Direct DNA Sequencing of PCR-Amplified Vector Inserts Following Enzymatic Degradation of Primer and dNTPs

Polymerase chain reaction (PCR) is a convenient tool for the analysis of recombinant DNA directly from plaques or bacterial colonies (2), thus circumventing the time-consuming λ DNA- or plasmid DNA-isolation. Cloning vectors have binding sites for reverse- and forward-sequencing primers next to the multiple cloning site. This pair of sequencing primers, which also flanks the cloned DNA, can be used for amplification of the inserted DNA by PCR.

After amplification, the resulting PCR products can be employed directly for DNA sequencing. A number of alternatives have been reported. Some methods are based on single-stranded DNA generated by asymmetric PCR. Utilizing 32P-end-labeled primers and Taq DNA Polymerase, single-stranded PCR products can be sequenced directly from bacterial colonies (4) or from viral plaques (6). Amplification of DNA inserts from bacteriophage vectors, followed by sequencing of the single-stranded DNA with T7 DNA polymerase, is also reported (5).

Double-stranded PCR products, which can be generated more easily than single-stranded PCR products, need to be purified before sequencing because residual PCR primers and deoxyribonucleotides interfere with the sequencing reaction. Conventionally, interfering components of the PCR mixture are removed by filtration, chromatography or gel purification methods.

In order to avoid purification steps, some sequencing protocols employ specific exonucleases. The use of λ-exonuclease generates single-stranded DNA from double-stranded PCR products if one of the primers shows a 5' terminal phosphate. Thus it is an alternative method to asymmetric PCR to obtain single-stranded DNA for sequencing (3,7). For direct sequencing of double-stranded PCR products, the exonuclease activity of mung bean nuclease is used to remove residual PCR primers from the PCR mixture after amplification (1).

Here we describe the application of exonuclease I of *Escherichia coli*, which hydrolyzes single-stranded DNA such as residual PCR primers in the 3'→5' direction. In addition, excess deoxyribonucleotides in the PCR mixture are dephosphorylated with the thermostable alkaline phosphatase of shrimp. These two enzymes are active in the PCR-buffer and can be inactivated afterwards by heat treatment (15 min at 70°C). This treated PCR mixture (5 μL) are sufficient) can be used without any further purification for sequencing with commercially available sequencing kits.

PCR and Direct Sequencing

Amplification was carried out in a 40-μL PCR mixture that included one unit of *Taq* DNA Polymerase (Promega, Serva GmbH, Heidelberg, FRG), reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.8, 1.5 mM MgCl2, 0.1% Triton X-100), 30 pmol of each PCR primer and 40 μmol deoxyribonucleoside triphosphates (dNTPs). Primers used for amplification were 5'-CGTTGTAACACGCAGCCTG-3' and 5'-CACACAGGAACACAGCTATGAC-3' for plasmids with pUC/M13 sequencing primers (e.g., pUC, pGEM®) and 5'-CTTTGACGAACTCCCGTTGTAAAG-3' and 5'-GGTTGGCTTTAGTATTTCTCAGAGG-3' for λ phages gt10, respectively. Material from a single bacterial colony or viral plaque was directly transferred into the PCR mixture with a sterile needle and overlaid with one drop of mineral oil. Samples were incubated at 94°C for 3 min and then subjected to 30 cycles in a thermal cycler: 94°C for 30 s, 50°C for 30 s and 72°C for 90 s. After amplification, 5 μL of the PCR were analyzed by gel electrophoresis. The PCR product should be visible as a strong single DNA band.

For direct DNA sequencing, 5 μL of the amplified PCR mixture were incubated with 1 μL of *E. coli* exonuclease I (1 unit/μL) and 1 μL of shrimp alkaline phosphatase (2 units/μL) (United States Biochemical [USB], Bad Homburg, FRG) at 37°C for 15 min. Inactivation of both enzymes was carried out at 70°C for 15 min. Then 10–20 pmol of the sequencing primer (one of the PCR primers or an internal primer) were added to the treated PCR mixture and adjusted with water to a total volume of 10 μL. After denaturation of the double-stranded DNA (3 min at 100°C), the annealing with the sequencing primer was carried out by transferring the vial quickly to an ice/water bath (snap-cooling procedure). The annealed template/primer mixture was kept on ice until sequencing. One set of the sequencing reaction was carried out with 0.5 μL [32P]dATP (>1000 μCi/ mmol, redvue; Amersham, Braunschweig, FRG) using commercially available sequencing kits from USB or Pharmacia Biotech GmbH (Freiburg, FRG) following the instructions of the manufacturer for sequencing of double-stranded DNA templates.

