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Purifying and Analyzing Genomic DNA

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Methods for isolating and analyzing genomic DNA are presented here. This chapter is not an attempt to provide a comprehensive treatment of the subject. Instead, a compilation of basic, streamlined protocols for isolating high-quality genomic DNA from a variety of tissues and organisms is provided. Methods provided for analyzing genomic DNA by Southern blotting include general protocols for digestion of DNA with restriction enzymes, transfer of DNA onto solid supports, labeling of probes, blocking of repetitive DNA sequences, and nucleic acid hybridization. Suggestions for troubleshooting are also described for all methods. Methods for preparing, manipulating, and mapping HMW DNA (>100 kb) are presented in Chapter 2.

Overview of Genomic DNA Analysis

HISTORICAL PERSPECTIVE

The advent of recombinant DNA techniques in the early 1970s permitted an unprecedented level of detail in genetic investigations by providing tools for genetic manipulation that were fundamentally different from those previously available. The primary change was the ability to purify and amplify small, unique DNA fragments from a background of millions of virtually identical fragments. The discovery of restriction enzymes (Smith and Wilcox 1970; Heitman 1993), the development of cloning vectors (Cohen et al. 1973; Bolivar et al. 1977), and the isolation of modification enzymes (e.g., DNA ligases [Higgins and Cozzarelli 1979]) provided many of the necessary tools.

Another major development was the recognition that the two antiparallel strands in double-stranded DNA can be separated by breaking their hydrogen bonds and can then be reannealed under appropriate conditions of ionic strength, temperature, and DNA concentration. This reannealing step has the remarkable property that, under appropriate conditions, only truly complementary strands reanneal to reform the DNA duplex. Although reannealing formed the original basis for techniques for measuring gene copy number, the most important application for reannealing has been in the detection of unique DNA (or RNA) sequences in highly complex mixtures (Southern 1975).

Southern (1975) developed the classical application combining agarose gel electrophoresis and nucleic acid reassociation that typically bears his name. Southern blotting allows the structure of a given gene to be examined within a background of genomic DNA. Since higher organisms typically have DNA complexities exceeding 1×10^9 bp, this is a formidable task. Some of this technique's many uses are building restriction maps, measuring specific gene copy number, detecting DNA sequence variations including rearrangements and certain point mutations, measuring levels of methylation at specific target sites, deducing aspects of genome structure such as hypersensitivity to DNase I, and detecting amplifications. A critical application has been the development of DNA methods for genetic linkage analysis through the use of RFLPs (Botstein et al. 1980). Although PCR (see Chapter 3) has supplanted Southern blotting for certain applications (e.g., the detection of unique and/or polymorphic loci in genomic DNA), Southern blotting is often relied on to verify or extend the results obtained with PCR.

ISSUES OF CONTAMINATION AND SAFETY

Investigators should establish a separate set of pipetting devices and reagent solutions dedicated to work with genomic DNA samples. In particular, these materials should never be used for the manipulation of cloned DNA. This precaution is aimed at minimizing or eliminating problems caused by contamination with prokaryotic episomal DNA. The reason that even a minute amount of episomal DNA can cause so substantial a problem lies in the difference between the complexity of most eukaryotic genomes and that of high-copy-number plasmid DNA molecules. For example, 3 µg of human DNA contains 1×10^6 genome copies (haploid genome equivalents), whereas a 3-kb plasmid has the same number of molecules in 3 pg. Thus, if a human genomic DNA sample were contaminated with even a few picograms of plasmid DNA, the human DNA and the plasmid DNA could result in equivalent signals on a Southern blot.

When performing the DNA isolation protocols in this chapter, always remember that pathogens may be present in samples of human origin and possibly in samples from other mammals. Human blood, blood products, and tissues may contain occult infectious materials such as hepatitis B virus and HIV that may result in laboratory-acquired infections. Investigators working with EBV-transformed lymphoblastoid cell lines are also at risk of EBV infection. Any human blood, blood products, or tissues should be considered a biohazard and should be handled accordingly. Wear disposable gloves, protective clothing, and goggles; use mechanical pipetting devices; work in a laminar-flow hood or biological safety cabinet; protect against the possibility of aerosol generation (e.g., during centrifugation or mixing by vortexing); and disinfect all waste materials before disposal. Autoclave contaminated plasticware before disposal; autoclave contaminated liquids or treat with bleach (10% [v/v] final concentration) for at least 30 minutes before disposal. Consult the local institutional safety officer for specific handling and disposal procedures. Other safety precautions are noted throughout the text and discussed in the Appendix.

Isolation of Genomic DNA

The preparation of high-quality chromosomal DNA is essential for many applications of genome analysis, including Southern blotting and the generation of recombinant DNA libraries. Although quicker methods for DNA isolation may suffice for certain applications (e.g., rapid screening by PCR), the relatively impure DNA that results from such methods may degrade during prolonged storage. Since genome analysis methods are often applied to many samples over a time span measured in years, the extra time spent in preparing high-quality DNA at the outset is often well worth the effort. Such preparations will be stable for several years, eliminating the need to repeat these isolations, a problem of considerable difficulty if access to the appropriate tissues or cell lines is limited. Moreover, samples derived from unique materials (e.g., tumors) are frequently not replaceable.

