Raid and Economical Method for Purification of Plasmid DNA*

Atsuo Mori • Zen-ichi Ogita**

SUMMARY

A preparative procedure for obtaining highly purified plasmid DNA from bacterial cells is described. Rapid preparation of milligram quantities of plasmid DNA is achieved by ethidium-bromide (EtBr)-cleared lysate formation, polyethyleneglycol (PEG) precipitation, and chromatography through a BioGel A-50m column. The average yield of pBR322 DNA from 1 liter of amplified culture by this procedure is 1.8 mg and the preparation is highly pure. This method is applied to the cloning of the human β-globin gene cluster.

Key words: Rapid gene cloning, BioGel A50-m, Human β-globin gene cluster, electrophoretic separation of vector.

INTRODUCTION

Recently, plasmids have become the most frequently used vectors for cloning specific DNA sequences. Pure plasmid DNAs in either small or large quantities must be prepared for use as cloning vectors. Several techniques have been reported on the isolation of plasmid DNA. At present, a widely used method is to band the DNA from a "cleared lysate" in a cesium chloride gradient in the presence of ethidiumbromide; covalently closed circular (ccc) DNA is separated from open circular (oc) and chromosomal DNA by this procedure. Then, "cleared lysate" is ordinarily prepared by the method of Clewell and Helinsky1). Other methods are hydroxyapatite chromatography2), acridine yellow affinity chromatography3), and alkaline extraction4). However, the cesium chloride equilibrium density gradient centrifugation method is expensive and time consuming. A more rapid method for preparing highly purified plasmid DNA employs hydroxyapatite chromatography, but the plasmid DNA isolated by this method still contains about 0.5% chromosomal DNA as a contaminant. Moreover, the yield by these methods is not usually high. And through every process, we have the same difficult stage clearing the lysate formation. The yield of plasmid DNA depends on the yield of cleared lysate. Purification of plasmid DNA therefore requires the selective removal of chromosomal DNA. A very effective initial purification is achieved by the preparation of an ethidium bromide-cleared lysate, the method was developed by Mukhopadhyay and Mandel5). In their method, the average yield of

* 迅速で経済的なプラスマッドの精製法
** 森 篤雄, 萩田喜一, 富山医科薬科大学和漢薬研究所病態生化学部門
（Department of Pathogenic Biochemistry, Research Institute for Oriental Medicines, Toyama Medical and Pharmaceutical University, Sugitani, Toyama 930-01, Japan）
（Accepted June 25, 1985, Received July 10, 1985）
plasmid DNA is 2~2.5 mg from 1 liter of amplified culture and the preparation is highly pure, containing only about 0.005% chromosomal DNA. Final separation of plasmid DNA from low molecular weight RNA is achieved by using polyethylene glycol 6000 and by a BioGel A-50m column.

By a combination of a few simple, effective techniques and using high pressure liquid chromatography we have invariably isolated up to 1.8 mg of plasmid DNA from 1 liter of amplified culture within three days. This quantity is enough to use as a cloning vector.

**MATERIALS AND METHODS**

The bacterial strains used were *Escherichia coli* strain HB101 containing the plasmid pBR322 and the same strain HB101 containing the cosmid pJB8 combined with a β-globin gene cluster. The strains HB101 containing pBR322 and pJB8 were obtained from Dr. Y. Yoneda and Dr. Y. Fukumaki respectively.

The following chemicals were used: chloramphenicol, lysozyme, ampicilne and tetracycline, which were purchased from Sigma Chemical Company, St. Louis, Missouri; Bacto-tryptone, Yeast extract, and sodium deoxycholate, which were Difco products; Brij 58, which was purchased from Wako Pure Chemical Industries, Ltd, Osaka, Japan; ethidium bromide (EtBr), which was purchased from Aldrich Chemical Company, Inc, Milwaukee; restriction endonucleases EcoRI, BamHI, etc., which were purchased from Nippon Gene Co. Ltd, Toyama, Japan.

