Fast Plasmid DNA Sequencing Using a Thermal Cycler and High Temperature Alkali Denaturation

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This report describes a modification of the Chen and Seeburg plasmid sequencing procedure (2). Chen and Seeburg demonstrated that the collapse of closed covalent circular plasmid DNA into networked DNA favored double-stranded sequencing; however, the method was sensitive to genomic DNA and RNA contamination. Complete alkaline denaturation (not thermal) and RNA degradation were essential for high resolution sequencing and achieving a low background. Current modifications of this protocol work well only when high-quality double-stranded plasmid is obtained as a template (1). This involves multiple steps to remove genomic DNA and RNA contamination by either LiCl precipitation, RNase digestion, CsCl ultracentrifugation, specialized resins or polyethylene glycol purification (3,4). These are time-consuming, or they necessitate the use of expensive kits for plasmid preparation if quite a few plasmids are to be sequenced. Our results show that a 10-min plasmid preparation step when combined with a high temperature alkali denaturation step similar to that described by Musich and Chu (3) produces high resolution sequencing results. Incubation of reaction components in a thermal cycler and utilization of tube strips permit rapid throughput under identical reaction conditions and greatly enhances reproducibility.

Plasmids in XL2-Blue (Stratagene, La Jolla, CA, USA) are grown in LB medium with appropriate antibiotics overnight at 37°C and are processed by the 10-min method of Zhou et al. (5). Briefly, 1.5 mL of an overnight culture are collected at 15 000× *g* for 20 s, 50–100 µL of supernatant should be allowed to remain for resuspending the bacterial pellet by vortex mixing and 300 µL of lysis buffer (0.1 *N* NaOH, 0.5% sodium dodecyl sulfate in 1× TE buffer [10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0]) are added to the suspension. After 5 s of vortex mixing, 150 µL of 3 M Na acetate (pH 5.2) are added and vortex mixed for 5 s. It is important to denature and neutralize the reaction without delay by vortex mixing to avoid the genomic DNA contamination. After centrifugation at 15 000× *g* for 2–3 min, the supernatant is transferred to 900 µL of ethanol and mixed well, and the plasmid is recovered at 15 000× *g* for 2–3 min and rinsed twice in 70% ethanol. The residual ethanol is removed by pipetting and air-drying. Then the steps in Table 1 are followed.

We found there is no difference in the sequencing result from RNase A-digested, phenol-purified plasmids and CsCl-purified plasmids (data not shown) if the high temperature alkali denaturation step is used instead of the room temperature alkali denaturation originally recommended. Results are more reproducible if a thermal cycler and PCR tube strips are used for maintaining precise temperature control and for making the various reactions easier to manipulate during denaturation, chain elongation/termination and loading, thus saving time and the possibility of handling errors. Double-stranded plasmid templates can be prepared and sequenced by this method through the gel electrophoresis step in a single working day, and the autoradiographic

