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RAPID PURIFICATION OF PLASMID DNA BY A SINGLE CENTRIFUGATION IN A TWO-STEP CESIUM CHLORIDE-ETHIDIUM BROMIDE GRADIENT

S. J. Garger, O. M. Griffith*,†, and L. K. Grill

Zoecon Corporation, 975 California Avenue,
Palo Alto, California 94304

*Beckman Instruments, Inc., Spinco Division,
1050 Page Mill Road, Palo Alto, California 94304

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SUMMARY: A procedure for rapid, preparative purification of plasmid DNA is described and compared with a conventional equilibrium centrifugation method. A discontinuous, two-step CsCl-ethidium bromide gradient is used, with the starting position of the plasmid-containing extract being at the bottom of the tube. During centrifugation in a fixed angle rotor, covalently closed circular plasmid DNA is separated from contaminating protein, RNA, and chromosomal DNA in 5 hr. Plasmids purified by this method are considerably less contaminated with RNA than when purified by a 48-hr equilibrium run in a homogeneous gradient, as determined by agarose gel electrophoresis and 5'-end-labeling studies. Plasmid DNA purified in two-step gradients can be used directly for restriction endonuclease analysis and DNA sequencing.

The widespread use of bacterial plasmids as cloning vehicles in recombinant DNA research has led to the development of many plasmid DNA extraction and purification methods (1-9). Rapid microscale techniques have been devised in order to screen large numbers of individual bacterial colonies for recombinant DNA segments (7,8). However, larger quantities of plasmid DNA, free from contaminating protein, RNA, and chromosomal DNA, are often needed for detailed restriction endonuclease mapping, DNA sequencing, and in certain cloning situations.

We describe here a procedure for the rapid isolation of large quantities of highly purified plasmid DNA from discontinuous, two-step CsCl-ethidium bromide gradients. Compared with conventional equilibrium gradient methods

† To whom correspondence should be addressed.

Abbreviations used: SDS, sodium dodecyl sulfate; TE buffer, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA; n, refractive index.

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which require centrifugation for 24-60 hr, this method greatly diminishes the time required for tight DNA banding and considerably reduces the amount of RNA contaminating the final plasmid preparation.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Media

Plasmid pBR322 was maintained in Escherichia coli strain C-600. Bacterial cells were grown in LB (Luria-Bertani) media supplemented with 10 μg/ml tetracycline (10).

Plasmid DNA Extraction

Bacteria were extracted with alkali according to a modified method of Birnboim and Doly (7). Plasmid DNA was first amplified in a one-liter culture of bacteria by the addition of 200 μg/ml chloramphenicol to the logarithmically growing cells. After overnight incubation at 37°C, bacteria were pelleted and resuspended in 24 ml of glucose buffer (50 mM glucose, 25 mM Tris-HCl, pH 8.0, and 10 mM EDTA), then mixed with 4 ml of glucose buffer containing 20 mg/ml lysozyme (freshly prepared) and incubated for 10 min at room temperature. Lysis was achieved by the addition of 55.2 ml of 1% SDS in 0.2 N NaOH. The mixture was gently swirled and immediately placed in an ice-water slurry for 5 min. Protein, chromosomal DNA, and high molecular weight RNA were precipitated by the addition of 28 ml of potassium acetate (3 M potassium, 5 M acetate), pH 4.8 (9). After an additional 15 min in an ice-water slurry, the insoluble contaminants were removed by centrifugation (JA-20 rotor, 10,000 rpm, 10 min). The supernatant was extracted with an equal volume of phenol, chloroform, and isoamyl alcohol (25:24:1). After a brief centrifugation (JA-20 rotor, 6000 rpm, 5 min), nucleic acids were precipitated from the aqueous phase for 10 min at room temperature by the addition of a 0.6 volume of isopropyl alcohol. The total nucleic acid precipitate (70 mg) was recovered by centrifugation (JA-20 rotor, 10,000 rpm, 15 min) and resuspended in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA).

