discard the supernatant.

3. Resuspend pellet in 567 µl TE buffer by repeated pipetting. Add 30 µl of 10% SDS and 3 µl of 20 mg/ml proteinase K to give a final concentration of 100 µg/ml proteinase K in 0.5% SDS. Mix thoroughly and incubate 1 hr at 37°C.

   The solution should become viscous as the detergent lyses the bacterial cell walls. There should be no need to predigest the bacterial cell wall with lysozyme.

4. Add 100 µl of 5 M NaCl and mix thoroughly.

   This step is very important since a CTAB–nucleic acid precipitate will form if salt concentration drops below about 0.5 M at room temperature (Murray and Thompson, 1980). The aim here is to remove cell wall debris, denatured protein, and polysaccharides complexed to CTAB, while retaining the nucleic acids in solution.

5. Add 80 µl of CTAB/NaCl solution. Mix thoroughly and incubate 10 min at 65°C.

6. Add an approximately equal volume (0.7 to 0.8 ml) of chloroform/isoamyl alcohol, mix thoroughly, and spin 4 to 5 min in a microcentrifuge.

   This extraction removes CTAB–protein/polysaccharide complexes. A white interface should be visible after centrifugation.

7. Remove aqueous, viscous supernatant to a fresh microcentrifuge tube, leaving the interface behind. Add an equal volume of phenol/chloroform/isoamyl alcohol, extract thoroughly, and spin in a microcentrifuge for 5 min.

   With some bacterial strains the interface formed after chloroform extraction is not compact enough to allow easy removal of the supernatant. In such cases, most of the interface can be fished out with a sterile toothpick before removal of any supernatant. Remaining CTAB precipitate is then removed in the phenol/chloroform extraction.

8. Transfer the supernatant to a fresh tube. Add 0.6 vol isopropanol to precipitate the nucleic acids (there is no need to add salt since the NaCl concentration is already

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high). Shake the tube back and forth until a stringy white DNA precipitate becomes clearly visible. At this point it is possible to transfer the pellet to a fresh tube containing 70% ethanol by hooking it onto the end of a micropipet that has been heat-sealed and bent in a Bunsen flame. Alternatively, the precipitate can be pelleted by spinning briefly at room temperature.

If no stringy DNA precipitate forms in the above step, this implies that the DNA has sheared into relatively low-molecular-weight pieces. If this is acceptable, i.e., if DNA is to be digested to completion with restriction endonucleases for Southern blot analysis, chromosomal DNA can often still be recovered simply by pelleting the precipitate in a microcentrifuge.

9. Wash the DNA with 70% ethanol to remove residual CTAB and respin 5 min at room temperature to repellet it. Carefully remove the supernatant and briefly dry the pellet in a lyophilizer.

10. Redissolve the pellet in 100 µl TE buffer.

This may take some time (up to 1 hr) since the DNA is of high molecular weight. 15 µl of this DNA will typically digest to completion with 10 U EcoRI in 1 hr, which is sufficient to be clearly visible on an agarose gel, or to give a good signal during Southern hybridization.

REMOVAL OF POLYSACCHARIDES FROM EXISTING GENOMIC DNA PREPS

SUPPORT PROTOCOL

Steps 4 through 10 of the basic protocol can be adapted for removing polysaccharides and other contaminating macromolecules from existing bacterial chromosomal DNA preparations. Simply adjust the NaCl concentration of the DNA solution to 0.7 M and add 0.1 vol CTAB/NaCl solution. A white interface after the chloroform/isoamyl extraction indicates that contaminating macromolecules have been removed. The CTAB extraction step (steps 5 and 6) can be repeated several times until no interface is visible.

MINIPREP OF BACTERIAL GENOMIC DNA

SHORT PROTOCOL

1. Grow bacterial strain to saturation.
2. Spin 1.5 ml for 2 min in microcentrifuge.
3. Resuspend in 567 µl TE buffer, 3 µl of 20 mg/ml proteinase K. Mix and incubate 1 hr at 37°C. ↑ 30µl 10% SDS
4. Add 100 µl of 5 M NaCl. Mix thoroughly.
5. Add 80 µl of CTAB/NaCl solution. Mix. Incubate 10 min at 65°C.
6. Extract with an equal volume of chloroform/isoamyl alcohol. Spin 5 min in microcentrifuge.
7. Transfer aqueous phase to a fresh tube. Extract DNA with phenol/chloroform/isoamyl alcohol. Spin 5 min in microcentrifuge.
8. Transfer aqueous phase to a fresh tube. Extract DNA with 0.6 vol isopropanol. Wash precipitate with 70% ethanol. Remove supernatant and briefly dry pellet in lyophilizer.
9. Resuspend pellet in 100 µl TE buffer.