Two kinds of media were used: TB medium was 1% Bacto-triptone, and 0.5% NaCl; LB medium was 1% Bacto-triptone, 0.5% Yeast extract, and 1% NaCl. Solutions were: TES buffer: 0.05 M Tris-HCl, 0.005 M EDTA, and 0.05 M NaCl (pH8.0); Lysis mixture: 1% Brij 58, 0.4% Sodium deoxycholate, 0.062 M EDTA, and 0.05 M Tris-HCl (pH8.0); and TBE buffer for electrophoresis: 0.09 M Tris-Borate and 0.0025 M EDTA2Na (pH8.3).

**Growth of Bacteria and Amplification of Plasmids.**

*Escherichia coli* HB101 containing the plasmid pBR322 was grown in TB and LB media at 37°C on a shaker up to an OD660 of 1.0~1.2. Plasmid amplification was achieved by further growing the above culture for 12~16 hrs. in the presence of 150 μg/ml of chloramphenicol under identical conditions of temperature and shaking. The cells were then harvested, washed once with Tris-HCl buffer, and were used on the same day for isolation of plasmid DNA as described below.

**Detailed Procedure for Isolating of Plasmid DNA from DNA from Escherichia coli.**

Sterile glassware and solutions were used. The EtBr containing steps were performed with gloves and sterile glassware were wrapped in aluminium foil to intercept direct light. All the operations were done quickly at 0~4°C. The procedure consists of the following steps:

1. **Preparation of EtBr-cleared lysate**

Cells collected from 1 liter of amplified culture were divided among two polyethylenetubes (50 ml), and were suspended in 7 ml of cold Tris-HCl (pH 8.0) buffer containing 25% sucrose respectively. Then 1.5 ml of a EtBr solution (5 mg/ml) was added to the suspension and mixed well and 1.5 ml of lysozyme solution (5 mg/ml) was added in the same way. After 7 min 3 ml of 0.25 M EDTA was added and the mixture was then incubated for 5 min. Finally, 12 ml of lysis mixture was added and the preparation mixed thoroughly but gently, and incubated for 30 min with occasional, gentle stirring. The slightly viscous, total lysate was then centrifuged at 31,000 × g for 40 min. The clear, reddish supernatant was removed with a pipette. This reddish EtBr-cleared lysate was used in the subsequent purification.

2. **Deproteinization of the EtBr-cleared lysate**

The EtBr-cleared lysate was reduced to 1% with respect to SDS and heated at 65°C for 30 min. After that the mixture was cooled to room temperature and was extracted twice with TES buffer saturated.

The colorless, aqueous layer (all the EtBr color remained in the phenol layer) from the last extraction was mixed with 8% (w/v) polyethylene glycol (PEG) 6,000. Into this mixture, diethylether was added. Traces of phenol in the aqueous layer were removed by extraction with an equal volume of diethylether.

(3) Removal of RNA by Polyethylene Glycol 6,000 and BioGel column

After removing the ether, the solution was added to 0.5M NaCl and stirred for 3 hrs. The precipitate containing the plasmid was collected by centrifugation at 4,000g for 20 min, and was redissolved in TES buffer. The resulting solution was passed through a BioGel A-50m column which was connected to high pressure liquid chromatography equipment (HPLC Pharmacia Fine Chemicals) equilibrated in 0.01M Tris-HCl, 0.01M NaCl, 0.001 M EDTA, 0.025% NaN₃ (pH8.5) at room temperature. The column size was 2.2×20 cm. The plasmid was concentrated by ethanol precipitation. Until then, the 99.5% ethanol was stored at -20°C.

Analysis of Plasmid DNA by Agarose Gel Electrophoresis

Plasmid DNA precipitation from each step was electrophoretically analyzed by using agarose gel. Electrophoresis was carried out at a constant 75 volts for 1.5 hrs. in 1.0% agarose gel (Tris-borate buffer with 0.5μg/ml of EtBr). In this case the gels were stained with EtBr, but EtBr was omitted from the Tris-Borate buffer. The gels were photographed on a short wave UV trans-illuminator.

