Cycle Sequencing of Filamentous Phage DNA Using a Biotinylated Primer and ΔTaq DNA Polymerase

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ABSTRACT

We describe a rapid nonradioactive, double-stranded phage DNA sequencing method using ΔTaq DNA polymerase in cycle reaction for phage peptide-display library screening. This procedure is specific, rapid, sensitive and safe for the sequencing of large numbers of phage peptide-display colonies. In addition, thermal cycle sequencing with this chemiluminescent image detection protocol provides an inexpensive method for completing a large amount of sequencing reactions. Consequently, this method is especially useful for those extensively working on peptide mimics using phage display libraries.

INTRODUCTION

Phage display libraries have become an important tool in biotechnology. Phage peptide-display libraries have been extensively used in investigating unknown peptide fragments with certain specific bioactivities (1,5,6). Although limited-length DNA fragments need to be sequenced (usually spanning from 18–45 bp of interest), sequencing large numbers of phage clones for this purpose is an arduous task. Mutational studies, holding fixed consensus residues identified by comparing a large number of phage sequences, adds to the volume of sequencing required. Subsequently, it has become an important topic to develop a simple yet reliable sequencing method, especially when large numbers of clones need to be examined (2).

To offset the prolonged exposure to radionucleotides during large phage-sequence studies, we have developed a nonradioactive ΔTaq cycle sequencing protocol, displaying the following features: (i) many samples can be handled at a time—one can start with 50–100 single-phage colonies. Rather than using 96-well plates for phage proliferation, we found that single microtube culturing eliminated cross contamination among phage colony samples; (ii) The DNA template preparation method is clean—no contaminated DNA and RNA from host cells is observed on 1% agarose gel; (iii) The method is fast—the optimized cycling sequencing protocol achieves double-stranded (ds) phage DNA sequencing in less than 90 min, with reliable and reproducible results; (iv) One can directly sequence ds phage DNA starting with a very low amount of DNA; (v) The method leads to a fast exposure time: 8–20 min; (vi) ΔTaq DNA polymerase generates a much lower background than that by the Taq enzyme; and (vii) The method eliminates radioactive materials without sacrificing sensitivity and resolution. The described four-lane sequencing approach has advantages over a two-lane sequencing strategy (2) in that the percentage of readable clones is significantly increased (100%) compared with an estimated 76% from a two-lane approach (2). In addition, cross-contamination risk from the two-lane protocol is high, and more hidden hands-on work is involved. Another practical advantage is sample size. When DNA template is less abundant, the two-lane sequencing method renders weaker band reading than that using our four-lane strategy. We chose ΔTaq for use in these studies because of the high fidelity in the polymerization reactions. It has been genetically engineered to eliminate the 3′–5′ proofreading exonuclease activity associated with Taq DNA polymerase and thus, in our opinion, is the preferred form for high-temperature deoxy sequencing reactions and for high-yield primer extension reactions.

MATERIALS AND METHODS

Phage DNA Preparation

We inoculated 1 mL Terrific Broth containing 100 μg kanamycin/mL and 20 μg tetracycline/mL with each single-phage pH115 colony in a 1.5-mL microcentrifuge tube, incubated at 300 rpm at 37°C overnight and then centrifuged at 13 000× g at 4°C for 10 min. Supernatant was then transferred to clean tubes, to which 2 μL of RNase A (12 U/μL; USB/Amersham, Cleveland, OH, USA) and 2 μL DNase I (10 U/μL; Boehringer Mannheim; Indianapolis, IN, USA) were added. The solution was incubated at 37°C for 60 min followed by polyethylene glycol (PEG) 8000/NaCl (16.7%/3.3 M) precipitation. The pellet was resuspended in 0.2 mL of Tris-buffered saline (TBS), extracted by adding 0.2 mL of phenol: chloroform (3:1)/tube 3× followed by an equal volume extraction with chloroform. DNA was precipitated with 0.1 vol of 5 M sodium acetate (pH 5.5) and 2.5 vol of 100% ethanol. The pellet was washed with 1 mL of 90% ethanol/tube and air-dried.

Primer Design and Sequencing

Primer 1797 was designed using the Oligo™ 5.0 Primer Analysis Software (National Biosciences, Plymouth, MN, USA) with the full-length sequence of the FUSE 5 phage vector (2). The primer was synthesized on a Cyclon Oligo Synthesizer 450 (PE Applied Biosystems, Foster City, CA, USA). Total primer length is 27-mer with melting temperature (Tm) = 78°C, 3′-GTAGCATTCCACAGACAGCCCTCATA-5′ (52% G+C, 48% A+T). The synthesized primer was then cartridge-purified (PE Applied Biosystems). Biotinylation was performed according to protocols (USB/Amersham). ΔTaq Version 2.0 DNA Polymerase (USB/Amersham) was used in a cycle sequencing reaction according to previously described protocol (4). The sequencing cycle reaction was completed at 95°C, 30 s; 72°C, 60 s for 47 cycles using an MJ Research Thermal Cycler (Watertown, MA, USA). Samples were stored at -20°C, unless electrophoresed immediately. For electrophoresis, separation was completed as previously described (3). Typically, gels were electrophoresed with a 6% denaturing sequencing gel at 70 W constant power until the gel temperature reached 48°–50°C.

