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A rapid method for the isolation of circular DNA using an aqueous two-phase partition system

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ABSTRACT

A method of isolating circular plasmid DNA from cleared lysates of E.coli is described. Purification is achieved by virtue of the rapid re-annealing kinetics of supercoiled DNA. After a brief denaturation step, double stranded plasmid DNA is separated from denatured chromosomal DNA and RNA in a two-phase partition system using dextran and polyethylene glycol. The method is much more rapid than the conventional dye-centrifugation technique and plasmid DNA of comparable purity and yield is obtained.

INTRODUCTION

Molecular cloning has become a widely used technique for the isolation of specific nucleic acid sequences and bacterial plasmids are the vector of choice for many cloning experiments. However, methods of preparing plasmid DNA have evolved very little since the development of caesium chloride-ethidium bromide centrifugation of cleared bacterial lysates (1). While this technique provides a high degree of purification it is expensive in both centrifuge time and materials and there is a clear application for a rapid method of purification. We have developed a technique which yields essentially pure plasmid DNA within minutes of obtaining a phenolized cleared bacterial lysate:

The technique depends on the fact that plasmid DNA, being a closed circular molecule, displays mono-molecular re-annealing kinetics (2). After denaturation of a mixture of plasmid and E.coli chromosomal DNA, plasmid DNA renatures very rapidly while chromosomal DNA remains single stranded. A two phase partition system is then used to purify duplex plasmid DNA away from single stranded chromosomal DNA and bacterial RNA. This technique has proved particularly useful in the screening of large numbers of transformed clones to determine the restriction enzyme pattern of their plasmid DNA. Since there is no inherent limitations on the scale of the procedure

it may also prove useful in the preparation of very large quantities of plasmid DNA.

MATERIALS AND METHODS

Poly-ethylene glycol 6000 (PEG) was obtained from BDH Chemicals Ltd. (England) and Dextran 500 (lot No. 3447) was purchased from Pharmacia Fine Chemicals (Sweden). E. Coli ribosomal RNA, transfer RNA and chromosomal DNA were obtained from Miles Laboratories Ltd. (England). The restriction enzyme Hae III was a gift from Dr. B. Griffin (Imperial Cancer Research Fund, London, England) and SV40 DNA was a gift from Dr. R. Kamen (Imperial Cancer Research Fund, London, England). Plasmid DNA was prepared from late exponential phase cultures treated for 16 hr with chloramphenicol at 170 µg/ml to amplify the plasmid DNA. Cleared lysates were prepared by the Triton X-100 lysis procedure of Katz et al. (3). The cleared lysates were then treated with Proteinase K at 100 µg/ml for 30' at 37°C. Finally, they were extracted twice with phenol and twice with chloroform-isoamyl alcohol (99 to 1) and precipitated with 2 volumes of ethanol. (Chloramphenicol was obtained from Sigma and Proteinase K from Merck)

In a typical experiment to purify plasmid DNA for restriction enzyme analysis, the phenolized cleared lysate of a 5 ml culture was resuspended (after ethanol precipitation) in 0.2 ml of TE-buffer (10 mM Tris pH 8.0, 1 mM EDTA) in a Microfuge tube. This gives a plasmid DNA concentration of roughly 25 µg/ml. The sample was heated for 2 minutes at 100°C to denature chromosomal DNA and rapidly cooled in dry ice-ethanol. Then 500 µl of dextran 500 (16.8% W/W), 250 µl of PEG (18.4% W/W) and 20 µl of 0.5 M sodium phosphate buffer pH 6.8, were added at 4°C. The volume was then adjusted to 1 ml with water and the whole stirred with a vortex mixer. It is important to note that the exact concentrations of the components of the two-phase system (this was determined by freeze druing a weighed aliquot of the stock solution), the pH of the buffer and the temperature (4°C) are critical factors in determining the separation achieved (for reviews of two-phase separation techniques see references 4 and 5). Separation of the polymer phase was accelerated by centrifugation in a Beckman Microfuge for 1 minute at 4°C. Then 450 µl of the upper, PEG rich, phase was recovered and transferred to a fresh tube. After addition of NaCl to 0.2 M, DNA was collected by precipitation with two volumes of ethanol. This procedure also precipitates the phosphate and a small amount of PEG but this does not interfere with subsequent restriction enzyme analysis. (An alternative procedure which avoids co-precipitation of phosphate and PEG