RESULTS AND DISCUSSION

The above procedure for large scale purification of plasmid DNA is simple and rapid. We routinely obtained more than 1.8 mg of plasmid DNA per liter of cells grown to late log phase followed by addition of chloramphenicol for pBR322 DNA. The Result of a typical purification is shown in Figure 1. In this gel and buffer system, low molecular weight RNA migrates more rapidly, and larger chromosomal fragments, more slowly. This plasmid was chosen because it has become the standard plasmid for recombinant DNA experiments and has been completely sequenced. Now we also use this plasmid for any recombinant DNA experiments. And it was found that this plasmid is suitable for analysis with restriction endonucleases and nick translation. Agarose gel electrophoresis shows (Figure 1) that plasmid DNA and low molecular weight RNA were the major nucleic acid present after PEG precipitation. Before PEG precipitation, traces of phenol in aqueous layer was removed. At fast 8% (w/v) PEG was mixed with aqueous layer and into this mixture equal volum of ether was added and mixed well. After half an hour solution was clearly separated into two layers. Using this method, the amount of bubbles which are supposed to be created between these two layers became very small. Then ether layer was able to removed easily. PEG precipitation did not always remove all of the RNA, but the residual RNA was easily removed by BioGel A-50m.
column chromatography (Figure 2).

Then all of the fractions were collected for an hour and those containing plasmid DNA were identified by determination of their absorbance at 260nm. Appropriate fractions were pooled and the amount of DNA was estimated (1 OD260=50µg/ml). The plasmid DNA was then concentrated by precipitating with 2 volumes of chilled (-20°C) 99.5% ethanol (at least 2 hrs. at −80°C).

Judging from the electrophoretic pattern, the quantity of covalently closed circular plasmid was much more than that of other plasmids. Mukhopadhyay and Mandel (5) have reported that the presence of EtBr prevents the lysate of bacterial cells from being viscous, and helps pack the pellet better during high-speed centrifugation. Our results were similar too. The isolation of DNA in the presence of a high concentration of EtBr was, for both quality and quantity, much better than that of the plasmid DNA prepared by other methods currently in use. In every method currently in use, it takes at least 2 days to cultivate bacteria including plasmid DNA. This time can’t be reduced. But using our method, one can reduce the time required to isolate the plasmid DNA. With our method, purified plasmid DNA can be obtained within only 8 hrs. The total period was 3 days.

It was also found that the yield of cells in the LB medium was much greater than that of cells in the TB medium.

To clone the human β-globin gene cluster our method was applied. To clone the human β-globin gene cluster, it was combined with cosmid vector pJB8 on a BamHI site. The bacterial strain HB101 containing the recombinant DNA was grown selectively on LB medium supplemented with ampicilnine and amplified by the addition of chloramphenicol. The recombinant DNAs were collected, according to the method just described. It was found that the human β-globin gene was cloned effectively (Figure 3).

Fig. 2. An elution profile showing the separation of plasmid pBR322 from low molecular weight RNA on a BioGel A-50m column.

500µl fractions were collected at a rate of 0.2ml/min at room temperature in an elution buffer of 0.01M Tris-HCl pH8.5, 0.01M NaCl, 0.001M EDTA, and 0.025% NaN$_3$.
Fig. 3. An elution profile showing the separation of cosmid pJB8 from low molecular weight RNA on a BioGel A-50m
500μl fractions were collected at a rate of 0.2ml/min at room temperature in an elution buffer of 0.01M Tris-HCl pH8.5, 0.01M NaCl, 0.001M EDTA, and 0.025% NaN₃.

REFERENCES

ACKNOWLEDGEMENT
The authors express their sincere appreciation to Dr. C.L. Markert for discussing this work with them and they are very thankful to Dr. J.G. Scandalios for his suggestions during preparation of the manuscript.

従来からいくつかのプラスマドの精製法が発表されているが、その中の Mukhopadhyay らの方法と Lillis らの方法を組み合わせさらに改良を加えることによって、プラスマドを短時間に効率よく精製する方法を確立した。培地1000mlで培養した大腸菌からのプラスマドpBR322の平均収量は1.8mgであった。我々が確立したこの方法で、ヒトβ鎖ヒトニン遺伝子群の組み込まれたプラスマドを回収したが、効率よく短時間に遺伝子のクローニングができることを確認したのでここに報告する。