The protocols in this chapter provide a framework for isolating high-quality genomic DNA from a variety of organisms, including human beings, small mammals, animal model organisms, plants, yeast, and bacteria. All of these protocols yield HMW DNA (~50–150 kb), which remains of high quality (i.e., not degraded into smaller fragments) for several years when stored as specified below.

For each organism or tissue source, a specific procedure is provided for releasing free chromosomal DNA from its cellular or nuclear location. The first task in each of these protocols is the removal of cell walls or the breakage of membranes that would otherwise impede the entry of enzymes and detergents needed for nuclear lysis and release of DNA. Cells are typically lysed in an SDS solution containing sucrose. Sucrose is included to increase viscosity and thus minimize shearing of the DNA. Once the DNA is released, steps are taken to prevent degradation of the DNA by nucleases and to permanently remove nucleases and other proteins from the preparation. Residual proteins may inhibit enzymatic reactions (e.g., digestion with restriction enzymes) used in the analysis of the resulting DNA. Sequestration of Mg^{++} by EDTA serves to inhibit nuclease activity, whereas proteinase K is typically included to degrade cellular proteins. After the cells are lysed and nucleases are inhibited, samples are handled in basically the same maner for each DNA source. Standard techniques for DNA purification by extraction with phenol, recovery of DNA by precipitation with ethanol or isopropanol, and measurement of DNA concentration are presented in the Appendix. On p. 37, some suggestions are provided for troubleshooting if the DNA yield or purity is low.

DNA FROM MAMMALIAN SOURCES

Suspension Cultures

The protocol on pp. 6–8 provides a standard method for isolating DNA from suspension cultures (e.g., EBV-transformed lymphoblastoid cell lines). The method provided here for cell lysis (Wolff et al. 1992) is used throughout the other mammalian DNA preparations.

Collection of samples from patients and their family members can be one of the limiting steps in performing a genetic study in human beings. Establishment of permanent lymphoblastoid cell lines provides unlimited access to these samples and allows cell lines to be frozen for long-term storage. Whole blood is collected, white blood cells are isolated by fractionation of the blood sample, and EBV is used to transform the B lymphocytes. Protocols for transforming lymphoblastoid cell lines with EBV and maintaining EBV-producing cell lines are provided on pp. 9–11.

Adherent Cell Lines

Adherent cell lines (e.g., fibroblasts) can be processed in two ways. In the protocol on p. 12, adherent cells are treated with trypsin to release them from the dish surface and then lysed by using the basic lysis protocol on pp. 6–8. When multiple fibroblast cell lines are being processed, it may be easier and more convenient to bypass this treatment with trypsin and to lyse the cells while they are still attached to the dish, a process called "sliming" (p. 13).

Peripheral Blood

Several procedures, which differ only in the way in which nucleated cells are isolated, can be used to prepare DNA from peripheral blood. Centrifugation of blood in collection tubes concentrates the nucleated white blood cells at the red cell/serum interface (this layer of white blood cells is called the buffy coat layer). Centrifugation of whole blood on Ficoll/Hypaque gradients (Böyum 1968) or in commercially available cell preparation tubes (Vacutainer CPT; Becton Dickinson Labware) results in much cleaner cell preparations but is unnecessary for DNA isolation. Lysis of whole cells and isolation of nuclei is an easy way to get rid of the nonnucleated red blood cells that make up the majority of whole blood. The protocol for DNA isolation on p. 14 entails lysis of whole blood and isolation of nuclei, which can then be lysed as described in the basic lysis protocol on pp. 6–8.

Fresh or Frozen Tissue

The protocol on p. 15 can be used to isolate DNA from fresh or previously frozen tissue. The first step is to '"snap freeze" the fresh tissue in liquid nitrogen. This frozen tissue is then ground into a fine powder in the presence of liquid nitrogen so that the lysis buffer will have uniform access to all cells in the tissue. Cells are then lysed as described in the basic lysis protocol on pp. 6–8.

Genetic analysis frequently takes place in model organisms such as the mouse or the rat. Almost any cell source can be used to prepare DNA but many investigators use solid tissues such as the liver or the spleen, since they are rich in nucleated cells. Isolation of DNA from these tissues should be performed exactly as described on p. 15. The drawback to this procedure is that the animal must be sacrificed to obtain these solid tissues. Sacrifice of the animal can be avoided by obtaining DNA from a tail clipping, thus allowing genetic analysis to be performed as well as further propagation of the animal. Isolation of DNA from tail clippings can be performed by one of two methods. The first is to snap freeze the tail clipping and to proceed as described on p. 15; the second is provided on p. 16.