is cetyl trimethyl ammonium bromide (CTAB) precipitation (8)). Pellets were washed twice in 100% ethanol, dried and taken up in a small volume of H_2O. Restriction analysis was performed using the enzyme Hae III with subsequent electrophoresis on 3.5% acrylamide gels (6). Electron microscopy was performed on DNA spread by the aqueous procedure of Davies et al. (7).

RESULTS

The principal components in a phenol extracted, cleared lysate of E. coli are ribosomal RNA and transfer RNA; plasmid DNA, when present, forms a relatively small fraction of the total nucleic acid. In addition to plasmid DNA there is normally a roughly equivalent amount of chromosomal DNA. This depending on the efficiency of the chloramphenicol amplification step and the effectiveness of the final centrifugation step in the lysis procedure. In order to determine the potential purification of plasmid DNA achievable in a two phase partition system, a model experiment was performed using purified E. coli RNA and DNA. The two phase system was optimized as described by Alberts (5) and the results are presented in Table 1a. As would be expected from previous results (5,6) only double stranded nucleic acids were found to any significant extent in the upper PEG rich phase. Thus partition under these conditions should produce a substantial purification of plasmid DNA away from the principal contaminant E.coli ribosomal RNA.

We reasoned that additional purification would result from heat denaturation of the nucleic acids prior to the partition, since the closed circular plasmid DNA will re-anneal rapidly under these conditions (2), and hence remain in the PEG phase. (A similar approach has been used to purify other rapidly re-annealing species (9,10). This proved to be correct (Table 1a) and, after heat treatment, chromosomal DNA was almost totally removed from the PEG phase. A significant amount of transfer RNA remains in the PEG phase, irrespective of heat treatment, presumably because of the large amount of intramolecular base pairing which causes rapid re-annealing. For most purposes the presence of transfer RNA presents no problem and we decided to attempt plasmid purification using this procedure.

Table 1b shows the degree of purification that can be achieved when an artifical mixture of plasmid DNA, chromosomal DNA and ribosomal RNA are subjected to phase separation. Using one cycle of purification a 17-fold

enrichment was obtained, and after a second partition of the PEG phase with dextran this was increased to 35 fold. The yield of plasmid DNA was 79% after one cycle of purification and 61% after two cycles. For restriction analysis the degree of purity and yield of plasmid DNA after a single phase extraction was more than adequate, and all subsequent preparations were purified in this way.

The use of this technique to purify plasmid DNA from bacterial lysates is illustrated in Fig. 1. A series of bacterial clones, each containing PMB9 plasmid with a different eukaryotic DNA sequence inserted at the ECoRI site, were grown up and chloramphenicol amplified. Phenol

TABLE 1a

Distribution of various E.coli nucleic acids after two-phase partition

Sample	% of input in upper phase (PEG)	
	Native	After heating at 100°C for 2 minutes
Ribosomal RNA	1.8	1.6
Transfer RNA	37	37
Chromosomal DNA	61	2.5
^{3}H Plasmid (PMB9) DNA	79	78

A 1 ml phase partition system was used (see Materials and Methods) and the input of each, non-isotopically labelled, nucleic acid was about six OD_{260} units. The ^{3}H labelled PMB9 DNA was prepared by standard dye-centrifugation procedure and had a specific activity of 200 cpm per µg; 1,000 cpm was used in each partition.

TABLE 1b

Distribution of an artifical mixture of nucleic acids after two-phase partition

	% of total OD_{260} units recovered in upper polymer phase	% recovery of ^{3}H PMB9	- fold purification
Samples subjected to a single two-phase partition	4.7	79	16.7
The same samples after a second two-phase partition	1.7	61	35.1

The artificial mixture contained, by weight 50 parts chromosomal DNA, 50 parts ribosomal RNA and 1.4 parts ^{3}H PMB9 (comprising 5 µg). A 1 ml phase partition system was used and the second partition was performed by adding 450 µl of dextran and 20 µl of phosphate buffer to the PEG phase. The theoretical maximum purification is 72.3 fold.

