Alternative Method for Isolation of Double-Stranded Template for DNA Sequencing


The increased use of plasmid DNAs in recombinant DNA technology, coupled with the growing popularity of double-stranded DNA sequencing, necessitates the development of rapid, inexpensive and efficient procedures for the isolation of highly purified plasmids. Numerous miniprep procedures have been described for preparing plasmid DNAs (1,2,4,5,8,9). A quick and efficient alternative method for the preparation of large quantities of purified plasmid DNA is presented in this report. Following alkaline lysis of the bacterial cells, polyethylene glycol-6000 (PEG-6000) (13% final concentration) is used to precipitate the nucleic acid. The plasmid is then further enriched by selective solubilization using 2.75 M LiCl. Any remaining chromosomal DNA, RNA and cellular debris are removed at this step. This method does not involve any phenol-chloroform extractions or treatment of the samples with ribonuclease and requires only two hours for completion of the protocol following growth of the bacterial cells. The quality and yield of the plasmid obtained with this method is comparable to that isolated by cesium chloride-ethidium bromide gradient centrifugation (CsCl-EtdBr) or purified through QIAGEN QIAprep® columns (Qiagen, Chatsworth, CA, USA). The plasmid obtained is amenable to digestion with various restriction endonucleases, can be used for cloning with high efficiency and is also suitable as a template for dideoxy sequencing. This inexpensive procedure, which includes a PEG-precipitation step, represents a modification of the alkaline lysis method (1,5) and the LiCl solubilization method (3) (Table 1).

For denaturation of the DNA template prior to dideoxy sequencing, a volume of 18 µL of plasmid DNA, as isolated in Table 1, was mixed with 2 µL 2 M NaOH. After 5 min, the solution was neutralized by the addition of 8 µL 5.5 M LiCl. The DNA was precipitated with 75 µL of cold 100% ethanol at -70°C for 10 min and recovered by centrifugation at 4°C for 10 min at 14 000× g. The DNA pellet was rinsed with 100 µL of cold 70% ethanol, dried under a vacuum for 5 min and stored as a dry pellet at -20°C (stable for several weeks). For the sequencing reaction, the denatured plasmid template was resuspended in a mixture of annealing buffer and sequencing primer (primer T3) as described in the standard Sequenase® protocol provided by United States Biochemical (Cleveland, OH, USA) (7).

For comparison purposes, pBlue-Script® II SK(+) plasmid (Stratagene, La Jolla, CA, USA) was isolated by the method described above and two other
Table 1. The Procedure for the Rapid Isolation of Plasmid DNA

1. Inoculate a single bacterial colony into 1.5 mL of TB medium [17 mM KH$_2$PO$_4$, 72 mM K$_2$HPO$_4$, 1.2% (wt/vol) Bacto-Tryptone, 2.4% (wt/vol) bacto-yeast extract and 0.4% glycerol] in a 10–15-mL culture tube and incubate in the presence of the appropriate antibiotic. Incubate at 37°C in a shaker-incubator for 12–18 h.

2. Centrifuge the bacterial cells in a microcentrifuge tube at 14,000×g for 2 min.

3. Remove the supernatant by aspiration, resuspend the bacterial pellet in 100 µL of GTE buffer (50 mM glucose, 25 mM Tris-HCl, pH 8.0, and 10 mM EDTA) and incubate at room temperature for 5 min.

4. Chill the pellet on ice, add 200 µL of freshly prepared alkaline lysis solution (0.2 N NaOH, 1% sodium dodecyl sulfate [SDS]), mix by inversion and incubate on ice for 5 min. For bacterial cells, which are difficult to lyse, a 5-min pretreatment with lysozyme at room temperature is optional (1 mg/mL final concentration).

5. Neutralize the solution by adding 150 µL of 3 M sodium acetate, pH 4.8, mixing by inversion and incubating on ice for 5 min.

6. Centrifuge the mixture at 14,000×g for 10 min at 4°C and transfer the supernatant to a clean microcentrifuge tube.

7. Add 145 µL of 40% PEG-6000 to the supernatant and mix by inversion. Incubate on ice for 10 min.

8. Centrifuge the plasmid DNA at 4°C for 10 min at 14,000×g.

9. Remove the supernatant by aspiration and perform a second brief spin to collect and remove all the supernatant.

10. Dissolve the centrifuged plasmid DNA in 100 µL of deionized H$_2$O or TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5), add 100 µL 5.5 M LiCl and incubate on ice for 10 min.

11. Centrifuge the sample at 14,000×g for 10 min at 4°C. Discard the pellet and transfer the supernatant to a clean tube.

12. Precipitate the plasmid DNA by adding 0.6 vol of isopropanol. Incubate for 10 min at room temperature.

13. Centrifuge the DNA at 14,000×g for 10 min at 4°C. Remove supernatant.

14. Rinse the DNA pellet with 500 µL of cold 70% ethanol. Dry the DNA pellet under a vacuum for 5 min.

15. Dissolve the DNA pellet in 20 µL of TE buffer. Load 1 µL of total volume on a 1% agarose gel to analyze the quantity and quality of plasmid DNA.

Two important parameters in this procedure for obtaining supercoiled plasmid DNA (>95%) are to keep the samples on ice and to avoid vortex mixing. Additionally, this procedure efficiently removes bacterial RNA by a combination of alkaline treatment and selective precipitation with LiCl. This approach avoids the need for ribonuclease treatment and subsequent organic extractions.

Figure 1 compares the quality and conformation of plasmid pBluescript II SK+ DNA isolated by our modified PEG-purification method and plasmid isolated by CsCl–EtdBr-gradient centrifugation and the commercial QIAGEN QIAprep column. The plasmid DNA isolated by our procedure is primarily supercoiled and shows no chromosomal DNA contamination; the quality and yield are as good as or better than that produced by the two other methods compared in this analysis. The
plasmid isolated by our procedure can be used directly as a template for double-stranded DNA sequence analysis. Figure 2 shows nucleotide sequence data from the multiple cloning site region of plasmid pBluescript II SK(+). Sequencing reactions using template isolated by our procedure generated sequencing information of equal quality to those templates isolated by the CsCl or the QIAGEN procedures.

In conclusion, we report here a rapid and inexpensive protocol for plasmid isolation. Plasmid isolated by this method is of sufficient quality for use in DNA sequencing reactions and other molecular biological techniques. This procedure provides an attractive alternative to more expensive and/or time-consuming methods currently used to prepare plasmid DNA.

REFERENCES


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