Two-Step CsCl Gradient Centrifugation

A solution of CsCl (density = 1.470 g/ml, $\underline{n}$ = 1.3780) was made up in TE buffer. Eight milliliters of this solution were placed into 5/8 x 3 in. ("large") Beckman Quick-Seal® polyallomer tubes or 4.0 ml into 5/8 x 1-3/4 in. ("small") Beckman Quick-Seal polyallomer tubes. An aliquot containing 14 mg of the buffered total nucleic acids was adjusted to a density of 1.80 g/ml ($\underline{n}$ = 1.4080) by the addition of solid CsCl and ethidium bromide solution as follows. For the large tubes, 4.2 g of CsCl were dissolved in 2.4 ml of buffered total nucleic acid extract to obtain a weight of 6.6 g. Then 0.4 ml of ethidium bromide solution (10 mg/ml) was added to arrive at a final weight of 7.0 g. For the small tubes, 2.1 g of CsCl were dissolved in 1.2 ml of buffered total nucleic acid extract to obtain a weight of 3.3 g. Ethidium bromide solution (0.2 ml) was added to arrive at a final weight of 3.5 g. The dense nucleic acid-containing CsCl solution (4.0 ml final volume for the large tubes, 2.0 ml final volume for the small tubes) was layered beneath the less dense CsCl solution with a glass Pasteur pipette or syringe with a long cannula, being careful not to disturb the solution interface. The tubes were then filled to capacity with the less dense CsCl solution and sealed.

The Type 80 Ti or Type 75 Ti rotors were run at 65,000 rpm for 4 or 5 hr. The Type 70.1 Ti rotor was run at 60,000 rpm for 5 hr, and 50,000 rpm for 17 hr. The Type 80 Ti rotor was also run at 70,000 rpm for 3 hr. All centrifugations were performed at 20°C.

Equilibrium CsCl Gradient Centrifugation

Homogeneous CsCl-ethidium bromide gradients (final density 1.55 g/ml) containing 14 mg buffered total nucleic acid extract were centrifuged at 40,000 rpm for 48 hr at 20°C using the Type 50 Ti rotor (9).

Recovery of Plasmid DNA from CsCl Gradients

Plasmid DNA was collected from the gradients by puncturing the side of the tubes with an 18-gauge needle. Ethidium bromide was removed by repeated washing with H_2O-saturated isoamyl alcohol. Plasmid DNA was diluted with 3 volumes of TE buffer and precipitated with 2 volumes of cold ethanol.

Gel Electrophoresis

Horizontal, 1% agarose (Bio-Rad) gels (20 × 13.5 × 0.5 cm) were used for nucleic acid analysis. Electrophoresis buffer contained 40 mM Tris, 2 mM EDTA, adjusted to pH 7.8 with acetic acid. Gels were electrophoresed at 100 V for 2.5 hr. After electrophoresis, gels were stained with ethidium bromide (1 μg/ml), observed with short wavelength UV light and photographed using Wratten no. 9 and 23A filters and Polaroid type 55 film.

^{32}P-5'-End-Labeling

Ten micrograms of CsCl-purified plasmid DNA were digested with 25 U of BamHI enzyme (New England Biolabs) for 1.5 hr at 37°C. The 5'-phosphates were removed with 500 U of bacterial alkaline phosphatase (Bethesda Research Laboratories) for 1 hr at 65°C. Phosphatase was removed by phenol/chloroform extraction and the samples precipitated with 2 volumes of cold ethanol. The 5'-hydroxyl ends were labeled with T4 polynucleotide kinase (P. L. Biochemicals) and [γ-^{32}P]ATP (New England Nuclear) in a 45-min reaction at 37°C (11).

