

Preparative Separation of DNA-Ethidium Bromide Complexes by Zonal Density Gradient Centrifugation

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Ethidium bromide-DNA complexes separated by rate-zonal sedimentation through a density gradient can be readily visualized and purified with little cross-contamination. The method is simple, rapid, and efficient.

Restriction enzyme fragmentation and recombinant DNA technology offer powerful tools with which to study gene structure, organization, and expression. Frequently in these studies the need arises to purify on a preparative scale a specific DNA molecule from a mixture.

The two most commonly used procedures in such purification are isopycnic centrifugation in dye-salt gradients and gel electrophoresis. Dye-buoyant density centrifugation allows excellent separation and purification of covalently closed circular (CCC) DNA molecules from DNA of other conformations (1-2). However, the procedure does not permit the separation of different kinds of DNA molecules if they have a similar conformation.

Electrophoresis through polyacrylamide or agarose gels separates nucleic acids primarily on the basis of size and conformational difference (3-5). However, any DNA passed through a gel is contaminated with fragments of the gel matrix. The presence of gel material often interferes with the further

analysis or processing of recovered DNA (e.g., in electron microscopy, ligation, sequencing, or endonuclease digestion). Complete elimination of contaminating gel is laborious and involves some loss of DNA (6-10).

A third procedure, rate-zonal sedimentation through a stabilizing gradient, separates molecules on the basis of difference in size or conformation. Rate-zonal centrifugation is basically simple and is suitable for handling relatively large amounts of material. The method has not been used widely for separation and purification of DNA molecules, in part because the separated bands of material are not observed directly until after removal from the tube. Commonly the molecules of interest are identified by collecting individual fractions and examining each for radioisotope content, absorbance, transforming ability, etc. Because the fractionation is usually blind, the resolving power and simplicity inherent in the centrifugation itself is lost.

Fluorescence by ethidium bromide-nucleic acid complexes irradiated with ultraviolet light provides a simple means of visualizing the position of DNA molecules in the centrifuge tube, and allows removal of fractions of interest with the least possible cross-contamination and with instruments

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² Abbreviations used: CCC, covalently closed circular; DEPC, diethylpyrocarbonate; PEG, polyethylene glycol; TES buffer, 30 mM Tris-HCl, 5 mM Na₂-EDTA, 50 mM NaCl (pH 8.0).

TABLE 1
PLASMID DNA

Plasmid	Size (kb)	Reference
pBR322	4.365	(20,21)
pSF2124	11.3	(22)
pSC105	16.2	(19)
pMIL12	13.8	(23,24)
pRBH022	9.6	(24)

no more sophisticated than a syringe or Pasteur pipet. Furthermore the position of nucleic acids is readily visualized without disturbing the gradient, so an optimal centrifugation time can be chosen by examining the tube during the course of sedimentation. By using vertical rotors the centrifugation time can be reduced to make the entire procedure fast and efficient. If DNA molecules can be separated by centrifugation, the procedure offers the distinct advantage over gel electrophoresis that the separated bands are not contaminated with the gel matrix.

In this paper we show that the use of zonal centrifugation in purification of nucleic acid molecules separable by size or conformational difference can be simple, rapid, and efficient. When applicable it is generally the method of choice.

MATERIALS AND METHODS

Strains. Plasmids were maintained in *Escherichia coli* K12 strain RH202. RH202 is F^- *thi lacY tonA supE44 hsdS* (11). Plasmids are listed in Table 1.

Plasmid DNA isolation. Strains were grown at 37°C in 1% casamino acids, 0.4% glucose, 2 µg/ml thiamine HCl, and salts (12). The medium was supplemented with ampicillin (20 µg/ml), tetracycline (10 µg/ml), or kanamycin (20 µg/ml), as appropriate. At $A_{600} = 0.60$ chloramphenicol was added to 200 µg/ml to all strains except that containing pSC105. Cultures were shaken for another 15–20 h. Then diethylpyrocar-

