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Simple methods for preparation of plasmid DNA yielding long and accurate sequence data

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DNA sequencing results largely depend on the quality of templates. Sequencing with fluorescent primers is especially sensitive to the quality of the DNA. Usual readable length is about 200–300 bp for plasmid DNA isolated either by the miniprep method of Serghini *et al.* (1) or by the alkaline lysis methods of Birnboim and Doly (2). To obtain sequence data up to 500 bp, we must use templates purified either by equilibrium centrifugation in CsCl-ethidium bromide gradients (3) or by binding to silica-based resins (4). We have found that addition of $MgCl_2$ and/or chilling at −80°C improve the quality of the plasmid DNA preparation. Templates purified by either of the following methods provide reproducible fluorescent sequencing results comparable to those obtained by resin-purified plasmid DNA.

Method 1 (Mg miniprep): Cells were harvested by spinning 1.5 ml of ovemight culture at 12,000×*g* for 30 sec and were resuspended in 0.1 ml of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). After addition of 0.1 ml of phenol/chloroform/isoamyl alcohol (25:25:1), the mixture was vigorously vortexed and centrifuged at 12,000×*g* for 5 min. The aqueous phase was transferred to a second tube, and 0.1 ml of TNEM (10 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 1 mM EDTA, 0.1 M $MgCl_2$) containing 100 μg/ml RNase A (Boehringer, Mannheim, Germany) was added, and then the mixture was centrifuged at 12,000 × *g* for 5 min. The supernatant was transferred to a third tube, and plasmid DNA was precipitated with 0.1 ml of 10 M ammonium acetate and 0.8 ml of cold ethanol on ice for 15 min. The precipitated plasmid DNA was collected by centrifugation at 12,000×*g* for 5 min, washed with 70% ethanol, dried under vacuum, and finally dissolved in 25 μl of distilled water.

Method 2 (alkaline lysis-chilling-Mg miniprep): Cells were harvested as above and resuspended in 0.2 ml of resuspension solution (50 mM Tris-HCl, pH 7.5, 10 mM EDTA). After addition of 0.2 ml of lysis solution (0.2 M NaOH, 1% SDS), the tube was inverted several times until the mixture became clear. The mixture was vigorously mixed with 0.2 ml of neutralization solution (2.55 M potassium acetate, pH 4.8) and placed at −80°C for 3 min (the final pH was approximately 5). Then 0.6 ml of phenol/chloroform/isoamyl alcohol (25:25:1) was mixed and the tube was centrifuged at 12,000×*g* for 2 min to precipitate cell debris and chromosomal DNA. The aqueous phase was transferred to a second tube, and plasmid DNA was precipitated with 0.9 ml of isopropanol at −80°C for 15 min. Then 0.2 ml of resuspension solution, containing 100 μg/ml RNase A and 0.1 M $MgCl_2$, was added. The mixture was incubated at 37°C for 5 min and centrifuged at 12,000×*g* for 5 min. After addition of 0.1 ml of 10 M ammonium acetate and 1.2 ml of ice-cold ethanol to the supernatant, the tube was kept on ice for 15 min. The plasmid DNA was collected by centrifugation, washed with 70% ethanol, dried under vacuum, and dissolved in 25 μl of distilled water.

Plasmid used was pCR™II (in a TA cloning kit from Invitrogen, San Diego, CA). The insert in the clone was a human mitochondrial DNA fragment from nucleotide position 3171 to 3557. Numbering of the mitochondrial DNA was according to Anderson *et al.* (5). The yield of plasmid DNA was approximately 5 μg for both methods. The concentration of the plasmid DNA was estimated on a 1% agarose gel using a CsCl-purified plasmid pGEM-3Z f(+) (0.2 μg/μl), supplied by Applied Biosystems (Foster City, CA), as a control. Concentration of the plasmid DNA was adjusted to 0.2 μg/μl with distilled water, and 11 μl (for a Prism sequencing kit from Applied Biosystems) or 8.6 μl (for an Express ready reaction kit from Applied Biosystems) was used for the sequencing reaction in a Molecular Biology LabStation Catalyst 800 (Applied Biosystems). Reaction conditions were according to the manufacturer's recommendation

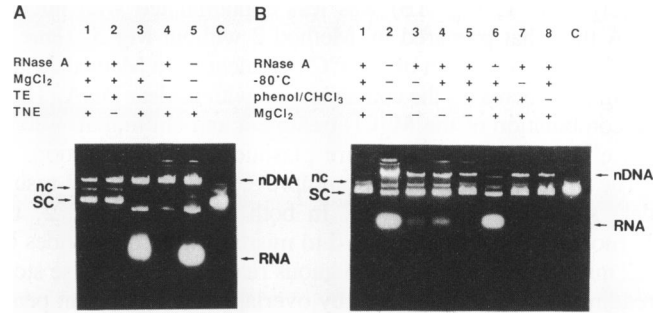

Figure 1. Preparation of plasmid DNA under different conditions. **A**, Method 1; **B**, Method 2. Presence (+) or absence (−) of reagents in the purification procedure is indicated for each lane. Abbreviations used are: nDNA, nuclear DNA; nc, nicked DNA; C, the resin purified control; SC, supercoiled plasmid DNA; TE, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA; TNE, 10 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 1 mM EDTA; $MgCl_2$, 0.1 M $MgCl_2$.