Autoradiography and Densitometry

Kodak X-Omat film was exposed at room temperature to the ^{32}P-5'-end-labeled nucleic acids separated by electrophoresis. Densitometer tracings were made from the autoradiograph. The areas under the peaks were determined using a Hewlett-Packard 3390A integrator.

RESULTS AND DISCUSSION

Preparative amounts of plasmid DNA can be banded in CsCl-ethidium bromide gradients using the two-step gradient method outlined above. Figure 1 shows typical plasmid DNA banding in both the small and large tubes after centrifugation for 5 hr at 65,000 rpm in either the Type 80 Ti or Type 75 Ti rotors. Plasmid DNA moved through the gradient, away from the contaminating RNA, and banded near the center of the tube. Protein pelleted against the centripetal wall of the tube, and high molecular weight RNA pelleted at the bottom of the tube. Similar banding positions were seen when two-step gradients were run under the other centrifugation conditions shown in Table 1. However, when the large tube was run for 3 hr at 70,000 rpm, there was

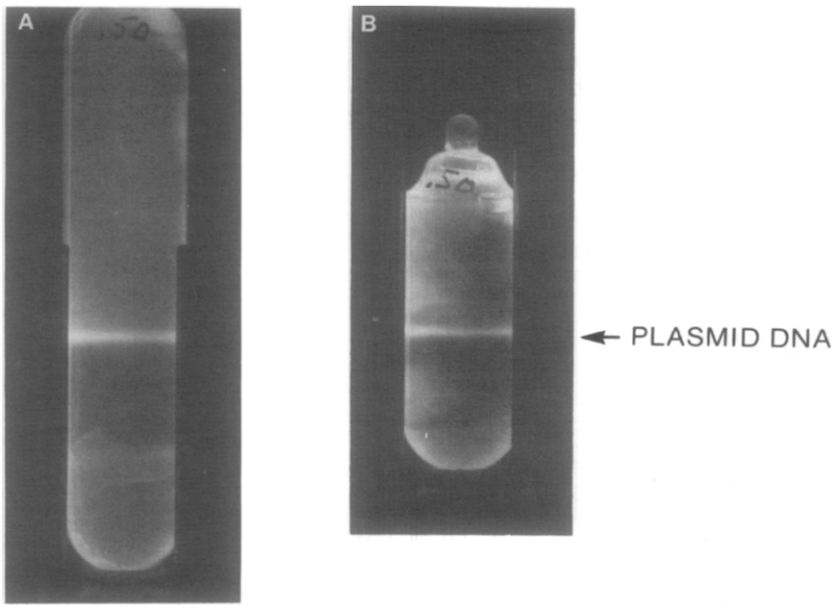

Figure 1. Ethidium bromide-stained plasmid DNA viewed with short wavelength UV light. Total nucleic acid extract (14 mg) prepared by alkaline extraction was centrifuged for 5 hr at 65,000 rpm in the Type 80 Ti rotor as described in Materials and Methods. (A) Large Quick-Seal polyallomer tube; (B) small Quick-Seal polyallomer tube.

insufficient banding of the plasmid DNA, and this tube was judged unsuitable for use with the 3-hr protocol.

Visualization of small plasmid DNA restriction fragments by ethidium bromide staining of agarose gels is often made difficult by the presence of co-migrating RNA. The purified plasmid samples were screened for contaminating RNA by agarose gel electrophoresis (Fig. 2). RNA was visible only when gel lanes were overloaded with plasmid DNA (Fig. 2, IIA-J) or when total nucleic acid extracts were electrophoresed (Fig. 2, I). All of the purified plasmid DNA preparations were sufficiently free of RNA that they could be used directly for restriction endonuclease mapping without ribonuclease treatment.

