Chapter 6

Preparation and Analysis of Eukaryotic Genomic DNA

Cell DNA

I am the singular
in free fall.
I and my doubles
carry it all:

life's slim volume
spirally bound.
It's what I'm about,
it's what I'm around.

Presence and hungers
imbue a sap mote
with the world as they spin it.
I teach it by rote

but its every command
was once a miscue
that something rose to,
Presence and freedom

re-wording, re-beading
strains on a strand
making I and I more different
than we could stand.

Les Murray

DOUBLE-STRANDED DNA IS A REMARKABLY INERT CHEMICAL. Its potentially reactive groups are buried within the central helix, tied up in hydrogen bonds. Its base pairs are protected on the outside by a formidable casing of phosphates and sugars and are reinforced internally by strong stacking forces. With such robust shielding and scaffolding, DNA outlasts most other intracellular components in locations as disparate as modern day crime scenes and ancient burial sites. The same chemical durability endows libraries of genomic DNA with both permanence and value, thereby enabling genetic engineering and sequencing projects, both large and small.

Despite its chemical stability, double-stranded DNA is nevertheless physically fragile. Long and snaky, with little lateral stability, high-molecular-weight DNA is vulnerable to hydrodynamic shearing forces of the most modest kind (please see Table 12-1 in Chapter 12). Double-stranded DNA behaves in solution as a random coil that is stiffened by stacking interactions between the base pairs and electrostatic repulsion between the charged phosphate groups in the DNA backbone. Hydrodynamic flow — resulting from pipetting, shaking, or stirring — generates drag on the stiffened coil and has the capacity to shear both strands of the DNA. The longer the DNA molecule, the weaker the force required for breakage. Genomic DNA is therefore easy to obtain in fragmented form but becomes progressively more difficult to isolate as the desired molecular weight increases. DNA molecules >150 kb are prone to breakage by forces generated during procedures commonly used to isolate genomic DNA.

The method described in Protocol 1 involves digesting eukaryotic cells or tissues with proteinase K in the presence of EDTA (to sequester divalent cations and thereby inhibit DNases) and solubilizing membranes and denaturing proteins with a detergent such as SDS. The nucleic acids are then purified by phase extractions with organic solvents. Contaminating RNA is eliminated by digestion with an RNase, and low-molecular-weight substances are removed by dialysis. This method can be scaled to yield amounts of DNA ranging from less than ten to more than hundreds of micrograms of DNA. However, shearing forces are generated at every step, with the result that the DNA molecules in the final preparation rarely exceed 100–150 kb in length. DNA of this size is adequate for Southern analysis on standard agarose gels, as a template in polymerase chain reactions (PCRs), and for the construction of genomic DNA libraries in bacteriophage λ vectors.

The successful construction of libraries in higher-capacity vectors and the analysis of genomic DNA by pulsed-field gel electrophoresis require DNAs >200 kb in size, which are well beyond the reach of methods that generate significant hydrodynamic shearing forces. Protocol 2 describes a method for isolating and purifying DNA that generates molecules of a size suitable for these specialized purposes. An alternative method for preparing genomic DNA in agarose plugs is described in Chapter 5, Protocol 13.

In this chapter, we also describe ways to isolate genomic DNA from different samples of cells and tissues (Protocol 3) and from many samples grown in microtiter dishes (Protocol 4). Other protocols describe the preparation of DNA from mouse tails (Protocol 5), the rapid isolation of mammalian DNA (Protocol 6), and the rapid isolation of yeast DNA (Protocol 7). Finally, we describe how to analyze purified genomic DNAs by Southern blotting and hybridization (Protocols 8 through 10). Note also that a number of commercial kits are available for purifying genomic DNA.

Protocol 1

Isolation of High-molecular-weight DNA from Mammalian Cells Using Proteinase K and Phenol

THIS PROCEDURE IS DERIVED FROM A METHOD ORIGINALLY described by Daryl Stafford and colleagues (Blin and Stafford 1976). It is the method of choice when large amounts of mammalian DNA are required, for example, for Southern blotting (Protocol 8) or for construction of genomic libraries in bacteriophage λ vectors (Chapter 2, Protocol 19). Approximately 200 μg of mammalian DNA, 100–150 kb in length, is obtained from 5×10^7 cultured aneuploid cells (e.g., HeLa cells). The usual yield of DNA from 20 ml of normal blood is ~250 μg.

