

# General Method for the Isolation of Plasmid Deoxyribonucleic Acid

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Plasmid deoxyribonucleic acid (DNA) ranging from  $5 \times 10^6$  to  $65 \times 10^6$  daltons may be isolated from chromosomal DNA by the preferential precipitation of the higher-molecular-weight chromosomal DNA in the presence of sodium lauryl sulfate and a high concentration of NaCl.

Most of the techniques currently employed for the isolation of plasmid deoxyribonucleic acid (DNA) are based upon their supercoiled, covalently closed circular (CCC) configuration within the bacterial cell. Thus, ethidium bromide density gradient centrifugation (CsCl-Et Br; 1), nitrocellulose adsorption (3), and alkaline sucrose sedimentation (6) are all dependent upon certain characteristics unique to CCC-DNA. The Brij lysis technique, or cleared lysate method (2), has also been widely employed to isolate plasmid DNA. This method is largely dependent upon the fact that plasmid DNA appears to be present in the cytoplasm of the cell as CCC-DNA and does not sediment with a Brij-resistant cellular fraction (presumably the cell membrane) to which the chromosome is firmly affixed. Hirt (8) has described a method for the separation of polyoma DNA from cellular DNA by the preferential precipitation of the higher-molecular-weight cellular DNA in the presence of sodium lauryl sulfate (SLS) and a high concentration of NaCl. We have modified slightly the Hirt procedure and have found it generally applicable to the separation of plasmid DNA from the bacterial chromosome. We believe this method deserves attention since it is applicable to a wide variety of bacterial species and it is not essential that plasmid DNA be present as CCC molecules, and this method facilitates the isolation and identification of replicative intermediates.

A number of strains of *Escherichia coli* K-12 harboring plasmids of known molecular sizes ranging from  $5.4 \times 10^6$  to  $65 \times 10^6$  daltons were grown to the late logarithmic stage of growth in 30 ml of a minimal salts medium supplemented with 0.5  $\mu$ g of thymine per ml, 250  $\mu$ g of

deoxyadenosine per ml, and 3  $\mu$ g of <sup>3</sup>H-thymine (New England Nuclear Corp.; 20 Ci/mmol) per ml. Cells were harvested by centrifugation and suspended in 1 ml of 25% sucrose in 0.05 M tris(hydroxymethyl)aminomethane (Tris), pH 8.0. After addition of 0.2 ml of lysozyme (Sigma Chemical Co., 5 mg/ml in 0.25 M Tris, pH 8.0) the suspension was placed in an ice bath for 5 min. Ethylenediaminetetraacetate (EDTA, 0.4 ml, 0.25 M, pH 8.0) was added to the cells, and the suspension was chilled in an ice bath for an additional 5 min. Complete cellular lysis was accomplished by the addition of SLS to a final concentration of 1%. In the case of lysozyme-resistant species, the lysozyme and EDTA steps were, of course, unnecessary, and lysis was directly accomplished with SLS. After cellular lysis, 5 M NaCl was added to the viscous solution with gentle mixing to a final concentration of 1 M. The lysates were stored at 4 C overnight, after which they were centrifuged at  $17,000 \times g$  for 30 min in a refrigerated centrifuge. This step was found to remove essentially all (>99%) of the chromosomal DNA, but left any plasmid DNA in the supernatant fluid. Figure 1 shows the sedimentation properties in 5 to 20% neutral sucrose gradients of samples of the supernatant fluid from three plasmid-bearing *E. coli* strains and an *E. coli* F<sup>-</sup> strain. As seen in Fig. 1 (upper curve), the small amount of radioactive material remaining in the supernatant fluid from a control F<sup>-</sup> strain sedimented with a broad, ill-defined pattern, similar to that seen in our laboratory in Brij-lysed cells. The radioactive material present in the supernatant fluid from isogenic R-plasmid-bearing strains yielded well-defined, sharp peaks corresponding to CCC-DNA and open circular molecules of plasmid DNA. These plasmid DNA peaks were identical in their sedimen-

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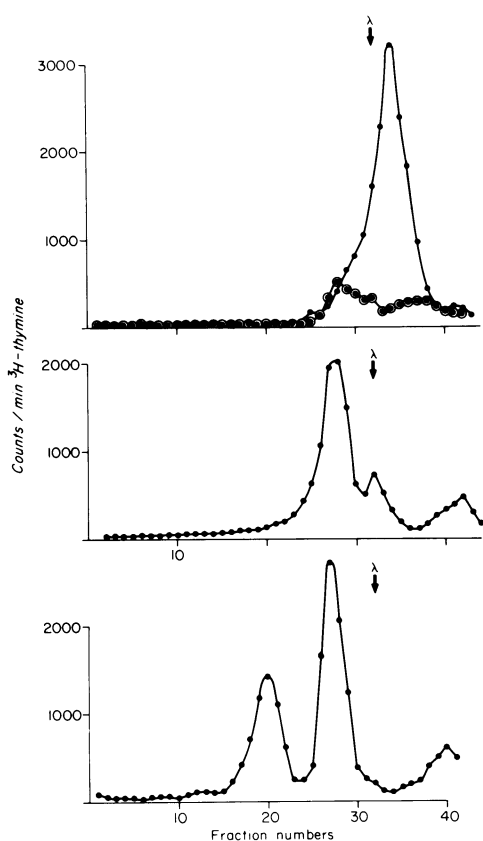