Minor quantities of contaminating RNA greatly reduce the specific activity of ^{32}P-end-labeled DNA required for Maxam-Gilbert DNA sequencing (12). The purified plasmid preparations were digested with BamHI endonuclease, phosphatase-treated, end-labeled, and electrophoresed as described in Materials and Methods. In order to estimate the relative incorporation of ^{32}P into the

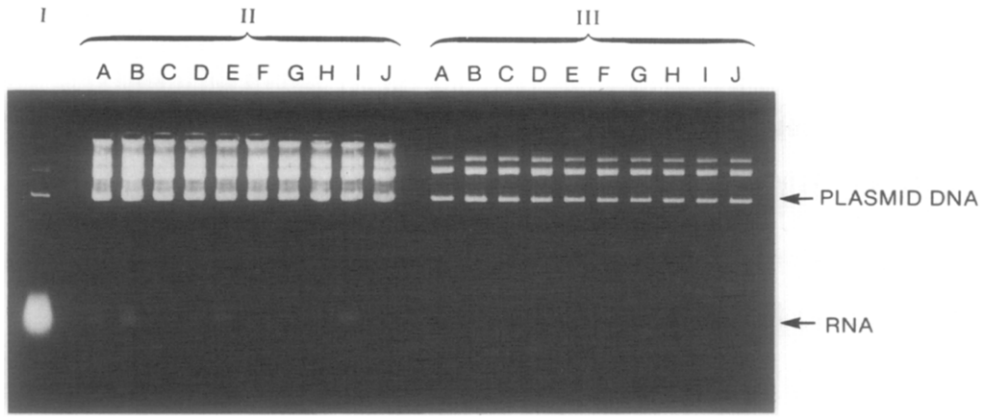

Figure 2. Analysis of CsCl-gradient purified plasmid DNA and total nucleic acid extract by agarose gel electrophoresis. (I) 5 μg of total nucleic acid extract before centrifugation; (II) 2 μg of purified nucleic acids per lane; (III) 250 ng of purified nucleic acids per lane. (A) 48 hr, 40,000 rpm, equilibrium gradient, large tube. Samples shown in lanes (B)-(J) were prepared by two-step gradients run as follows: (B) and (C) 17-hr, 50,000 rpm, small and large tube, respectively; (D) 3 hr, 70,000 rpm, small tube; (E) and (F) 5 hr, 65,000 rpm, small and large tube, respectively; (G) and (H) 5 hr, 60,000 rpm, small and large tube, respectively; (I) and (J) 4 hr, 65,000 rpm, small and large tube, respectively. Rotors used and electrophoresis conditions are described in Materials and Methods.

endonuclease-cleaved plasmid DNA and into contaminating RNA, a densitometer tracing was made of the autoradiograph shown in Figure 3. The areas under the peaks corresponding to the ^{32}P-labeled DNA and RNA were determined and compiled in Table 1 as percentages of the total ^{32}P-label incorporated. The range of percentages of ^{32}P shown as incorporated into DNA are minimum values,

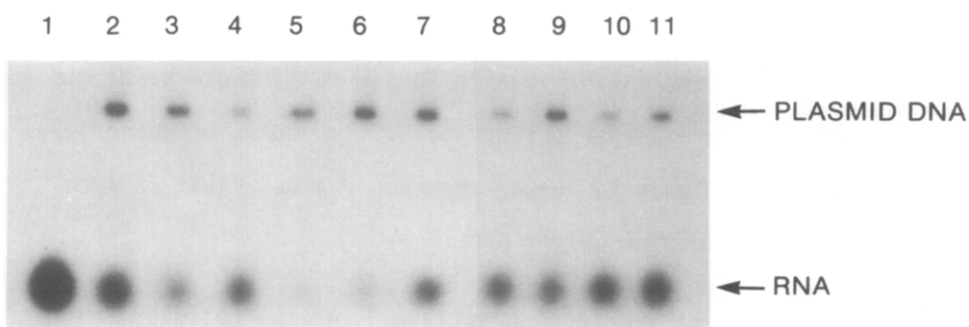

Figure 3. Autoradiograph of ^{32}P-5'-end-labeled plasmid DNA preparations (approximately 60,000 cpm of labeled nucleic acids per lane). (1) Total nucleic acid extract before centrifugation; (2) 48 hr, equilibrium gradient, 40,000 rpm. Samples in lanes (3)-(11) were prepared in two-step gradients using centrifugation conditions as shown in Table 1, lanes (3)-(11). See Materials and Methods for 5'-end-labeling, electrophoresis, and autoradiography conditions.