All of the materials listed below are required for purification of mammalian genomic DNA, irrespective of the type of sample. Additional materials that are needed for particular types of samples are listed under the subheads for the four Step 1 methods: lysis of cells growing in monolayers, lysis of cells growing in suspension, lysis of tissue samples, and lysis of blood cells in freshly drawn or frozen samples.

MATERIALS

CAUTION: Please see Appendix 12 for appropriate handling of materials marked with <!>.

▲ **WARNING** Primate tissues and primary cultures of cells require special handling precautions.

Buffers and Solutions

Please see Appendix 1 for components of stock solutions, buffers, and reagents. Dilute stock solutions to the appropriate concentrations.

Ammonium acetate (10 M) (used as an alternative to dialysis, Step 9)

Dialysis buffer (used as an alternative to ethanol precipitation, Step 9)

50 mM Tris-Cl (pH 8.0)
10 mM EDTA (pH 8.0)

Prepare four lots of 4 liters of dialysis solution and store at 4°C.

Ethanol (used as an alternative to dialysis, Step 9)

Lysis buffer

10 mM Tris-Cl (pH 8.0)
0.1 M EDTA (pH 8.0)
0.5% (w/v) SDS
20 μg/ml DNase-free pancreatic RNase

The first three ingredients of the lysis buffer may be mixed in advance and stored at room temperature. RNase is added to an appropriate amount of the mixture just before use. Adding RNase to the lysis buffer

eliminates the need to remove RNA from semipurified DNA at a later stage in the preparation. Pancreatic RNase is not highly active in the presence of 0.5% SDS, but when added at high concentrations, it works well enough to degrade most of the cellular RNA.

Phenol, equilibrated with 0.5 M Tris-Cl (pH 8.0) <!>

▲ **IMPORTANT** The pH of the phenol must be ~8.0 to prevent DNA from becoming trapped at the interface between the organic and aqueous phases (please see Appendix 8).

TE (pH 8.0)

Tris-buffered saline (TBS)

Enzymes and Buffers

Proteinase K (20 mg/ml)

For this protocol, we recommend the use of a genomic grade proteinase K that has been shown to be free of DNase and RNase activity. Please see Appendix 4.

Gels

Pulsed-field gel (please see Chapter 5, Protocols 17 and 18) or Conventional horizontal 0.6% agarose gel (Chapter 5, Protocol 1)

Nucleic Acids and Oligonucleotides

Bacteriophage λ DNA, intact

Purify λ DNA as described in Chapter 2, Protocol 11 or 12. The DNA is used as a size standard during gel electrophoresis (please see Step 11).

Centrifuges and Rotors

Sorvall centrifuge with H1000B and SS-34 rotors (or their equivalents)

Special Equipment

Cut-off yellow tips

Cut-off yellow tips can be generated rapidly with a scissors or a dog nail clipper (e.g., Fisher 05-401A). Alternatively, the pointed ends of the tips can be removed with a sharp razor blade. The cut-off tips should be sterilized before use, either by autoclaving or by immersion in 70% alcohol for 2 minutes followed by drying in air. Alternatively, presterilized wide-bore tips can be purchased from a number of commercial companies (e.g., Bio-Rad).

Dialysis tubing clips

Spectra Por closures from Spectrum Medical Industries, Houston, Texas.

Rocking platform or Dialysis tubing

Prepared as described in Appendix 8.

Shepherd's crooks (used as an alternative to dialysis)

Shepherd's crooks are Pasteur pipettes whose tip has been sealed in the flame of a Bunsen burner and shaped into a U with a hemostat. Wear safety glasses while molding the Shepherd's crooks. For further information, please see Steps 5–7 of Protocol 3.

Spectrophotometer or Fluorometer

Tube mixer or Roller apparatus

Vacuum aspirator equipped with traps

Water bath, preset to 50°C

Wide-bore pipettes (0.3-cm diameter orifice)

Wide-bore pipettes are available from several manufacturers. However, standard glass pipettes can be used if they are autoclaved in the wrong orientation without cotton plugs.

Cells and Tissues

Monolayers or suspensions of mammalian cells, or fresh tissue, or blood samples

METHOD

Below are four alternative versions of Step 1 used to lyse different types of cells or tissue samples. Use the version appropriate for the material under study and then proceed to Step 2 on page 6.9.