PROTOCOL

DNA Isolation from Tissue-culture Cell Lines Growing in Suspension

This protocol is from Wolff et al. (1992).

1. Grow a suspension culture of **lymphoblastoid cells.**

   Notes: Lymphoblastoid cells are typically grown in RPMI-1640 medium (Life Technologies 11875-093) with L-glutamine plus 10% FCS at 37°C. Within 48 hours of use in this protocol, the suspension culture should be split into one 75-ml flask and fed by adding 3 volumes of fresh medium to 1 volume of cells. A 50-ml culture should contain approximately 5×10^7 cells and yield approximately 300 µg of DNA.

   This protocol is specifically designed for lymphoblastoid cells since they are widely used, but it can be used with any suspension culture of mammalian cells.

   human blood, blood products, and tissues (see Appendix for Caution)

2. Transfer the lymphoblastoid cells and culture medium into a 50-ml conical centrifuge tube. Centrifuge in a Beckman GS-6R centrifuge (or equivalent centrifuge with swinging-bucket rotor) at 1200 rpm (330*g*) at room temperature for 10 minutes to recover the cells. Discard the medium.

3. Add 10 ml of PBS (pH 7.4) (without Ca^{++} or Mg^{++}) and resuspend the cell pellet by gently pipetting up and down. Recover the cells as described in step 2 and discard the PBS.

   Note: This washing step removes any serum remaining from the growth medium. Serum is rich in protein and can inhibit the subsequent digestion with proteinase K.

4. Add 1.2 ml of cell resuspension buffer and resuspend the cells by gently pipetting up and down. Add 8 ml of sucrose/proteinase K cell lysis buffer and mix thoroughly by gently inverting the tube several times. Incubate at 37°C overnight without agitation.

   Note: As the chromosomal DNA is released and stripped of complexed proteins, this mixture should become extremely viscous.

   Cell resuspension buffer

Component and final concentration	Amount to add per 1 liter
10 mM Tris-Cl	10 ml of 1 M (pH 7.4 at 25°C)
10 mM NaCl	2 ml of 5 M
1.5 mM $MgCl_2$	1 ml of 1.5 M
H_2O	0.987 liter

   Store at room temperature indefinitely.

Sucrose/proteinase K cell lysis buffer

Component and final concentration	Amount to add per 1 liter
27% sucrose	270 g
1x SSC	50 ml of 20x
1 mM EDTA	2 ml of 0.5 M (pH 8.0)
1% **SDS**	100 ml of 10%
H_2O	to make 1 liter
200 µg/ml proteinase K	200 mg

Combine all of the components except the proteinase K. Store at room temperature indefinitely. Just before use, warm the required volume of solution to 37°C and add the proteinase K.

SDS (see Appendix for Caution)

5. Use a pipette to transfer the lysate into a new 50-ml conical tube. Extract with 10 ml of **phenol:chloroform**:isoamyl alcohol (25:24:1) (see Appendix). Use phenol saturated with 1 M Tris-Cl (pH 8.0 at 25°C).

phenol, chloroform (see Appendix for Caution)

6. Precipitate the DNA in the aqueous phase with 1 volume of isopropanol (see Appendix), spool the DNA on a pasteur pipette (i.e., wind the DNA around the tip of a pasteur pipette), and wash the spooled DNA by repeatedly dipping it into a separate tube containing 70% ethanol. Do not dry the DNA.

Notes: When the DNA concentration is high, nucleic acid strands will form a visible precipitate, which collects into a compact mass of material that can easily be removed from the tube by spooling the DNA mass on a pasteur pipette. Spooling the DNA in this manner, instead of recovering it by centrifugation, separates the DNA from the bulk of the RNA, which has been copurified but remains in solution. This eliminates the need to add exogeneous RNase, which may be contaminated with nucleases. Washing the spooled DNA in 70% ethanol removes the majority of the salts.

No salt (e.g., sodium or ammonium acetate) is needed to precipitate the DNA in this step.

7. Place the DNA spooled on the pipette tip in 2 ml of TE (pH 7.4) in a 15-ml conical tube and allow it to sit until the DNA is released from the pipette tip. Once the DNA has been released, mix gently by low-speed vortexing or by flicking the tube with a finger to help the DNA dissolve.

Note: Although even gentle mixing may shear the DNA, the size of the DNA rarely falls below 50 kb, and the resulting preparations are excellent for most applications.

8. Extract with 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1). Use phenol saturated with 1 M Tris-Cl (pH 8.0 at 25°C).

9. Precipitate the DNA in the aqueous phase with 1/3 volume of 7.5 M ammonium acetate (pH 7.4) and 2.5 volumes of absolute ethanol (see Appendix), spool the DNA on a pasteur pipette, and wash the spooled DNA by repeatedly dipping it into a separate tube containing 70% ethanol. Do not dry the DNA.

10. Dissolve the DNA in TE (pH 7.4) at a concentration of approximately 200 µg/ml. Measure the DNA concentration (see Appendix) and adjust the final concentration to 200 µg/ml with TE (pH 7.4).