bonate (DEPC) was added to 0.3% to kill the cells (13). Harvested cells were washed in 10 mM Tris-HCl, 1 mM EDTA (pH 8.0) at 4°C to remove contaminating medium, small molecules, and traces of DEPC. Cells were resuspended (40 ml/liter cell-culture) in Tris-sucrose buffer (25% sucrose, 50 mM Tris-HCl (pH 8.0)). Lysis was achieved by adding 10 ml of 0.25 M EDTA (pH 8.0) (50 mM final concn) and 10 mg lysozyme with occasional shaking for 10 min at 0°C. Triton X-100 was added to a concentration of 0.15% by using 8.8 ml of lysis buffer (50 mM Tris-HCl, 1 mM EDTA 1% Triton X-100 (pH 8.0)). NaCl was then added to a concentration of 0.5 M using 6 ml of 5 M NaCl. The mixture was incubated at 0°C for 20 min or longer, then cleared by centrifugation at 25K (46,000g) for 30 min at 4°C using the Sorvall A641 fixed angle rotor in an OTD65 ultracentrifuge. Plasmid DNA was precipitated by adding 10% (w/v) polyethylene glycol (PEG) (14). After 6 h or overnight at 4°C, the precipitated DNA was collected by centrifugation at 3000 rpm (1000g) for 5 min at 4°C, and resuspended in 6.2 ml saturated CsCl (technical grade filtered through Norite, Kawecki Berylco) in TES buffer (30 mM Tris-HCl, 5 mM Na₂-EDTA, 50 mM NaCl (pH 8.0)). Contaminating PEG formed a thin surface layer after centrifugation at 7000 rpm (5900g) for 5 min at room temperature. This layer was removed by a spatula or by decanting the solution into a clean tube. Ethidium bromide, 0.64 ml, (2 mg/ml), and 1.6 ml TES buffer were added and the refractive index and the volume of the solution were adjusted to be $N_D^{25} = 1.3930$ ($p^{25} = 1.6307$) and 8.8 ml, respectively. Centrifugation was made in two tubes using the TV865 vertical rotor at 38K (126,500g) for 18 h at 20°C in the OTD65 ultracentrifuge. The CCC DNA band was removed using a Pasteur pipet attached to a pump. Ethidium bromide was removed by extraction with *n*-butanol. Plasmid DNA was purified from contaminating RNA and CsCl by exclusion chromatography through a 2 × 12-cm aga-

rose column (Bio-Gel A-50, 100–200 mesh), eluting with 25 mM Tris–HCl, 0.6 M NaCl, 1 mM EDTA buffer (pH 8). The first peak, comprising plasmid DNA, was precipitated with 2 vol cold 95% ethanol and washed with 70 and 95% ethanol. The DNA was then dissolved in $0.1\times$ SSC and stored over chloroform at 4°C. Generally 0.3 to 3.5 mg of plasmid DNA per liter culture was obtained. This corresponds to 20 to 60% of the material absorbing at 260 nm after the CsCl–ethidium bromide gradient.

Digestion with restriction enzymes and agarose gel electrophoresis. Fragments of DNA were generated by using the endonucleases *EcoRI* (15) or *BamHI* (16) as described. Agarose gel electrophoresis using 0.8% agarose has been described (4).

Separation and purification of DNA fragments. Linear sucrose gradients were prepared using 5–20% sucrose in 1 mM EDTA, 20 mM NaCl, 10 mM Tris–HCl (pH 8.0), or in the same solution except the NaCl concentration was 0.1 M (17). The DNA solution containing the different fragments (5–300 μ g or more in 0.1–0.8 ml) was mixed with 1–2 μ l of ethidium bromide (2 mg/ml in TES) and layered on the top of the gradient. Centrifugation was made in the SW27.1 swinging bucket rotor (Beckman), or in the TV850 or TV865 vertical rotors (Sorvall), in the OTD65 ultracentrifuge at 5°C. The centrifuge was programmed for slow start (Arc 1E) and stop (Reograd). After centrifugation, the band(s) of interest, visualized by long wavelength ultraviolet light, was carefully removed into a small tube by a Pasteur pipet attached to a pump. Alternatively a syringe was used to remove the desired band(s). In any case, care was taken to avoid the cross-contamination of the bands. The DNA was extracted two times with *n*-butanol and then precipitated with 2 vol of cold 95% ethanol. The DNA was washed with 70% and 95% ethanol, dried, and dissolved in $0.1\times$ SCC. To check the purity, a sample was analyzed by agarose gel electrophoresis.

Biohazard consideration. All experiments involving recombinant DNA molecules were carried out under P2-level conditions as specified in "Guidelines for research involving recombinant DNA molecules" (Fed. Register 41: (131), 27913, 1976), and as approved by the local biohazard committee.

RESULTS AND DISCUSSION

The location of discrete DNA species is easily visualized by fluorescence of the ethidium bromide–DNA complex. Figure 1 shows bands of λ plac5 and pSF2124 DNA in the course of sedimentation. Two micrograms of DNA in a band can be observed easily by eye when the tube is placed over a source of "black light." Even less DNA can be seen when shorter wavelength exciting light is applied. We routinely use light with an emission maximum of 366 nm rather than light of shorter wavelength in order to avoid dimer formation, nicking, and photobleaching (18). The fluorescence intensity decreases as the DNA progresses down the tube. If necessary to visualize the material, the amount of ethidium bromide added to the sample can be increased. The leading edge of each band is characteristically diffuse in comparison with the sharpness of the trailing edge. We attribute the spreading of the leading edge to diffusion and convection of the sample into the gradient before initiation of the run.