Lysis of Cells Growing in Monolayers

Additional Materials

Aspiration device attached to a vacuum line equipped with traps
Bed of ice large enough to accommodate 10–12 culture dishes
Erlenmeyer flask (50 or 100 ml)
Rubber policeman
Sorvall centrifuge, H1000B rotor (or equivalent) and centrifuge tubes cooled to 4°C
Tris-buffered saline (TBS), ice cold

1. Lyse cells growing in monolayer cultures.

   It is best to work with batches of 10–12 culture dishes at a time. Store the remaining culture dishes in the incubator until they are required.

   a. Take one batch of culture dishes, containing cells grown to confluency, from the incubator and immediately remove the medium by aspiration. Working quickly, wash the monolayers of cells twice with ice-cold TBS. This is most easily accomplished by gently pipetting ~10 ml of TBS onto the first monolayer. Swirl the dish gently for a few seconds and then tip the fluid into a 2-liter beaker. Add another 10 ml of ice-cold TBS and store the dish on a bed of ice. Repeat the procedure until the entire batch of monolayers has been processed.

   b. Tip the fluid from the first monolayer into the 2-liter beaker. Remove the last traces of TBS from the culture dish by aspiration. Add 1 ml of fresh ice-cold TBS and store the dish on a bed of ice. Repeat the procedure until the entire batch of monolayers has been processed.

   c. Use a rubber policeman to scrape the cells from the first culture dish into the 1 ml of TBS. Use a Pasteur pipette to transfer the cell suspension to a centrifuge tube on ice. Immediately wash the culture dish with 0.5 ml of ice-cold TBS, and combine the washings with the cell suspension in the centrifuge tube. Process the remaining monolayers in the same way.

   d. Recover the cells by centrifugation at 1500*g* (2700 rpm in a Sorvall H1000B rotor and swinging buckets) for 10 minutes at 4°C.

   e. Resuspend the cells in 5–10 volumes of ice-cold TBS and repeat the centrifugation.

   f. Resuspend the cells in TE (pH 8.0) at a concentration of 5×10^7 cells/ml. Transfer the solution to an Erlenmeyer flask.

      For 1 ml of cell suspension, use a 50-ml flask; for 2 ml, use a 100-ml flask, and so on. The density of cells grown as a monolayer culture (from Step a above) will vary with the cell type and culture conditions. As a rule of thumb, a confluent continuous culture (e.g., of HeLa or BHK cells) grown on a 90-mm culture dish contains on average 1×10^5 to 3×10^5 cells/cm^2.

   g. Add 10 ml of lysis buffer for each milliliter of cell suspension. Incubate the suspension for 1 hour at 37°C, and then proceed immediately to Step 2 (p. 6.9).

      Make sure that the cells are well dispersed over the inner surface of the Erlenmeyer flask when the lysis buffer is added. This dispersal minimizes the formation of intractable clumps of DNA.

Lysis of Cells Growing in Suspension

Additional Materials

Aspiration device attached to a vacuum line equipped with traps
Erlenmeyer flask (50 or 100 ml)
Sorvall centrifuge, H100B rotor (or equivalent) and centrifuge tubes or bottles cooled to 4°C
TE (pH 8.0), ice-cold
Tris-buffered saline (TBS), ice cold

1. Lyse cells growing in suspension cultures.

   a. Transfer the cells to a centifuge tube or bottle and recover them by centrifugation at 1500*g* (2700 rpm in a Sorvall H100B rotor and swinging buckets) for 10 minutes at 4°C. Remove the supernatant medium by aspiration.

   b. Wash the cells by resuspending them in a volume of ice-cold TBS equal to the volume of the original culture. Repeat the centrifugation. Remove the supernatant by aspiration and then gently resuspend the cells once more in ice-cold TBS. Recover the cells by centrifugation.

   c. Remove the supernatant by aspiration and gently suspend the cells in TE (pH 8.0) at a concentration of 5×10^7 cells/ml. Transfer the suspension to an Erlenmeyer flask.