FIG. 1. Sedimentation of the radioactive material remaining in the supernatant fluid after NaCl precipitation. Bacterial cells labeled with  $^3\text{H}$ -thymine were lysed with SLS and mixed with NaCl to a final concentration of 1 M, as described in the text. After storage overnight, the lysates were centrifuged at  $17,000 \times g$  for 30 min. Samples of 100  $\mu\text{l}$  of the supernatant fluid were mixed with an equal volume of TE (0.01 M Tris, 0.001 M EDTA, pH 8.1) to lower the NaCl concentration, and layered onto 5 to 20% (wt/vol) linear sucrose gradients containing 0.5 M NaCl, 0.01 M potassium phosphate, pH 7.0.  $^{14}\text{C}$ - $\lambda$  DNA was added as a sedimentation marker (34S;  $30 \times 10^6$  daltons). Gradients were centrifuged at 40,000 rpm at 15 C for 60 min in an SW41 rotor of a Beckman L2-50 preparative ultracentrifuge. After centrifugation, 12-drop fractions were collected from a hole punctured in the bottom of the tube, and the fractions were counted as described previously (10). Top: Supernatant fluid from *E. coli* J5  $F^-$  (●) and from the same strain harboring the non-conjugative plasmid RSF1010 (Su, Sm;  $5.4 \times 10^6$  daltons) (●). Middle: Supernatant fluid from *E. coli* J5 carrying the R plasmid RSF10 (Cm, Km, Sm;  $26 \times 10^6$  daltons). Bottom: Supernatant fluid from *E. coli* J5 carrying the R-plasmid, R144 (Km, col I;  $65 \times 10^6$  daltons).

tation properties to plasmid DNA isolated by both the Brij-cleared lysate and CsCl-EtBr methods. The identity of the DNA fractions seen in the supernatant fluid of salt-precipitated lysates as plasmid DNA was further confirmed by electron microscopy.

In many instances the plasmid isolation procedure described in this paper offers several distinct advantages over other available methods. First of all, it is not essential that the plasmid be present as CCC-DNA; the technique has permitted us to isolate any plasmid DNA species within the range of  $5 \times 10^6$  to  $65 \times 10^6$  daltons from a variety of bacterial hosts. We have not yet determined the upper limit of usefulness of the method. The  $65 \times 10^6$  dalton plasmid of R-factor R144 was isolated in the same quantity (or slightly greater) by the salt precipitation method as with the Brij method. Presumably, even though the method is based on selective precipitation according to size, it may be amenable to plasmids considerably larger than  $65 \times 10^6$  daltons. Since the majority of R plasmids under study, as well as F and Col I, are in the  $5 \times 10^6$  to  $65 \times 10^6$  dalton range, the method should have rather wide application.

A major advantage of the method reported here is with respect to the isolation and identification of replicative intermediates. As noted earlier, when not replicating, plasmid DNA appears to be present in the cell as CCC-DNA. However, during replication a membrane attachment site may be present and, in fact, attachment of noncircular, "intermediate" plasmid DNA species to a rapidly sedimenting cellular component has been observed (4, 7). Such replicative forms are usually not observed in the supernatant fluid of Brij-lysed cells. Such intermediates normally sediment as linear or open circular DNA in alkaline sucrose gradients or as bands of intermediate density or light density in CsCl-EtBr gradients of whole-cell lysates. There are relatively few methods available to more directly focus on such replicative forms. The minicell system has such possibilities (9) but involves somewhat tedious isolation steps and is restricted in that not all plasmids segregate into minicells and replication of those that do may be limited (9). Another method that may be used to characterize replicative intermediates directly is the mating system of Freifelder and Freifelder (6) for  $F_{lac}^+$  and used previously in this laboratory to study the R plasmid Rldrd 19 (4, 10). However, this experimental system is also limited in that it may be applied only to derepressed sex factors. In principle, the salt precipitation procedures can,

under appropriate conditions, be used to examine CCC, open circular, linear, and replicative intermediate forms of all plasmid classes, transmissible as well as non-conjugative. Indeed, as will be reported in a subsequent communication, the salt precipitation method has been successfully applied to the identification and isolation of replicative intermediates of several plasmids that had not been previously identified in Brij lysates.

A final advantage of the salt precipitation method is that it may be used with SLS and, therefore, can be more easily applied to examine plasmid components of bacterial species, other than *E. coli*, which are not amenable (or only marginally so) to Brij lysis. The salt precipitation method, for example, has been successfully employed in this laboratory to study plasmids in *Proteus*, *Salmonella*, *Vibrio cholerae*, *Streptococcus lactis*, and *Neisseria gonorrhoeae*.

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