since the relative efficiency of RNA end-labeling is greater than DNA end-labeling. Also, the concentration of 5'-hydroxyl ends available for labeling is much less for DNA than for RNA because of the difference in size between the two types of molecules.

The end-labeling studies showed that plasmid samples with significantly less contaminating RNA were obtained by the 5-hr, 65,000 rpm runs in the Type 80 Ti or Type 75 Ti rotors, where the low molecular weight RNA had not yet diffused sufficiently to contaminate the plasmid band. Plasmid purities similar to the 48-hr equilibrium method were achieved by running the Type 80 Ti or Type 75 Ti rotor for 4 hr at 65,000 rpm, the Type 80 Ti for 3 hr at 70,000 rpm (using the small tube), or the Type 70.1 Ti rotor for either 5 hr at 60,000 rpm or 17 hr at 50,000 rpm. With the exception of the 3-hr, 70,000 rpm run, the large tube with its longer pathlength provided better separations. Plasmid DNA prepared by the 5-hr, 65,000 rpm, two-step gradient method has been used, without further purification, for sequencing of the cloned tubulin genes of Chlamydomonas (K. Brunke, personal communication).

The high concentrations of CsCl used in the two-step gradient techniques produce a small precipitate of CsCl crystals during centrifugation. This amount of precipitate does not overstress the titanium fixed angle rotors used in this study. However, these high concentrations should never be run in any swinging bucket rotor, any aluminum fixed angle rotor, or for any length of time beyond those specified above. During longer runs, these gradients would reach equilibrium and much larger pellets of CsCl would be produced (13). As shown by the data in Table 1, longer runs would also result in more contamination by low molecular weight RNA.

The two-step gradient procedure greatly reduces the ultracentrifugation time required for plasmid DNA purification, thereby extending the useful life of both the rotors and the ultracentrifuge employed. Large quantities of different plasmids can be purified in less than one day. This procedure can be useful as a final plasmid DNA purification step following many

Table 1

	Centrifugation Conditions	Percent ^{32}P Label	
		Plasmid DNA	RNA
(1)	Total nucleic acid extract before centrifugation	<1	≈100
(2)	48 hr, equilibrium gradient, Type 50 Ti, 40,000 rpm, large tube	29-45	55-71
(3)	17 hr, two-step gradient, Type 70.1 Ti, 50,000 rpm, large tube	37-45	55-63
(4)	same as lane 3, small tube	25-40	60-75
(5)	5 hr, two-step gradient, Type 80 Ti or Type 75 Ti, 65,000 rpm, large tube	68-75	25-32
(6)	same as lane 5, small tube	60-66	34-40
(7)	3 hr, two-step gradient, Type 80 Ti, 70,000 rpm, small tube	35-40	60-65
(8)	5 hr, two-step gradient, Type 70.1 Ti, 60,000 rpm, small tube	30-35	65-70
(9)	same as lane 8, large tube	35-45	55-65
(10)	4 hr, two-step gradient, Type 80 Ti or Type 75 Ti, 65,000 rpm, small tube	25-35	65-75
(11)	same as lane 10, large tube	30-40	60-70

Comparison of ^{32}P incorporated into plasmid DNA and contaminating RNA as determined by densitometer tracings of autoradiographs such as the one in Figure 3. These percentage ranges represent three different plasmid preparations, and are relative due to different labeling efficiencies (see Results and Discussion).

different plasmid extraction protocols. It may also be possible to adapt this technique for the purification of other types of DNA (e.g., chromosomal, organelle).

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