      For 1 ml of cell suspension, use a 50-ml flask; for 2 ml, use a 100-ml flask, and so on. The density of cells grown in suspension (from Step a) will vary with the cell type and culture conditions. As a rule of thumb, a saturated suspension culture of a continuous cell line (e.g., of HeLa or BHK cells) grown in a 1-liter culture contains on average 1×10^6 cells/ml.

   d. Add 10 ml of lysis buffer for each milliliter of cell suspension. Incubate the solution for 1 hour at 37°C and then proceed immediately to Step 2 (p. 6.9).

      Make sure that the cells are well dispersed over the inner surface of the Erlenmeyer flask when the lysis buffer is added. This dispersal minimizes the formation of intractable clumps of DNA.

Lysis of Tissue Samples

Because tissues generally contain large amounts of fibrous material, it is difficult to extract genomic DNA from them in high yield. The efficiency of extraction is greatly improved if the tissue is reduced to powder before homogenization in lysis buffer. If a large amount of fresh tissue (>1 g) is available, powdering can be accomplished with a Waring blender.

Additional Materials

Beaker (25 ml)
Liquid nitrogen **<!>**
Polypropylene centrifuge tube (50 ml; Falcon or equivalent)
Waring blender equipped with a stainless steel container
or
Mortar and pestle, prechilled with liquid nitrogen

It is important to cool the mortar slowly by adding small amounts of liquid nitrogen over a period of time. Filling the mortar to the brim or suddenly immersing the grinding part of the pestle in liquid nitrogen can cause fracturing. Placing the mortar in an ice bucket filled with dry ice is a good way to precool the mortar before adding the liquid nitrogen. Be careful when grinding human and primate tissues as powdered aerosols are readily generated, especially when adding liquid nitrogen to the mortar.

1. Pulverize tissue samples.

   a. Drop ~1 g of freshly excised tissue into liquid nitrogen in the stainless-steel container of a Waring blender. Blend at top speed until the tissue is ground to a powder.

      Alternatively, smaller quantities of tissue can be snap-frozen in liquid nitrogen and then pulverized to a powder using a mortar and pestle precooled with liquid nitrogen.

   b. Allow the liquid nitrogen to evaporate, and add the powdered tissue little by little to ~10 volumes (w/v) of lysis buffer in a beaker. Allow the powder to spread over the surface of the lysis buffer, and then shake the beaker to submerge the material.

   c. When all of the material is in solution, transfer the suspension to a 50-ml centrifuge tube. Incubate the tube for 1 hour at 37°C, and then proceed to Step 2 (p. 6.9).

Lysis of Blood Cells in Freshly Drawn or Frozen Samples

Additional Materials

Acid citrate dextrose solution B (ACD) (for freshly drawn or frozen blood samples)
0.48% w/v citric acid
1.32% w/v sodium citrate
1.47% w/v glucose
Aspiration device attached to a vacuum line equipped with traps
EDTA (an alternative to ACD, for freshly drawn or frozen blood samples)
ACD, an anticoagulant that is used when preparing genomic DNA from whole blood, is superior to EDTA in preserving high-molecular-weight DNA (Gustafson et al. 1987). However, blood is more frequently collected in commercially available tubes that contain measured amounts of EDTA as an anticoagulant. In most hospitals in the United States, blood collection tubes are conveniently color-coded to indicate which contain anticoagulants and which do not: Purple-topped tubes contain anticoagulant, usually dried EDTA, whereas yellow-topped tubes do not. In molecular cloning, the former (purple) are used to collect blood from which genomic DNA will be extracted, whereas the latter (yellow) are typically used to collect blood that will be used as a source of lymphocytes for immortalization with Epstein-Barr virus. Such immortalized cells provide a renewable resource from which large amounts of DNA can be isolated for later use in, for example, genetic studies.
Phosphate-buffered saline (PBS, for frozen blood samples)
Sorvall H1000B rotor (or equivalent) and centrifuge tubes cooled to 4°C for freshly drawn blood samples
Sorvall SS-34 rotor (or equivalent) and centrifuge tubes cooled to 4°C for frozen blood samples
Water bath, at room temperature

1. Collect blood cells from freshly drawn or frozen samples. Human blood must be collected by a trained phlebotomist under sterile conditions.

To collect cells from freshly drawn blood

   a. Collect ~20 ml of fresh blood in tubes containing 3.5 ml of either acid citrate dextrose solution B (ACD) or EDTA (please see note to EDTA in the materials list).