ALTERNATE PROTOCOL

LARGE-SCALE CsCl PREP OF BACTERIAL GENOMIC DNA

This procedure is essentially a scale-up of the chromosomal miniprep described in the basic protocol, followed by additional purification on a cesium chloride gradient. This procedure may be used if large amounts of exceptionally clean genomic DNA are required, e.g., for the construction of genomic libraries.

Additional Materials

Cesium chloride
10 mg/ml ethidium bromide
CsCl-saturated isopropanol or H_2O-saturated butanol
3 M sodium acetate, pH 5.2

Beckman JA-20 rotor or equivalent
50-ml Oak Ridge centrifuge tubes
Wide-bored pipet
4-ml sealable centrifuge tubes
Beckman VTi80 rotor
3-ml plastic syringe with 15-G needle

Preparation and lysis of cells

1. Grow 100 ml culture of bacterial strain to saturation.
2. Pellet cells for 10 min at 4000 × *g* (e.g., in a Beckman JA-20 rotor at 6000 rpm). Discard supernatant.

   This, and the following steps, can be conveniently carried out using 50-ml Oak Ridge centrifuge tubes.

3. Resuspend cells gently in 9.5 ml TE buffer. Add 0.5 ml of 10% SDS and 50 µl of 20 mg/ml proteinase K. Mix thoroughly and incubate 1 hr at 37°C.

Precipitation and purification of DNA

4. Add 1.8 ml of 5 M NaCl and mix thoroughly.
5. Add 1.5 ml CTAB/NaCl solution. Mix thoroughly and incubate 20 min at 65°C.
6. Add an equal volume of chloroform/isoamyl alcohol. Extract thoroughly. Spin 10 min at 6000 × *g* (JA-20 rotor at 7000 rpm), room temperature, to separate phases.
7. Transfer aqueous supernatant to a fresh tube using a wide-bored pipet.

   The supernatant will probably be very viscous if the yield is high. An additional chloroform/isoamyl alcohol extraction, or a phenol/chloroform/isoamyl alcohol extraction, is optional but should not be necessary if the material is to be purified on a cesium chloride gradient.

8. Add 0.6 vol isopropanol and mix until a stringy white DNA pellet precipitates out of solution and condenses into a tight mass. Transfer the precipitate to 1 ml of 70% ethanol in a fresh tube, by hooking it on the end of a Pasteur pipet that has been bent and sealed in a Bunsen flame.
9. Spin the pellet 5 min at 10,000 × *g* (JA-20 rotor at 9900 rpm). Remove supernatant and redissolve the pellet in 4 ml TE buffer. This may take several hours to overnight—the DNA can be placed at 60°C to hasten the process.
10. Measure the DNA concentration on a spectrophotometer. Adjust concentration to 50 to 100 µg/ml. This will give 200 to 400 µg chromosomal DNA per 4 ml gradient.

    It is not advisable to spin larger quantities of chromosomal DNA on such a small gradient.

11. Add 4.3 g CsCl per 4 ml TE buffer. Dissolve. Add 200 µl of 10 mg/ml ethidium bromide. Transfer to 4-ml sealable centrifuge tubes. Adjust volume and balance tubes with CsCl in TE buffer (1.05 g/ml). Seal tubes. Spin 4 hr in a Beckman VTi80 rotor at 70,000 rpm, 15°C, or overnight at 55,000 rpm, 15°C.

12. Visualize gradient under longwave UV lamp. A single band should be visible. Remove band using a 15-g needle and a 3-ml plastic syringe.

    If the DNA is intact high-molecular-weight chromosomal DNA it will appear very viscous as the band is withdrawn from the gradient; hence, it is important to use a wide-bore needle to avoid mechanical shearing of the DNA. If the band appears right at the top of the gradient, then the gradient is too dense. Reduce the amount of CsCl added in step 11.

13. Remove the ethidium bromide by sequential extractions with CsCl-saturated isopropanol or water-saturated butanol, as described in *UNIT 1.7*.