Figure 1. Direct sequencing of a PCR-amplified vector insert. PCR was carried out with vector-specific primers directly from a bacterial colony that harbored a recombinant pGEM®-T plasmid (Promega). After treatment of the PCR product, the sequencing reaction was performed with one of the PCR primers (5'-CGTTGTAACACGCAGCCTG-3'). Electrophoresis was accomplished on a 6% polyacrylamide/8 M urea denaturing gel with 1X TBE buffer (100 mM Tris-base, 83 mM boric acid, 1 mM EDTA). The autoradiograph shows parts of the sequence of the chloroplast ATP synthase β gene (atpB) of *Lupinus polyphyllus* (left) and *Lupinus albus* (right).
The present report demonstrates that exonuclease I of *E. coli* and alkaline phosphatase of shrimp can be used to generate double-stranded sequencing templates directly from PCR products. The entire procedure can be carried out in a thermal cycler and is easy to perform, especially if a large number of probes has to be analyzed. No precipitation, purification or additional amplification by linear PCR is needed.

In conclusion, combination of the PCR-based amplification of cloned DNA and direct sequencing of the double-stranded PCR product makes this protocol a convenient tool for clone characterization.

REFERENCES

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Use of Agar in Selective Media May Give False Positives in a Functional Complementation Assay

Adenosylsuccinate lyase (ASL) [Enzyme Commission No. 4.3.2.2] catalyzes the removal of fumarate from 4-(N-succinyl)-5-aminoimidazole-4-carboxamide ribonucleoside 5′-phosphate to yield 5-amino-imidazole-4-carboxamide ribonucleoside 5′-phosphate in the multistep de novo biosynthesis of IMP and from adenosylsuccinate (SAMP) to yield AMP in the two-step conversion of IMP to AMP. The study of ASL is important since ASL deficiency has been linked to some types of autistic children (5). ASL is inefficient in the conversion of dideoxysAMP (ddSAMP) to ddAMP which is involved in the conversion of the anti-HIV drug dideoxynosine to ddATP (3) and an elevation of ASL in human breast and prostate tumors suggest potential as a tumor marker (4). We have shown the presence of multiple isoforms of ASL by isoelectro-focusing (2). In our continuing studies on ASL isoforms, we are utilizing functional complementation in order to determine if more than one ASL cDNA exists. The bacteria used for this study is *Escherichia coli* JK 268 (1), which is deficient in ASL and thus requires adenine for growth. To test whether a plasmid contains ASL cDNA, it is first transformed into *E. coli* JK 268. The transformed bacteria are then plated on an adenine-deficient plate to determine if growth is possible in the absence of adenine. In carrying out these studies, we found that some to all non-transformed *E. coli* JK 268 can grow on agar plates supposedly deficient in adenine. We determined that the substitution of agarose for agar results in a suitable selective media for an adenine-deficient test system.

*E. coli* JK 268 was grown in S.O.C. media to 0.4 OD, harvested, washed twice in minimum media and diluted in minimum media so that approximately 200 bacteria were applied to a plate. The minimum media was M9 minimum media, which contained tryptophan (50 μg/mL), thiamine (0.2 μg/mL), 0.1% casamino acids and 0.2% glycerol. For plating, 1.5% agar or 1.5% agarose were added. Purified grade agar was obtained from Fisher Scientific (Catalog No. A360-500; Pittsburgh, PA, USA). SeaKem® Agarose types LE, ME and HE were obtained from FMC BioProducts (Rockland, ME, USA). The number of colonies were counted after 20-24 h at 37°C.

We found that when *E. coli* JK 268 was plated on what was supposedly an adenine-deficient agar plate, there was little difference in the number of colonies obtained with and without added adenine (Table 1). This contrasted with the fact that there was no growth of *E. coli* JK 268 in liquid minimum media unless adenine was added. This indicated that agar may have been the source of the problem. Upon further investigation, we found that agar was indeed the source of the unwanted adenine since, when it was replaced with agarose, there was no growth of *E. coli* JK 268 unless adenine was added (Table 1). There was little difference in growth of *E. coli* JK 268 among the three types of agarose we tried, which differed only in electrodosmosis.

<table>
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<th>Type of Gel</th>
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Table 1. *E. coli* JK 268, Comparison of Types of Agarose

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