      The blood may be stored for several days at 0°C or indefinitely at –70°C before the DNA is prepared. Blood should not be collected into heparin, which is an inhibitor of the polymerase chain reaction (Beutler et al. 1990).

   b. Transfer the blood to a centrifuge tube and centrifuge at 1300*g* (2500 rpm in a Sorvall H1000B rotor and 50-ml swinging buckets) for 15 minutes at 4°C.

c. Remove the supernatant fluid by aspiration. Use a Pasteur pipette to transfer the buffy coat carefully to a fresh tube and repeat the centrifugation. Discard the pellet of red cells.

The buffy coat is a broad band of white blood cells of heterogeneous density.

d. Remove residual supernatant from the buffy coat by aspiration. Resuspend the buffy coat in 15 ml of lysis buffer. Incubate the solution for 1 hour at 37°C, and proceed to Step 2.

TO COLLECT CELLS FROM FROZEN BLOOD SAMPLES

a. Collect ~20 ml of fresh blood in tubes containing 3.5 ml of either acid citrate dextrose solution B (ACD) or EDTA (please see note to EDTA in the materials list).

The blood may be stored for several days at 0°C or indefinitely at –70°C before the DNA is prepared.

b. Thaw the blood in a water bath at room temperature and then transfer it to a centrifuge tube. Add an equal volume of phosphate-buffered saline at room temperature.

c. Centrifuge the blood at 3500*g* (5400 rpm in a Sorvall SS-34 rotor) for 15 minutes at room temperature.

d. Remove the supernatant, which contains lysed red cells, by aspiration. Resuspend the pellet in 15 ml of lysis buffer. Incubate the solution for 1 hour at 37°C, and then proceed to Step 2.

Method Continues with Step 2 Below

Treatment of Lysate with Proteinase K and Phenol

2. Transfer the lysate to one or more centrifuge tubes that fit into a Sorvall SS-34 rotor, or equivalent. The tubes should not be more than one-third full.

3. Add proteinase K (20 mg/ml) to a final concentration of 100 μg/ml. Use a glass rod to mix the enzyme solution gently into the viscous lysate of cells.

4. Incubate the lysate in a water bath for 3 hours at 50°C. Swirl the viscous solution from time to time.

5. Cool the solution to room temperature and add an equal volume of phenol equilibrated with 0.1 M Tris-Cl (pH 8.0). Gently mix the two phases by slowly turning the tube end-over-end for 10 minutes on a tube mixer or roller apparatus. If the two phases have not formed an emulsion at this stage, place the tube on a roller apparatus for 1 hour.

Blin and Stafford (1976) recommend the use of 0.5 M EDTA (pH 8.0) in the lysis buffer. However, the density of the buffer almost equals that of phenol, which makes separation of the phases difficult. The lysis buffer used here contains EDTA at a concentration of 0.1 M, which permits easier separation of the phenolic and aqueous phases while maintaining a high degree of protection against degradation of the DNA by nucleases and heavy metals.

6. Separate the two phases by centrifugation at 5000*g* (6500 rpm in a Sorvall SS-34 rotor) for 15 minutes at room temperature.

7. Use a wide-bore pipette (0.3-cm diameter orifice) to transfer the viscous aqueous phase to a fresh centrifuge tube.

   When transferring the aqueous (upper) phase, it is essential to draw the DNA into the pipette very slowly to avoid disturbing the material at the interface and to minimize hydrodynamic shearing forces. If the DNA solution is so viscous that it cannot easily be drawn into a wide-bore pipette, use a long pipette attached to an aspirator to remove the organic (lower) phase as follows:

   i. Before starting, make sure that the vacuum traps are empty and secure, so that phenol cannot enter the vacuum system.

   ii. With the vacuum line closed, slowly lower the pipette to the bottom of the organic phase. Wait until the viscous thread of aqueous material detaches from the pipette, and then carefully open the vacuum line and gently withdraw all of the organic phase. Close the vacuum line and quickly withdraw the pipette through the aqueous phase. Immediately open the vacuum line to transfer the residual phenol into the trap.

   iii. Centrifuge the DNA solution at 5000*g* (6500 rpm in a Sorvall SS-34 rotor) for 20 minutes at room temperature. Protein and clots of DNA will sediment to the bottom of the tube. Transfer the DNA solution (the supernatant) into a 50-ml centrifuge tube, leaving behind the protein and clots of DNA.