The DNA bands can be removed without cross-contamination unless they are very close together. As shown in Fig. 2, three DNA species removed from the same tube each showed a single prominent band when examined by agarose gel electrophoresis. The purified λ DNA showed a small amount of contamination by pSF2124 DNA (about 3% by weight) when examined by gel electrophoresis (Fig. 2). The other purified DNA species showed no detectable cross-contamination. No cross-contamination was detected in any other purified sample shown in the figures in this paper.

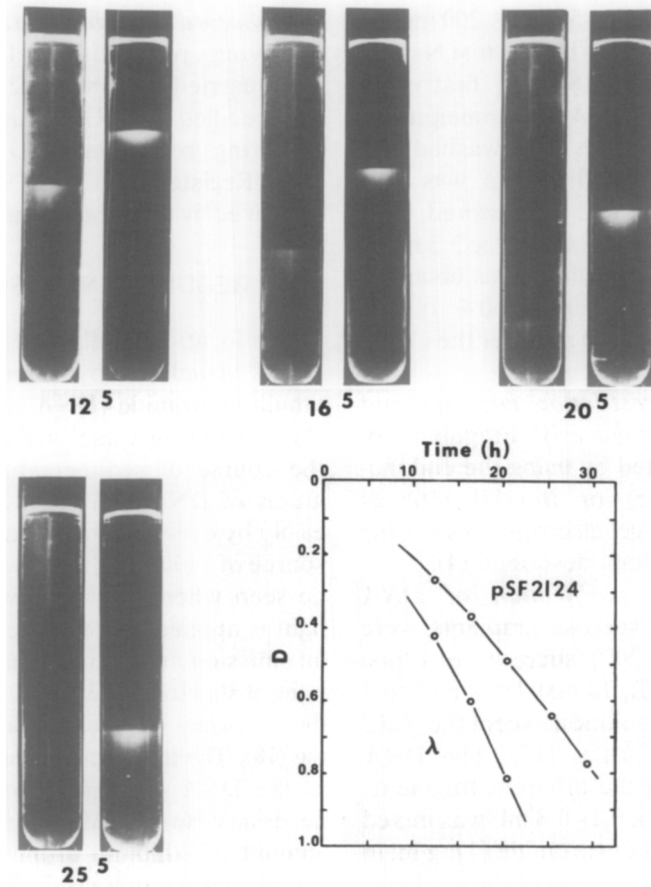


FIG. 1. Sedimentation of noncircular DNA molecules as a function of time. DNA samples were sedimented in an SW27.1 rotor at 25,000 rpm. Left tube in each pair contains 15 μ g of λ plac5 DNA (47.4kb); right tube, 15 μ g of *Eco*RI-digested pSF2124 DNA (11.3 kb). (D) represents relative distance. Plots are not linear because the gradients were not isokinetic and because the cumulative stopping time was not taken into account.

The purified DNA is routinely satisfactory as a substrate for all enzymes we have tried. Figure 3 demonstrates the separation of a cloned fragment of the chloroplastic ribosomal RNA genes of the alga *Euglena* from the plasmid cloning vehicle (pSF2124) and the purification of the two DNA species. The DNA fragment is free from contamination by the cloning vehicle. The pSF2124 DNA recovered separately from the same gradient is also uncontaminated and is completely digestible by the restriction endonuclease *Bam*HI (Fig. 3). In this experiment the applied DNA was quantita-

tively recovered in the two purified fractions. We routinely recover 85–100% of the applied DNA unless we deliberately discard DNA to avoid cross-contamination of very close bands.

The hybrid plasmid pSC105 contains a gene for kanamycin resistance cloned in pSC101 (19). pSC101 has a size of 8.8 kb, and the cloned fragment of 7.4 kb. As shown in Fig. 4, the *kan* DNA is completely separable from the pSC101 cloning vector. The recovered DNA was free of the cloning vector as judged by agarose gel electrophoresis, and also by the inability to

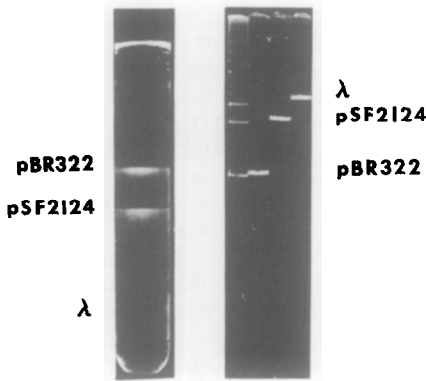


FIG. 2. Purification of the individual components from a mixture of different DNA molecules by zonal centrifugation. Twenty micrograms of λ plac5 DNA, 15 μ g *Eco*RI-digested pSF2124 DNA, and 10 μ g of *Bam*HI-digested pBR322 DNA were centrifuged in an SW27.1 rotor at 25,000 rpm for 20 h. The bands were removed separately and examined by agarose gel electrophoresis.

transform *E. coli* cells to Kan^r with or without ligation.