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Table 1. Comparison of the errors and ambiguities in the sequence data obtained by different methods

Methods	Error		Ambiguity	
Control (silica-based resin)	3*	(0.8%)	0	(0%)
Method 1 Complete**	2	(0.5%)	1	(0.3%)
Method 1 without $MgCl_2$	11	(2.8%)	12	(3.1%)
Method 2 Complete	2	(0.5%)	0	(0%)
Method 2 without $MgCl_2$	13	(3.4%)	11	(2.8%)
Method 2 without −80°	4	(1.0%)	6	(1.6%)

Data are based on the sequences shown in Figure 2.
Calculations:
(% Error) = (# Errors)/(Bases)×100, and
(% Ambiguity) = (# Ambiguities; typed out as N's in sequencing chart)/(# Bases)×100,
where (# Bases) = 387.
*Two deletions due to compression in GC-rich regions [GGGCC (correct) → GG−CC (error) at nucleotide position 3429, and CAGCG (correct) → CA−CG (error) at nucleotide position 3455 in the H-strand sequence of mitochondrial DNA] were observed not only in the control but in all the preparations. A misreading of CCCGG (correct) → CCGGG (error) at position 3247 was observed only in the control. Compared with the reported sequence of mitochondrial DNA (5), the insert DNA had a G→T transversion at nucleotide position 3423. This transversion was not counted, because this substitution is common among Mongolians and Caucasians.
**Reproducibility of Method 1 was examined by repeated experiments (n = 20): error = 2.9 ± 0.7 (0.75% ± 0.19%) and ambiguity = 3.1 ± 1.0 (0.80% ± 0.26%).

except the following PCR conditions: a three-step profile (96°C for 20 sec, 60°C for 20 sec, 70°C for 30 sec) for 25 cycles followed by a two-step profile (96°C for 10 sec, 70°C for 60 sec) for 15 cycles. The reacted samples were analyzed on 6% polyacrylamide gel at 27 watts by an automated DNA sequencer Model 373A from Applied Biosystems using an analysis program version 1.2.1. The sequences up to 500 bp were recorded. The insert of 387 bp was used for the calculation of errors and ambiguities. The distance between the sequencing primer and the cloning site of the pCRII plasmid was 94 bp. This region was excluded from the analysis. A control template was prepared with a silica-based resin in a Wizard™ miniprep kit (Promega, Madison, USA).

Figure 1 shows the purification results of plasmid DNA under different conditions. Compared with the sample prepared by Method 1 without $MgCl_2$ (lane 4, Fig. 1A), the sample prepared by Method 1 with $MgCl_2$ (lane 1, Fig. 1A) contained higher amount of plasmid DNA and was less contaminated with nuclear DNA. The beneficial effect of $MgCl_2$ treatment was also observed in Method 2. The sample prepared by Method 2 with $MgCl_2$ (lane 1, Fig. 1B) was less contaminated with nuclear DNA than that prepared by Method 2 without $MgCl_2$ (lane 5, Fig. 1B). The omission of −80°C treatment from Method 2 (lane 7, Fig. 1B) increased the contamination with nuclear DNA. Thus, the combination of the $MgCl_2$ treatment and chilling at −80°C was effective in obtaining pure plasmid DNA preparations.

The Mg^{2+} treatment notably improved the sequencing results (data submitted, not shown). In both Methods 1 and 2, the absence of $MgCl_2$ treatment led to misreading of nucleotides (G was misread as C) and to ambiguous readings due to false stops (reading as N), characterized by overlapping of different peaks at one site.

Table 1 summarizes the errors and ambiguities in the sequence results for the various template preparations. The error rate (0.5%) and the ambiguity rate (0.3%) for the templates prepared by Method 1 were comparable to those (error rate 0.8%; ambiguity rate 0%) for the control template. By omitting the $MgCl_2$ treatment from Method 1, the error rate and the ambiguity rate were drastically increased (2.8% and 3.1%, respectively). Low levels of errors and ambiguities of Method 1 were confirmed with repeated experiments (n = 20): error = 0.75% ± 0.19% and ambiguity = 0.80% ± 0.26%. The effect of $MgCl_2$ treatment was also evident in Method 2 (error rate of 0.5% and ambiguity rate of 0% for the complete Method 2 *versus* error rate of 3.4% and ambiguity rate of 2.8% for Method 2 without $MgCl_2$). The sequencing result from the template prepared by Method 2 without chilling at −80° was relatively free from misreadings (error rate 1.0%), but the frequency of ambiguous readings was high (1.6%). Thus, the combination of both the $MgCl_2$ treatment and the chilling at −80°C included in Method 2 improved the quality of the sequence results.

Method 1 was more rapid (less than 30 min to complete for 12 samples) than Method 2 (1.5 h to complete). In the present methods, we dissolved the final plasmid preparations in destilled water, and immediately used for sequencing. For the purposes other than sequencing, the final preparation should be stored in a buffer containing EDTA. The modified miniprep procedures, presented here, provide simple inexpensive means to obtain DNA suitable for *Taq* cycle sequencing reactions with fluorescence-labeled primers.

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REFERENCES

1. Serghini,M.A., Ritzenthaler,C. and Pinck,L. (1989) *Nucleic Acids Res.* **17**, 3604.
2. Bimboim,H.C. and Doly,J. (1979) *Nucleic Acids Res.* **7**, 1513.
3. Sambrook,J., Fritsch,E. and Maniatis,T. (1989) In *Molecular Cloning: A Laboratory Manual* (Second edn), Cold Spring Harbor, New York, 1.42.
4. Hengen,P. (1994) *Trends Biol. Sci.* **19**, 182−183.
5. Anderson,S., *et al.* (1981) *Nature* **290**, 457−465.