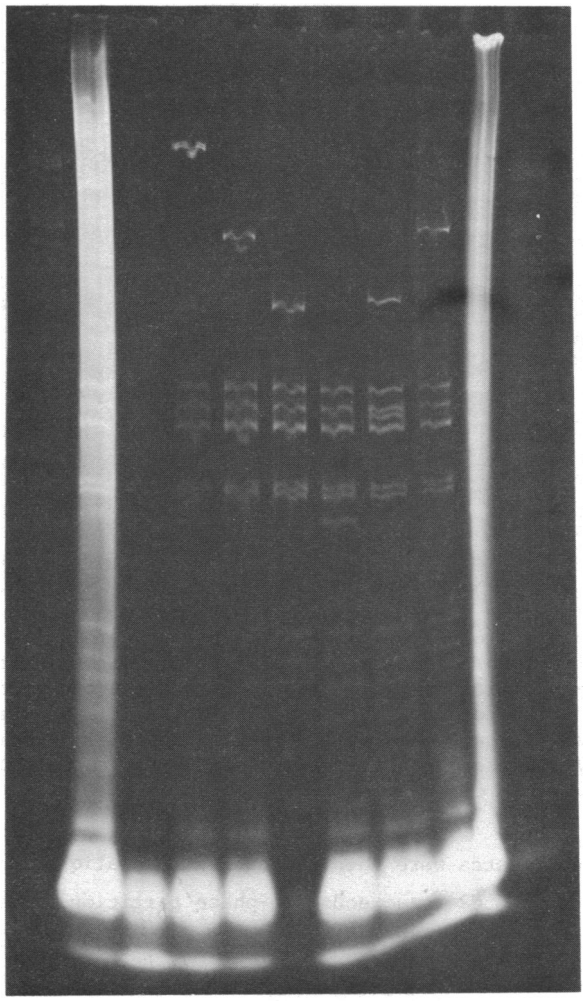

Figure 1. The total nucleic acid from phenolized, cleared lysates of eight bacterial cultures (20 ml), each containing a fragment of eukaryotic DNA of undetermined length, was resuspended in 1 ml of distilled H_2O. Two hundred microlitre aliquots of each preparation were subjected to a single two phase partition and ethanol precipitated. Each pellet was then dissolved in 20 µl of H_2O, restricted with Hae III and run on 3.5% acrylamide gel (slots b to h). One hundred microlitre aliquots of the preparations run in slots b and h were ethanol precipitated and restricted without purification (slots a and i). The outside slots were loaded with an SV40 marker DNA digested to completion with Hin III.