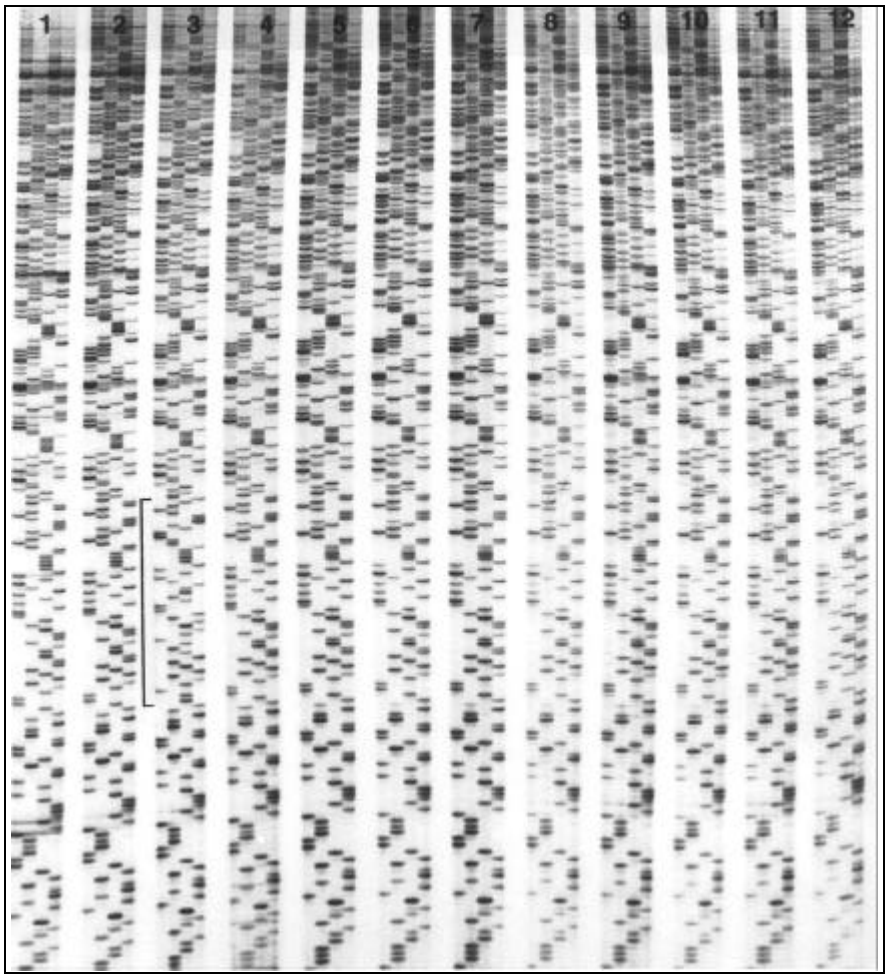

Figure 1. Mutation screening for fluorophore-swapped GREEN LANTERN™-1 Vector (Life Technologies, Gaithersburg, MD, USA). 12 candidate clones were sequenced with a 20-mer gene-specific primer in pcDNA3 vector (Invitrogen, San Diego, CA, USA). The mutated region is bracketed in the third set of sequencing reactions. It spans 49 nucleotides (nt) and is 60 nt from the 3′ end of the primer. Loading order: G, A, T, C. Sequences are easily read from nt 13 through nt 250 in a single loading and can be extended to nt 450–500 with a second loading.

Table 1. Protocol

1) The plasmid is dissolved in 60 μL of distilled water, and a 10–15-μL aliquot of plasmid (3–5 μg) is denatured in 0.2 *N* NaOH and 1 mM EDTA (pH 8.0) at 65°C for 5 min.
2) One-tenth volume of 3 M Na acetate (pH 5.2) and 3 vol of ethanol are added to precipitate the denatured plasmid at -20°C for 10 min. It is recovered at 15 000× *g* for 10 min, washed once with 70% ethanol and air-dried.
3) The template is mixed with 10 pmol of primer, 2 μL Sequenase® Version 2.0 reaction buffer (Amersham, Arlington Heights, IL, USA) in 10-μL volume, heated at 65°C for 15 min, then annealed at 37°C for 15 min and stored on ice.
4) 2 μL of 1:8 diluted Sequenase Version 2.0 are added on one side of the tube and 3.3 μL of the master solution (1 μL of dithiothreitol, 2 μL of diluted labeling mixture and 0.3 μL of [α-^{33}P]dATP [NEN Life Science Products, Boston, MA, USA]) on the other side of the tube. Every 45 s, one tube is mixed by a brief spin and incubated at room temperature for 5 min.
5) A 0.2-mL PCR tube strip (4 tubes per strip; Robbins Scientific, Sunnyvale, CA, USA) with 2.5 μL of ddNTP/dNTP mixture on the bottom is placed in a Model PTC-100™ Thermal Cycler (MJ Research, Watertown, MA, USA), which is set at 40°C.
6) 3.5 μL of the labeled mixture are added to the tube strip by pipetting up and down twice and incubating at 40°C for 5 min.
7) 4 μL of stop solution are added to each tube. The reactions can be stored at -20°C before loading.
8) Before loading, they are denatured in a thermal cycler at 95°C for 5 min and then quickly cooled on ice.
9) They are loaded on a 0.4-mm, 6% sequencing gel (1× TBE [89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.0] , 7.5 M urea) and run at 70 W constant power in an STS45 sequencing gel apparatus (IBI/Kodak, New Haven, CT, USA). The upper tank buffer is 1× TBE and the lower tank buffer is 1× TBE containing 0.1 M Na acetate (pH 5.2).

results can be obtained the next day. An example of a set of sequence verification reactions for mutant screening by this method is shown in Figure 1.

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