8. Repeat the extraction with phenol twice more and pool the aqueous phases.

9. Isolate DNA by one of the following two methods.

To isolate DNA in the size range of 150–200 kb

a. Transfer the pooled aqueous phases to a dialysis bag. Close the top of the bag with a dialysis tubing clip, allowing room in the bag for the sample volume to increase 1.5–2-fold during dialysis.

b. Dialyze the solution at 4°C against 4 liters of dialysis buffer. Change the buffer three times at intervals of ≥6 hours.

Because of the high viscosity of the DNA solution, dialysis generally takes ≥24 hours to complete.

To isolate DNA that has an average size of 100–150 kb

a. After the third extraction with phenol, transfer the pooled aqueous phases to a fresh centrifuge tube and add 0.2 volume of 10 M ammonium acetate. Add 2 volumes of ethanol at room temperature and swirl the tube until the solution is thoroughly mixed.

b. The DNA immediately forms a precipitate. Remove the precipitate in one piece from the ethanolic solution with a Shepherd's crook (a Pasteur pipette whose end has been sealed and shaped into a U; please see Steps 5–7 of Protocol 3). Contaminating oligonucleotides remain in the ethanolic phase.

c. If the DNA precipitate becomes fragmented, abandon the Shepherd's crook and collect the precipitate by centrifugation at 5000*g* (6500 rpm in a Sorvall SS-34) for 5 minutes at room temperature.

d. Wash the DNA precipitate twice with 70% ethanol, and collect the DNA by centrifugation as described in Step c.

e. Remove as much of the 70% ethanol as possible, using an aspirator. Store the pellet of DNA in an open tube at room temperature until the last visible traces of ethanol have evaporated.

Do not allow the pellet of DNA to dry completely; desiccated DNA is very difficult to dissolve.

f. Add 1 ml of TE (pH 8.0) for each 0.1 ml of cells (Step 1). Place the tube on a rocking platform and gently rock the solution for 12–24 hours at 4°C until the DNA has completely dissolved. Store the DNA solution at 4°C.

10. Measure the concentration of the DNA.

It is often difficult to measure the concentration of high-molecular-weight DNA by standard methods such as absorbance at 260 nm. This is because the DNA solution is frequently nonhomogeneous and is usually so viscous that it is impossible to withdraw a representative sample for analysis. These problems can be minimized by withdrawing a large sample (10–20 µl) with an automatic pipetter equipped with a cut-off yellow tip. The sample is then diluted with ~0.5 ml of TE (pH 8.0) and vortexed vigorously for 1–2 minutes. The absorbance of the diluted sample can then be read at 260, 270, and 280 nm in the standard way.

A solution with an A_{260} of 1 contains ~50 µg of DNA/ml. Note that estimates of purity of nucleic acids based on OD_{260}:OD_{280} ratios are unreliable (Glasel 1995) and that estimates of concentration are inaccurate if the sample contains significant amounts of phenol. H_2O saturated with phenol absorbs with a characteristic peak at 270 nm and an OD_{260}:OD_{280} ratio of 2 (Stulnig and Amberger 1994). Nucleic acid preparations free of phenol should have OD_{260}:OD_{280} ratios of ~1.2. For further information, please see Appendix 8.

More accurate measurement of DNA concentrations can be made by fluorometry in the presence of fluorescent dyes such as SYBR Gold and Hoechst 33258, which bind DNA without intercalating and with specificity to double-stranded DNA (for further details, please see Appendix 8). For a method of fluorometric measurement of DNA concentrations using Hoechst 33258, please see the panel on **ADDITIONAL PROTOCOL: ESTIMATING THE CONCENTRATION OF DNA BY FLUOROMETRY** on the following page.

11. Analyze the quality of the preparation of high-molecular-weight DNA by pulsed-field gel electrophoresis (Chapter 5, Protocol 17 or 18) or by electrophoresis through a conventional 0.6% agarose gel (Chapter 5, Protocol 1). Use unit-length and/or linear concatemers of λ DNA as markers. A method to generate linear concatemers of λ DNA is described in Chapter 5, Protocol 16.

Do not be concerned if some of the DNA remains in the well, since DNA molecules >250 kb have difficulty entering the gel. This problem can usually be solved by embedding the DNA in a small amount of melted agarose (at 55°C) and transferring the molten solution to the well of a preformed agarose gel. The transfer should be done before the gel is submerged in electrophoresis buffer.