Notes: DNA at a concentration of 200 µg/ml is viscous enough to minimize shearing but can still be pipetted accurately.

Pure DNA samples that are free of contaminating nucleases can be stored at 4°C for several years. However, since slow degradation may occur at this temperature, −80°C is recommended for prolonged storage. If a nonfrost-free freezer is available, DNA can also be stored at −20°C.

Comments

■ Poor recovery of DNA should be evident during the first precipitation step. If the DNA does not precipitate from solution at this point, the following steps can be used to recover the DNA.

1. Add an additional 10 ml of isopropanol to the DNA solution and mix by inverting the tube.

   Note: This large excess of isopropanol should turn the solution cloudy.

2. Centrifuge in a Beckman GS-6R centrifuge at 3000 rpm (2060*g*) at room temperature for 10 minutes to separate the mixture into two phases.

3. Use a Pipetman to transfer the lower phase (which is the clear viscous solution containing the DNA) into a 1.8-ml microcentrifuge tube. Discard the upper phase.

4. Centrifuge in a microcentrifuge at 12,000*g* at room temperature for 1 minute. Remove and discard any remaining isopropanol (upper phase).

5. Precipitate the DNA in the lower phase with 1/3 volume of 7.5 M ammonium acetate (pH 7.4) and 2.5 volumes of absolute ethanol (see Appendix). For visible DNA precipitates, spool the DNA on a pasteur pipette. For DNA that does not form a visible precipitate, recover the DNA by centrifugation. Wash with 70% ethanol, but do not dry the DNA.

6. For visible DNA precipitates, dissolve the DNA in 0.5 ml of TE (pH 7.4). For DNA that does not form a visible precipitate, dissolve the DNA in 0.2–0.5 ml of TE (pH 7.4). Measure the DNA concentration (see Appendix) and adjust the final concentration to 200 µg/ml with TE (pH 7.4).

PROTOCOL

Transforming Lymphoblasts with EBV

This protocol is from L. Jerominski (pers. comm.).

1. Collect **blood** in a yellow-top acid citrate dextrose tube (8.5-ml ACD tube; Becton Dickinson Labware).

   Notes: Keep the blood at room temperature for the transformation. Blood stored at 4°C does not transform very well. To obtain the best results, cultures should be prepared from the blood as soon as possible.

   Blood must be collected under sterile conditions by a trained phlebotomist. The remainder of this protocol should also be performed under sterile conditions.

   Human blood, blood products, and tissues (see Appendix for Caution)

2. Use a 10-ml syringe fitted with an 18-gauge needle to transfer 8 ml of blood into a cell preparation tube (Vacutainer CPT; Becton Dickinson Labware).

3. Centrifuge in a Beckman GS-6R centrifuge (or equivalent centrifuge with swinging-bucket rotor) at 2500 rpm (1200–1500*g*) at room temperature for 20 minutes.

4. Remove as much plasma as possible without aspirating the cells. Use a cotton-plugged pasteur pipette to transfer the white blood cell layer into a 15-ml centrifuge tube.

   Note: White blood cells will form a white layer just above the polyester gel of the cell preparation tube.

5. Add 10 ml of RPMI-1640 medium with L-glutamine but no added serum.

6. Centrifuge in a Beckman GS-6R centrifuge (or equivalent centrifuge with swinging-bucket rotor) at 1000 rpm (200*g*) at room temperature for 10 minutes. Discard the supernatant.

7. Resuspend the cells in 4 ml of RPMI-1640 medium with L-glutamine plus 15% FCS.

8. Inoculate the cells with 400 µl of **EBV** (from p. 11), and then add 2 µl of cyclosporin A solution (1 mg/ml in H_2O; Sandoz Pharmaceutical Co.). Transfer the cells and medium into a T-25 flask and incubate at 37°C. Do not disturb the flask for 10 days.

   EBV (see Appendix for Caution)

9. At the end of 10 days, feed the cells by adding 4 ml of RPMI-1640 medium with L-glutamine plus 15% FCS.

10. Incubate the cells at 37°C. Do not disturb the flask for an additional 7 days.

11. At the end of 7 days, feed the cells by adding 10 ml of RPMI-1640 medium with L-glutamine plus 15% FCS.

12. Incubate the cells at 37°C. Monitor their growth every 2 days. If the culture is doing well, lots of cell clumps will begin to appear and the medium will become very acidic and turn yellow. Once the culture is well established, split the culture into one T-75 flask and feed by adding 3 volumes of fresh medium to 1 volume of cells.

Note: Cells can be used to isolate DNA as described on pp. 6–8 or they can be stored in liquid nitrogen for years.

PROTOCOL

Maintaining B95-8 and IA-3 EBV-producing Cell Lines

B95-8 and IA-3 are **EBV**-transformed marmoset B-cell lines developed by B. Sugden, who provided the maintenance guidelines presented here (pers. comm.).