Detection of DNA Sequence

DNA was transferred to Hybond®. N+ nylon membrane (Amersham, Arlington Heights, IL, USA) at room
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For 30 min. Streptavidin-alkaline phosphatase conjugate (SAAP, 1 mg/mL; USB/Amersham) was added in a 1:2000 to 1:4000 vol directly to the blocking buffer and incubated for 10 min at RT. The membrane was washed 3 times with buffer A (0.1 M NaCl, 10 mM Tris-HCl, 0.1% SDS, pH 10.0) and once with buffer B (0.1 M NaCl, 50 mM Tris-HCl, pH 10.0).

Exposure of Membrane to Film
The membrane was developed by adding 0.01 mL Lumi-Phos™ 530 (Boehringer Mannheim) per cm² and incubating for 3 min at RT. The membrane was stored in the dark at RT for 2 h, then exposed to Hyperfilm™ (Amer sham) (or equivalent) in close contact for 8–20 min at RT.

RESULTS AND DISCUSSIONS

Figure 1 represents a typical gel showing the nonradioactive cycle direct-sequencing method with high-quality band resolution. On a 42 × 62 cm film, one can expose up to 30 samples with 4 lanes rather than 2 lanes for each sample. We have compared two approaches in phage DNA preparation. One uses asymmetric polymerase chain reaction (PCR), the other uses enzymatic digestion with DNase I and RNase A. Although one can use asymmetric PCR to amplify a target DNA fragment directly from a single colony, we feel for many entry-level operators, it is not an easy task to control all parameters, especially the transferring step from the end product after asymmetric PCRs to the next cycle sequencing. It is easier to start with inoculating 1 mL of media with each single colony in a 1.5-mL microcentrifuge tube and let them grow overnight. We have found that it is much more convenient to let the cells make their own single-stranded (ss)DNA during the overnight incubation. When using PCR (asymmetric or symmetric), one more day is required for two successive PCRs. Two primers rather than one also need to be prepared. Furthermore, PCRs introduce potential mutations, which is especially true with 2 successive reactions. This problem is minimized by culturing cells for ssDNA. Although overgrow-

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Figure 1. Typical sequencing gel from nonradioactive ΔTaq phage DNA sequencing. Representative phage DNA sample was sequenced using the biotinylated 1797 primer, with described procedure (see Materials and Methods). High band resolution is still obtained even after overexposure (15 min for this sample). Typically, we only read 200–250 bp, but readability would extend to 1000 bp under the conditions described. Similar resolution is observed for 32P (Sequenase® Version 2.0 Kit; USB/Amersham) after 5-h exposure time (data not shown).
mers to 27-mers to generate a $T_m$ value above 76°C. Our primer has a calculated $T_m$ value of 78°C. Therefore we can merge the annealing step and the extension step into one step by using the same temperature through the entire cycle-sequencing reaction. This minimizes possible nonspecific annealing during the reaction, dramatically reducing the nonrelevant background bands and requiring less time for this step. This effectively excludes the ramp time between annealing and extension, and shortens the ramp time between the denaturing and annealing steps. We have found that the design of a sequencing primer should reflect a $T_m$ value higher than 76°C. We set up two different cycle sequencing programs: one was set up as 95°C, 30 s; 63°C, 15 s; 72°C, 60 s; total 48 cycles; the other was set up as 95°C, 30 s; 72°C, 75 s; 50 cycles, the total readable sequences was beyond 1000 bp. To obtain sharp bands during the transfer, it was better to use 3MM filter paper (Whatman, Clifton, NJ, USA) than the normal laboratory paper towel. Since the sequence of interest in the plIII15 phage display library is only 45-bp-long, we only needed to transfer that portion of the bands to the membrane, thus saving transfer material. By developing only the sequence in the region desired, we were able to handle large numbers of samples. When we developed the whole sequencing gel, the readability is around 200–250 bp/run.

While the two-lane sequencing strategy suggested for phage display work has some advantages (2), our experience indicates that the readable clone percentage (76%) is much lower than ours (100%). This means that when 784 clones are prepared for sequencing, about 200 clones are unreadable and hence completely wasted. In addition, cross-contamination risk from the two-lane protocol is still high, and more hidden hands-on work is involved. When the amount of DNA template is less, the two-lane sequencing method inefficiently renders faint bands as opposed to our 4-lane strategy.

**REFERENCES**


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