ADDITIONAL PROTOCOL: ESTIMATING THE CONCENTRATION OF DNA BY FLUOROMETRY

Measuring the concentration of DNA using fluorometry is more sensitive than spectrophotometry, allowing the detection of nanogram quantities of DNA. In this assay, DNA preparations of known and unknown concentrations are incubated with the fluorochrome Hoechst 33258. Absorption values for the unknown sample are compared with those observed for a known series, and the concentration of the unknown sample is estimated by interpolation.

Additional Materials

NaCl (4 M)

Sodium phosphate (0.5 M, pH 7.4)

Fluorometry buffer

2 M NaCl

50 mM sodium phosphate

Prepare 500 ml and sterilize the solution by filtration through a 0.45-μm filter.

Hoechst 33258 dye (0.2 mg/ml in H_2O)

The concentrated solution of dye can be stored at room temperature in a foil-wrapped test tube.

High-molecular-weight DNA solution, reference standard (100 μg/ml in TE)

A DNA solution of known concentration is required to construct a standard curve. Because the binding of Hoechst 33258 dye to DNA is influenced by the base composition, the DNA used to construct the standard curve should be from the same species as the test sample.

Fluorometer, either fixed wavelength or scanning model

Method

1. Turn on the fluorometer 1 hour before the assay is carried out to allow the machine to warm up and stabilize.

   When bound to high-molecular-weight double-stranded DNA, Hoechst 33258 dye absorbs maximally at 365 nm and emits maximally at 458 nm.

2. Prepare an appropriate amount of diluted dye solution (50 μl of concentrated dye solution per 100 ml of fluorometry buffer). Each tube in the DNA assay requires 3 ml of diluted Hoechst 33258 dye solution. Transfer 3 ml of diluted dye solution to an appropriate number of clean glass tubes. Include six extra tubes for a blank and the standard curve.

3. Prepare a standard curve by adding 100, 200, 300, 400, and 500 ng of genomic DNA from the reference stock solution to individual tubes. Mix and read the absorbance on the prewarmed fluorometer of each tube immediately after addition of the DNA.

4. Add 0.1 μl (i.e., 1 μl of a 1:10 dilution), 1.0 μl, and 10 μl of the preparation of genomic DNA, whose concentration is being determined, to individual tubes containing diluted dye solution. Immediately read the fluorescence.

5. Construct a standard curve plotting fluorescence on the ordinate (*y* axis) and weight of reference DNA (in ng) on the abscissa (*x* axis). Estimate the concentration of DNA in the unknown sample by interpolation.

   If the reading for the unknown genomic DNA solution falls outside that of the standard curve, read the fluorescence of a more concentrated sample or make an appropriate dilution of the sample and repeat the assay.

   Binding of Hoechst 33258 is adversely influenced by pH extremes, the presence of detergents near or above their critical micellar concentrations, and salt concentrations above 3 M. If these conditions or reagents are used to prepare the genomic DNA and improbable results are obtained in the fluorometry assay, precipitate an aliquot of the DNA preparation with ethanol, rinse the pellet of nucleic acid in 70% ethanol, dissolve the dried pellet in TE, and repeat the assay.

   If the preparation of test DNA is highly viscous, sampling with standard yellow tips may be so inaccurate that the dilutions of unknown DNA will not track with the standard curve. In this case, the best solution is to withdraw two samples (10–20 μl) with an automatic pipetter equipped with a cut-off yellow tip. Each sample is then diluted with ~0.5 ml of TE (pH 8.0) and vortexed vigorously for 1–2 minutes. Different amounts of the diluted samples can then be transferred to the individual tubes containing diluted dye solution. The results obtained from the two sets of samples should be consistent.

   Use scissors or a dog nail clipper (e.g., Fisher) to generate cut-off yellow tips. Alternatively, the tips can be cut with a sharp razor blade. Sterilize the cut-off tips before use, either by autoclaving or by immersion in 70% alcohol for 2 minutes followed by drying in air. Presterilized, wide-bore tips can be purchased from a number of commercial companies (e.g., Bio-Rad).

   The assay can be used to measure the concentration of DNAs whose sizes exceed ~1 kb. Hoechst 33258 binds poorly to smaller DNA fragments.