1. Grow **B95-8** or **IA-3** cells in T-25-ml flasks in RPMI-1640 medium with L-glutamine plus 5% bovine calf serum at 37°C. Passage the cultures twice a week (on Mondays and Thursdays or on Tuesdays and Fridays) by transferring 2 ml of cells into a new flask containing 6 ml of fresh medium.

   Note: Passaging the cells twice a week should allow them to reach a density of 1.2×10^6 to 1.5×10^6 cells/ml.

   EBV (see Appendix for Caution)

2. When freshly prepared virus is needed, prepare it at the same time that the culture is passaged as follows:

   a. Transfer the remaining cells in culture (i.e., ~6 ml of cells remaining in the original flask after passaging the cells) into a 15-ml conical tube. Centrifuge in a Beckman GS-6R centrifuge (or equivalent centrifuge with swinging-bucket rotor) at 1000 rpm (200*g*) at room temperature for 10 minutes.

      Note: Virus production is dependent on the cell density. Therefore, there should be at least 1×10^6 cells/ml in a culture used for making virus.

   b. Discard the cell pellet. Pass the supernatant through a sterile 0.22-µm filter to remove all remaining marmoset cells from the virus. The remaining solution is the viral stock.

      Note: Filtered virus can be stored at 4°C for up to 1 week.

3. Once a month, test the B95-8 or IA-3 cultures for virus production by immunofluorescence staining with viral capsid antigen. A good culture should be 5–10% positive. Discard any culture that is less than 5% positive and start a new culture from a frozen aliquot of the cells.

   Note: During the time that virus production is high (i.e., when the culture is 5–10% positive), aliquots of the culture can be stored at in liquid nitrogen for use in preparing new cultures if virus production declines.

PROTOCOL

DNA Isolation from Adherent Fibroblasts Using Trypsin

This protocol is from Cox et al. (1990).

1. Grow **fibroblasts** in the appropriate growth medium at 37°C until the cells become 90–100% confluent.

   Notes: A 10-cm tissue-culture dish at 90–100% confluence should contain approximately 2 x 10^7 cells and yield approximately 120 µg of DNA.

   The growth medium used throughout this protocol varies with the particular type of fibroblast.

   human blood, blood products, and tissues (see Appendix for Caution)

2. When cell densities reach the desired level, transfer the dish(es) into a laminar-flow hood and remove the medium by aspiration.

3. Add 8 ml of PBS (pH 7.4) (without Ca^{++} or Mg^{++}) to each dish and swirl gently. Remove the PBS by aspiration.

   Note: This washing step removes any serum remaining from the growth medium. Serum will inactivate the trypsin.

4. Add 2 ml of 0.25% trypsin in HBSS without Ca^{++} or Mg^{++}, and swirl gently to mix. Incubate at 37°C for 5 minutes.

   Note: Sheets of cells should lift off the dish when the dish is swirled at the end of this incubation.

5. Tilt the dish and break up the clumps of cells by pipetting the cells up and down with a pasteur pipette.

6. Transfer the cells and trypsin solution into 8 ml of growth medium containing 10% FCS in a 15-ml conical tube and mix by inverting the tube.

   Note: The serum in the medium inactivates the trypsin.

7. Centrifuge in a Beckman GS-6R centrifuge (or equivalent centrifuge with swinging-bucket rotor) at 1200 rpm (330*g*) at room temperature for 10 minutes to recover the cells. Discard the medium.

8. Resuspend the cells in 10 ml of PBS (pH 7.4) (without Ca^{++} or Mg^{++}). Recover the cells as described in step 7 and remove the PBS by aspiration.

   Note: This washing step removes the serum. Serum is rich in protein and therefore can inhibit the subsequent digestion with proteinase K.

9. Follow steps 4–10 on pp. 6–8.

PROTOCOL

DNA Isolation from Adherent Fibroblasts ("Sliming")

This protocol is from K. Fraser (pers. comm.).

1. Grow **fibroblasts** in the appropriate growth medium at 37°C until the cells become 90–100% confluent.

   Notes: A 10-cm tissue-culture dish at 90–100% confluence should contain approximately 2 x 10^7 cells and yield approximately 120 µg of DNA.

   The growth medium used throughout this protocol varies with the particular type of fibroblast.

   human blood, blood products, and tissues (see Appendix for Caution)

2. When cell densities reach the desired level, transfer the dish(es) into a laminar-flow hood and remove the medium by aspiration.

3. Add 8 ml of PBS (pH 7.4) (without Ca^{++} or Mg^{++}) to each dish and swirl gently. Remove the PBS by aspiration.

   Note: This washing step removes any serum remaining from the growth medium. Serum is rich in protein and therefore can inhibit the subsequent digestion with proteinase K.

4. Add 1.5 ml of sucrose/proteinase K cell lysis buffer (for preparation, see p. 7) to each dish. Swirl each dish, tilting the dish slightly to allow the lysis buffer to come into contact with the entire surface.