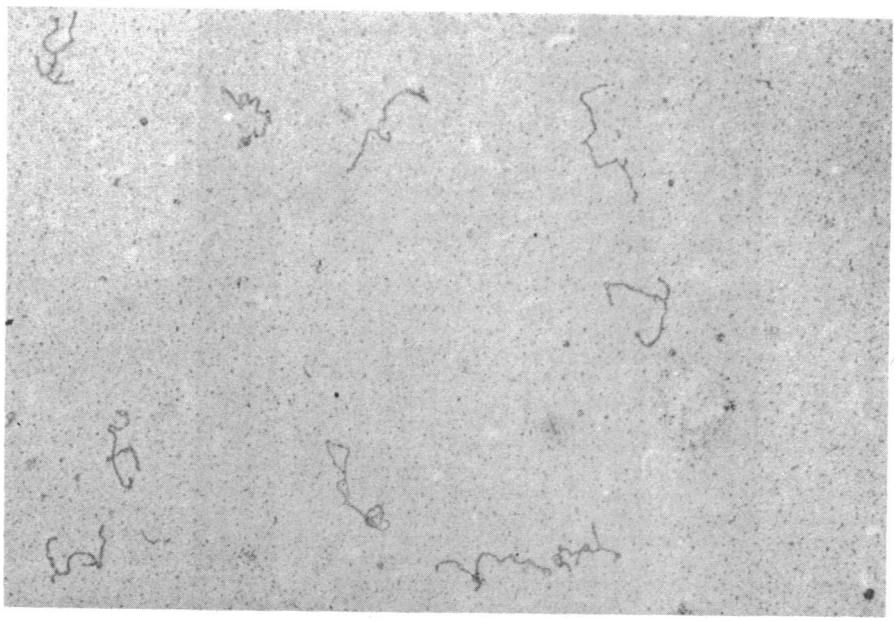

Figure 2. A phenolized cleared lysate of E.coli containing the plasmid PMB9 was subjected to a single two phase partition. Samples removed before and after purification were prepared for electron microscopy under the aqueous conditions of Davies et al. (8). The sample removed before purification showed a large amount of granular material, assumed to be RNA, which completely obscured DNA molecules (results not shown). The field shown is quite typical of the purified preparation.

extracted cleared lysates were then prepared and an aliquot of each preparation was subjected to aqueous two-phase partition, restricted with Hae III and analyzed by electrophoresis on 3.5% acrylamide gels. For two of the preparations, an aliquot of unpurified nucleic acid was similarly analyzed (Fig. 1). The majority of the nucleic acid in the unpurified samples is RNA, which totally obscures the smaller Hae III restriction fragments of the plasmid DNA. There is also a significant amount of chromosomal DNA, giving rise to a restriction enzyme pattern which partially obscures the large restriction enzyme fragments of the plasmid DNA. After two-phase partition plasmid DNA appears to be pure except for a significant amount of tRNA which runs near the end of the gel. Gel electrophoresis, however, is not sensitive enough to detect a low level contaminant of heterogeneous size (such as restriction fragments of chromosomal DNA). Therefore, similar samples were analyzed by electron microscopy. Before phase partition the presence of large amounts of RNA made it impossible to quantitate the various species. After phase

partition (Fig. 2), in a total of 95 molecules examined, there were 92 supercoils, and 3 linear molecules of lengths different from that of the plasmid DNA, this indicates a very high degree of purification with respect to chromosomal DNA.

DISCUSSION

The great virtue of the aqueous two-phase separation technique presented here is the speed with which a quite highly purified preparation of plasmid DNA can be prepared with a minimum expense in materials and equipment. Once a phenolized cleared lysate has been obtained, the entire procedure takes less than one hour. This compares with two or three days of centrifugation to obtain caesium chloride purified plasmid, which subsequently has to be purified to remove ethidium bromide and other components of the lysis and centrifugation solutions. The small scale on which the procedure can be performed means that plasmid DNA can be prepared from a few ml. of E.coli and this reduces the potential hazards associated with growing large amounts of an uncharacterized eukaryote cloned DNA. Another small scale plasmid enrichment procedure has been reported (11) but this requires an RNAase step and contaminating chromosomal DNA is not removed. Our technique can easily be scaled up to prepare very large quantities of plasmid DNA in which case the alkali denaturation procedure of Summers and Szybalski (9) is convenient since no boiling step is required.

The procedure is of course very suitable for dealing with large numbers of samples. We have found this particularly useful when screening individual "cDNA" clones to determine the size of the eukaryotic DNA insert. This being a common requirement of the cloning procedure, even if size selected cDNA is used for the cloning. (Fig. 1 illustrates such a screen). However, the technique clearly has application in all situations where contamination by E.coli tRNA and a small amount of E.coli chromosomal DNA constitutes no problem. The data in Table 1b is particularly impressive in view of the very high ratio of chromosomal DNA to plasmid DNA in these samples (49 to 1.4). This is much higher than that actually found in chloramphenicol amplified cleared lysates (which we estimate as being about 1 to 1). This indicates that the method can probably be used to purify plasmids, which cannot be chloramphenicol amplified (especially if two cycles of phase separation are used). Also of course it is applicable to any closed circular or spontaneously re-annealing DNA and we have recently used this technique to purify mitochondrial DNA (C.J. Coote and

R. Ohlsson, unpublished results).

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