All of the above separations were carried out in the SW27.1 swinging bucket rotor.

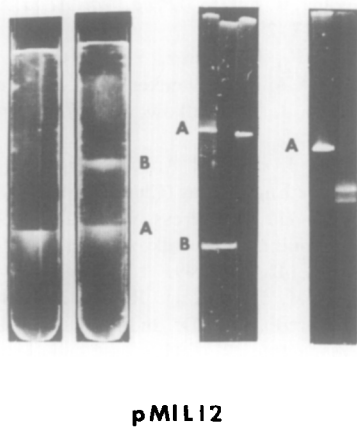


FIG. 3. Purification of a cloned DNA fragment and of the plasmid carrier, and cleavage of the purified DNA by endonuclease *Bam*HI. Fifteen micrograms of *Eco*RI-digested pSF2124 DNA (left tube) and 20 μ g of *Eco*RI-digested pMIL12 DNA (right tube) were sedimented in parallel SW27.1 tubes for 24 h at 25,000 rpm. The cloned fragment "B" (2.48 kb *Eco*RI fragment R of *Euglena gracilis bacillaris* chloroplast DNA (26)) and the carrier plasmid (pSF2124) were removed separately and examined by agarose gel electrophoresis, as shown in the middle. The purified carrier plasmid (A) was digested with *Bam*HI and the products were examined by electrophoresis as shown in the gels at right.

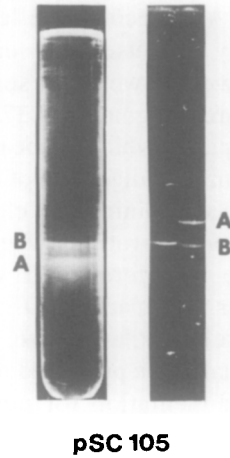


FIG. 4. Isolation of a specific cloned DNA fragment. Fifteen micrograms of *Eco*RI-digested pSC105 DNA was centrifuged in an SW27.1 rotor for 27 h at 25,000 rpm. The cloned fragment (band B) was removed and examined by agarose gel electrophoresis.

Similar results can be achieved in vertical rotors in a much shorter time (Fig. 5A). Figure 5A also shows that the procedure is useful for separating DNA molecules differing in conformation. The supercoiled and nicked forms of circular plasmid DNA separate well during centrifugation and can be purified easily as demonstrated in Fig. 5B.

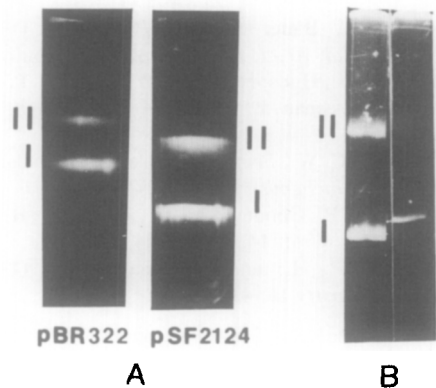


FIG. 5. Preparative separation of DNA molecules in a vertical rotor (A) pBR322 DNA, 7.5 μ g, and 10 μ g of pSF2124 DNA were centrifuged in a TV865 rotor for 1 h at 35,000 rpm. Bands corresponding to form I (supercoiled) and form II (nicked) DNA are designated. (B) Agarose gel electrophoretic patterns of total pSF2124 DNA (as applied to the gradient in (A)) and of the purified form I DNA recovered from the gradient.

Small DNA molecules are less likely to band sharply because they diffuse more readily. However, we have separated and recovered three fragments of 375, 1500, and 3000 base pairs which appear following restriction enzyme digestion of a derivative of pBR322 containing a cloned DNA sequence. For separations where diffusion is likely to interfere with clean band separation, or for separating DNA molecules that are closely similar in size, gel electrophoresis remains the preferred method. The utility of sedimentation for purification of DNA segments of larger size is obvious, particularly for molecules too large to separate by gel electrophoresis. Purification of ethidium bromide-DNA complexes separated by centrifugation through a stabilizing gradient provides DNA which is undamaged and uncontaminated, and suitable for processing with enzymes, transformation, and sequencing. The method is simple, fast and efficient.

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