   Note: As the cells lyse, this solution should become extremely viscous. This process should take approximately 30 seconds.

5. Combine the lysate from all dishes from the same cell line by pouring them into a 15- or 50-ml conical centrifuge tube. Incubate at 37°C overnight without agitation.

6. Follow steps 5–10 on pp. 7–8.

PROTOCOL

DNA Isolation from Peripheral Blood

This protocol is from L. Jerominski (pers. comm.).

1. Collect **blood** in yellow-top acid citrate dextrose tubes (8.5-ml ACD tubes; Becton Dickinson Labware).

   Note: Blood must be collected under sterile conditions by a trained phlebotomist.

   human blood, blood products, and tissues (see Appendix for Caution)

2. Transfer 20 ml of blood into a 50-ml conical tube. Add 20 ml of chilled 2x sucrose solution for nuclei isolation and mix gently by inverting the tube.

   Note: A 20-ml sample of blood should yield approximately 400 µg of DNA.

   2x Sucrose solution for nuclei isolation

Component and final concentration	Amount to add per 1 liter
0.637 M sucrose	218 g
20 mM Tris base	2.4 g
10 mM $MgCl_2 \cdot 6H_2O$	2.03 g
2% Triton X-100	20 ml
H_2O	to make 1 liter

   Combine the components. Adjust the pH to 7.6 with **concentrated HCl**. Store at room temperature indefinitely. Chill to 4°C before use.

   concentrated HCl (see Appendix for Caution)

3. Centrifuge in a Beckman GS-6R centrifuge (or equivalent centrifuge with swinging-bucket rotor) at 2500 rpm (1400*g*) at 4°C for 30 minutes. Decant the supernatant.

4. Add 1 volume of chilled 1x sucrose solution for nuclei isolation (a 1:1 dilution of the 2x solution in H_2O) and resuspend the nuclei pellet by pipetting up and down with a 25-ml disposable pipette.

   Note: This pellet is sticky and difficult to resuspend, but an attempt should be made to break up the pellet at least to some degree.

5. Repeat step 3.

6. Add 0.25 volume of sucrose/proteinase K cell lysis buffer (for preparation, see p. 7) and pipette up and down to partially break up the pellet. Incubate at 37°C overnight.

   Note: This pellet will dissolve during the incubation.

7. Follow steps 5–10 on pp. 7–8.

PROTOCOL

DNA Isolation from Fresh and Frozen Tissues

This protocol is from Wolff et al. (1992).

1. Place 100 mg to 2 g of frozen **tissue** in a mortar half filled with liquid nitrogen. (If the tissue is fresh, snap freeze it in **liquid nitrogen**.) Grind the frozen tissue into a fine powder using a clean pestle that has been chilled in liquid nitrogen. Be careful to avoid propelling chunks of frozen tissue out of the mortar by overly vigorous grinding.

   Notes: Frozen tissue should be stored at −80°C or −135°C until used for this procedure.

   It is important that the mortar be a tenth to half filled with liquid nitrogen throughout the grinding process. As the liquid nitrogen boils away, it can carry with it a fine powder of frozen material. This problem becomes particularly acute when the liquid nitrogen is nearly gone.

   Be sure to wear thermal gloves during this procedure.

   This protocol can be used to isolate DNA from solid tissues such as the liver or the spleen of various animals. Procedures for the humane treatment of animals must be observed at all times. Check with the local animal facility for guidelines.

   The DNA yield will vary widely, depending on the source of the tissue.

   human blood, blood products, and tissues; liquid nitrogen (see Appendix for Caution)

2. Pour the pulverized tissue and liquid nitrogen into a 15-ml conical centrifuge tube and allow the remaining liquid nitrogen to boil away.

3. For each 100 mg of tissue, add 1 ml of sucrose/proteinase K cell lysis buffer (for preparation, see p. 7). Mix gently by inverting the tube. Incubate at 37°C overnight.

   Note: As chromosomal DNA is released, this mixture should become extremely viscous. Any small chunks of tissue that were not completely pulverized should soften and dissolve completely during the overnight incubation.

4. Follow steps 5–10 on pp. 7–8.

PROTOCOL

DNA Isolation from a Mammalian Model Organism: The Mouse

This protocol is from N. Lonberg (pers. comm.).

1. Clip 0.5–1 inch from the end of the mouse's tail, and place the clipping in 1 ml of sucrose/proteinase K cell lysis buffer (for preparation, see p. 7). Incubate at 55°C overnight.

   Notes: The tail should dissolve. As the chromosomal DNA is released and stripped of complexed proteins, the resulting solution should become extremely viscous.

   Procedures for the humane treatment of animals must be observed at all times. Check with the local animal facility for guidelines.

   This protocol should yield approximately 50–100 μg of DNA.

2. Follow steps 5–10 on pp. 7–8.

DNA FROM ANIMAL MODEL ORGANISMS

The methods for lysis are modified slightly in these protocols to accommodate differences between these model organisms and mammals, but the final purification and recovery steps are essentially identical to those used above for mammalian DNA.