14. Dialyze overnight against 2 liters TE buffer to remove CsCl.

15. Transfer DNA solution to a fresh tube. If required, precipitate chromosomal DNA as described above (steps 8 and 9) by adding 1/10 vol of 3 M sodium acetate and 0.6 vol isopropanol, and resuspend at desired concentration.

SHORT PROTOCOL

LARGE-SCALE CsCl PREP OF BACTERIAL GENOMIC DNA

1. Grow 100 ml culture of bacterial strain to saturation.
2. Spin 10 min at 4000 × *g*.
3. Resuspend pellet in 9.5 ml TE buffer, 0.5 ml of 10% SDS, and 50 µl of 20 mg/ml proteinase K. Mix and incubate 1 hr at 37°C.
4. Add 1.8 ml of 5 M NaCl. Mix thoroughly.
5. Add 1.5 ml CTAB/NaCl solution and mix. Incubate 20 min at 65°C.
6. Extract with an equal volume of chloroform/isoamyl alcohol. Spin 10 min at 6000 × *g*, room temperature.
7. Transfer aqueous phase to a fresh tube. Extract with phenol/chloroform/isoamyl alcohol if necessary. Spin as in step 6.
8. Transfer aqueous phase to a fresh tube. Precipitate DNA with 0.6 vol isopropanol. Wash precipitate with 70% ethanol. Remove supernatant and resuspend pellet in 4 ml TE buffer.
9. Measure DNA concentration. Adjust concentration to give 50 to 100 µg/ml. Add 4.3 g CsCl per 4 ml TE buffer. Add 200 µl of 10 mg/ml ethidium bromide. Transfer to sealable centrifuge tubes. Spin 4 hr at 70,000 rpm, 15°C.
10. Visualize gradient with UV light. Remove band.
11. Extract ethidium bromide with CsCl-saturated isopropanol.
12. Dialyze overnight against 2 liters TE buffer.

REAGENTS AND SOLUTIONS

***CTAB/NaCl solution** (10% CTAB in 0.7 M NaCl)*

Dissolve 4.1 g NaCl in 80 ml water and slowly add 10 g CTAB (hexadecyltrimethyl ammonium bromide) while heating and stirring. If necessary, heat to 65°C to dissolve. Adjust final volume to 100 ml.

COMMENTARY

Background Information

Most commonly used protocols for the preparation of bacterial genomic DNA consist of lysozyme/detergent lysis, followed by incubation with a nonspecific protease and a series of phenol/chloroform/isoamyl alcohol extractions prior to alcohol precipitation of the nucleic acids (Meade et al., 1984; Silhavy et al., 1982). Such procedures effectively remove contaminating proteins, but are not effective in removing the copious amounts of exopolysaccharides that are produced by many bacterial genera, and which can interfere with the activity of molecular biological enzymes such as restriction endonucleases and ligases. In this procedure, however, the protease incubation is followed by a CTAB extraction whereby CTAB complexes both with polysaccharides and with residual protein; both groups of contaminating molecules are effectively removed in the subsequent emulsification and extraction with chloroform/isoamyl alcohol. This procedure is effective in producing digestible chromosomal DNA from a variety of gram-negative bacteria, including those of the genera *Pseudomonas*, *Agrobacterium*, *Rhizobium*, and *Bradyrhizobium*, all of which normally produce large amounts of polysaccharides. If large amounts of exceptionally clean DNA are required, the procedure can be scaled up and the DNA purified on a cesium chloride gradient, as described in the alternate protocol. The method can also be used to extract high-molecular-weight DNA from plant tissue (Murray and Thompson, 1980).

Critical Parameters

The most critical parameter is the salt (NaCl) concentration of the solution containing the lysed bacteria prior to adding CTAB. If the NaCl concentration is <0.5 M then the nucleic acid may also precipitate; indeed, CTAB is frequently used for just this process (Murray and Thompson).

It is also important to maintain all solutions above 15°C, as the CTAB will precipitate below this temperature.

Anticipated Results

The typical yield from both the miniprep and the large-scale prep is 0.5 to 2 mg DNA per 100 ml starting culture (10^8 to 10^9 cells/ ml).

Time Considerations

The miniprep takes ~2 hr, including the 1-hr incubation. The large-scale prep takes slightly longer to reach the point where the CsCl gradients loaded. Subsequent steps will spread over 1 to 2 days, depending on the time of the CsCl gradient spin.

Literature Cited

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