The Fruit Fly *D. melanogaster*

DNA is isolated from 20 whole flies in the protocol on pp. 18–19. Flies can be stored at –20°C for several weeks before the DNA is isolated. For a discussion of the study of *D. melanogaster*, see Lindsley and Zimm (1992).

The Nematode *C. elegans*

The protocol on pp. 20–22 for isolating DNA from the nematode *C. elegans* is straightforward and can be performed rapidly. This protocol makes use of a rapidly growing culture of nematodes, mainly consisting of L1s (early larval stage). For a discussion of the study of *C. elegans*, see Hodgkin et al. (1995).

PROTOCOL

DNA Isolation from *D. melanogaster*

This protocol is from E. Wolff (pers. comm.).

1. Immobilize the flies of interest by anesthetizing them with **ether** or CO_2 or by chilling them to –20°C. Place 20 flies in a 1.5-ml microcentrifuge tube.

   diethyl ether (see Appendix for Caution)

2. Add 400 µl of homogenizing buffer to the flies. Use a glass rod to grind the flies. Incubate at 65°C for 30 minutes.

   Homogenizing buffer

Component and final concentration	Amount to add per 1 liter
100 mM Tris-Cl	100 ml of 1 M (pH 9.0 at 25°C)
100 mM EDTA	200 ml of 0.5 M (pH 8.0)
1% **SDS**	100 ml of 10%
H_2O	0.6 liter

   Store at room temperature indefinitely.

   SDS (see Appendix for Caution)

3. Add 56 µl of 8 M potassium acetate and mix. Place on ice for 30 minutes.

4. Centrifuge in a microcentrifuge at 12,000*g* at 4°C for 15 minutes.

5. Transfer the supernatant into a new 1.5-ml microcentrifuge tube. Discard the pellet.

6. Precipitate the DNA as follows:

   a. Add 200 µl of isopropanol and mix by inverting the tube. Allow to stand at room temperature for 5 minutes.

   b. Centrifuge in a microcentrifuge at 12,000*g* at room temperature for 5 minutes. Decant the supernatant.

   c. Wash the pellet by adding 400 µl of 70% ethanol, recentrifuging for 5 minutes, and discarding the supernatant.

   d. Air dry the DNA pellet.

7. Dissolve the DNA in 200 µl of TE (pH 7.4).

8. Extract with 1 volume of **phenol**:**chloroform**:isoamyl alcohol (25:24:1) (see Appendix). Use phenol saturated with 1 M Tris-Cl (pH 8.0 at 25°C).

phenol, chloroform (see Appendix for Caution)

9. Precipitate the DNA as follows:

   a. Add 1/3 volume of 7.5 M ammonium acetate (pH 7.4) and 2.5 volumes of absolute ethanol to the aqueous phase and mix by inverting the tube. Place on dry ice for at least 10 minutes.

   b. Centrifuge in a microcentrifuge at 12,000*g* at 4°C for 10 minutes. Decant the supernatant.

      Note: Since the amount of DNA is small (~10 µg), it should be recovered by centrifugation instead of by spooling.

   c. Wash the pellet by adding 400 µl of 70% ethanol, recentrifuging for 5 minutes, and discarding the supernatant.

   d. Air dry the DNA pellet.

10. Dissolve the DNA in 100 µl of TE (pH 7.4). Measure the DNA concentration (see Appendix) and adjust the final concentration to 100 µg/ml with TE (pH 7.4).

    Notes: Pure DNA samples that are free of contaminating nucleases can be stored at 4°C for several years. However, since slow degradation may occur at this temperature, −80°C is recommended for prolonged storage. If a nonfrost-free freezer is available, DNA can also be stored at −20°C.

    Since each fly yields approximately 0.5 µg (5 µl) of DNA, this protocol produces sufficient DNA for 5–10 lanes on a Southern blot. Approximately 10–20 µl (1–2 µg) of DNA should be used in each digestion for Southern analysis. Inclusion of 2 µl of DNase-free RNase A (1 mg/ml solution; for preparation, see p. 29) during digestion with restriction enzymes will remove RNA from the preparation.

PROTOCOL

DNA Isolation from *C. elegans*

This protocol is from B. Kimmel (pers. comm.).

1. Select a large NGM dish (10 cm) in which the *E. coli* lawn has just cleared (i.e., has become translucent). (This population will consist mostly of nematodes in the L1 stage.) Wash the nematodes off the surface of the dish by adding 5–10 ml of 200 mM Tris-Cl/100 mM EDTA/400 mM NaCl to the dish and then using a 10-ml pipette to transfer the nematode-containing solution into a 13-mm x 100-mm glass test tube.

Notes: A 10-cm dish yields approximately 100 µl of packed nematodes. The DNA yield should be approximately 100 µg per 10-cm dish.

For a discussion of methods for working with *C. elegans*, see Sulston and Hodgkin (1988).

NGM

Component and final concentration	Amount to add per 1 liter
0.3% NaCl	3 g
2.5% peptone	25 g
1.7% agarose	17 g
H_2O	972 ml
5 µg/ml cholesterol	1 ml of 5 mg/ml (in ethanol)
1 mM $CaCl_2$	1 ml of 1 M
1 mM $MgSO_4$	1 ml of 1 M
25 mM $KHPO_4$	25 ml of 1 M (pH 6.0)

Combine the NaCl, peptone, agarose, and H_2O. Sterilize by autoclaving. Allow to cool to 65°C, and then add the cholesterol, $CaCl_2$, $MgSO_4$, and $KHPO_4$. Store at 4°C for up to 3 weeks.

200 mM Tris-Cl/100 mM EDTA/400 mM NaCl

Component and final concentration	Amount to add per 1 liter
200 mM Tris-Cl	200 ml of 1 M (pH 7.5 at 25°C)
100 mM EDTA	200 ml of 0.5 M (pH 8.0)
400 mM NaCl	125 ml of 5 M
H_2O	475 ml

Store at room temperature indefinitely.

2. Centrifuge in a clinical centrifuge on setting 5 at room temperature for 3 minutes to recover the nematodes. Discard the supernatant.

3. Wash the pellet four times as follows:

   a. Add 10 ml of 200 mM Tris-Cl/100 mM EDTA/400 mM NaCl and pipette up and down.

   b. Centrifuge as described in step 2 and discard the supernatant.

   Note: Washing the nematodes reduces the amount of contaminating bacterial DNA carried over from the plate into the final product.

4. Transfer the washed nematodes into a 1.5-ml microcentrifuge tube and centrifuge in a microcentrifuge at 12,000*g* at room temperature for 1 minute. Remove as much liquid as possible by aspiration.

5. Place the nematode pellet on dry ice for 10 minutes and then store at –80°C until convenient to proceed to step 6.

   Note: Frozen nematode pellets can be stored for at least 3 weeks with no adverse effects.

6. Prepare the lysate as follows:

   a. Thaw the pellet by adding 0.5 ml of nematode lysis buffer equilibrated to 65°C.

   Nematode lysis buffer

Component and final concentration	Amount to add per 1 liter
100 mM NaCl	20 ml of 5 M
100 mM Tris-Cl	100 ml of 1 M (pH 8.5 at 25°C)
50 mM EDTA	100 ml of 0.5 M (pH 8.0)
1% **SDS**	100 ml of 10%
H_2O	to make 1 liter
1% (v/v) **β-mercaptoethanol**	10 ml of 14.3 M
100 µg/ml proteinase K	100 mg

   Combine all of the components except the β-mercaptoethanol and proteinase K. Store at room temperature indefinitely. Add the β-mercaptoethanol and proteinase K just before use.

   SDS, **β-mercaptoethanol** (see Appendix for Caution)

   b. Immediately pipette up and down through a cut-off P-1000 pipette tip and then mix by inverting the tube several times.

   Note: Cut the pipette tips to widen the opening to approximately 2 mm. Be sure to mix the contents thoroughly and disperse the nematode bodies.

   c. Incubate at 65°C for 1 hour, inverting the tube every 20 minutes.

   Note: The nematodes should dissolve.

7. Extract the lysate twice with 1 volume of **phenol** (see Appendix). Use phenol saturated with 1 M Tris-Cl (pH 8.0 at 25°C). If a white interface remains, repeat the extraction until this material is completely removed.

   phenol (see Appendix for Caution)

8. Extract with 1 volume of **chloroform** to remove residual phenol.

   chloroform (see Appendix for Caution)

9. Precipitate the DNA in the aqueous phase with 1/3 volume of 7.5 M ammonium acetate (pH 7.4) and 2.5 volumes of absolute ethanol (see Appendix). Do not dry the DNA pellet.

10. Add 400 µl of nematode lysis buffer (equilibrated to 65°C) to the DNA and allow the pellet to rehydrate at 65°C for 30 minutes.

11. Flick the tube with a finger to loosen the pellet and then mix by inverting the tube. Incubate at 65°C for an additional 2–4 hours, inverting the tube every 30 minutes.

    Note: The pellet should dissolve completely.

12. During the last 30 minutes of incubation, add 40 µl of DNase-free RNase A (1 mg/ml in TE [pH 7.4]) and allow the incubation to continue for 30 minutes.

13. Repeat steps 7–9, but allow the final pellet to air dry for 5 minutes.

14. Dissolve the DNA in 200 µl of TE (pH 7.4). Measure the DNA concentration (see Appendix) and adjust the final concentration to 100 µg/ml with TE (pH 7.4).

    Note: Pure DNA samples that are free of contaminating nucleases can be stored at 4°C for several years. However, since slow degradation may occur at this temperature, –80°C is recommended for prolonged storage. If a nonfrost-free freezer is available, DNA can also be stored at –20°C.