

Dye terminator C 1 μ l
Taq polymerase 4 units

9. Prepare the individual reactions to contain 12 μ l DNA template, 7.25 μ l reaction premix, and 1 μ l primer (10 μ M).

10. Cover with one drop of mineral oil and thermal cycle as follows: denature at 96° for 30 sec, anneal at 50° for 15 sec, and extend at 60° for 4 min.

11. Repeat for a total of 25 cycles. *Note:* The thermal cycle of this protocol is optimized for traditional M13 forward and reverse sequencing primers. Other primer sequences may work most effectively at a different annealing temperature. Also, the extension temperature is lower than the temperature optimum of *Taq* polymerase, to allow the incorporation of the highly modified dye-labeled dideoxy terminators.

12. After the cycling, remove the dNTPs and decomposed dye via a spin column (Centrisep, Princeton Separations, Adelphia, NJ) according to the instructions of the manufacturer. Add 4 μ l of a 5:1 solution of formamide and 50 mM EDTA. Vortex and heat to 90° for 2 min. Apply to sequencing gels.

Acknowledgments

We thank J. Conroy, E. M. Prager, T. W. Quinn, and A. Sidow for helpful discussions and the National Science Foundation and the National Institutes of Health for financial support.

[29] Rapid Production of Single-Stranded Sequencing Template from Amplified DNA Using Magnetic Beads

By BARBARA H. BOWMAN and STEPHEN R. PALUMBI

Introduction

We present a powerful, general method for producing a pure preparation of single-stranded DNA (ssDNA) sequencing template from double-stranded DNA (dsDNA) amplified using the polymerase chain reaction (PCR). The amplified DNA is synthesized using one biotinylated and one nonbiotinylated primer and is bound to streptavidin beads via the biotin molecule. The two DNA strands are then dissociated, and the nonbiotinylated strand is released and recovered from the supernatant. The procedure presented here is a modification and simplification of the method origi-

nally proposed by Mitchell and Merril.¹ It produces ssDNA from templates of any amplifiable length, without strand bias and with sufficient yield for several sequencing reactions. This method utilizes the sensitivity and yield of double-strand PCR reactions, and it provides pure templates that give optimal sequencing results.

The double-stranded amplified DNA to be sequenced is generated in two separate, reciprocal reactions. In the first, the 5' primer is biotinylated² and the 3' primer is not; in the second, the reverse is true. This enables either of the two strands to be recovered in single-stranded form for sequencing. Each double-stranded DNA contains a biotin molecule at one end (Fig. 1a) and is captured on streptavidin-coated magnetic beads (Fig. 1b). Other PCR reaction components are removed by washing. The dsDNA is denatured by the addition of base (Fig. 1c), whereupon the strand lacking biotin is separated from the bound strand. The released strand can be recovered from the supernatant, while the biotinylated strand remains attached to the streptavidin-coated magnetic beads. Typical yields of ssDNA are sufficient for five sequencing reactions, which may be performed using either the PCR primer (Fig. 1d) or internal sequencing primers.

The streptavidin capture method is applicable to any PCR product, in that the efficiency of recovery of ssDNA does not appear to be affected by the length, base composition, or secondary structure of the amplified region. The method allows direct sequencing of amplified DNA, which for many applications is preferable to sequencing individual, cloned PCR products because direct sequencing obscures any random errors induced by *Taq* polymerase. The technique requires only a single round of amplification to produce each ssDNA sequencing template, thus limiting both the opportunity for contamination and the costs of a second amplification reaction. To minimize the number of primers that must be biotinylated, a single set of primers that anneal to highly conserved regions of the target molecule can be used to amplify DNAs from a wide variety of taxa.

This method is of particular use for sequencing long templates such as small subunit ribosomal DNA (rDNA). Such long molecules [over 1700 base pairs (bp) in the case of eukaryotic small subunit rDNA] exceed the length at which asymmetric amplification³ and double-stranded sequencing⁴ are reliable alternatives. For several reasons, sequencing ribosomal RNA (rRNA) genes from an amplified product is strongly preferred over

¹ L. G. Mitchell and C. R. Merril, *Anal. Biochem.* **178**, 239 (1989).

² C. Levenson and C. Chang, in "PCR Protocols" (M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds.), p. 99. Academic Press, San Diego, 1990.

³ U. B. Gyllensten and H. A. Ehrlich, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 7652 (1988).

⁴ B. Bachmann, W. Lüke, and G. Hunsmann, *Nucleic Acids Res.* **18**, 1309 (1991).

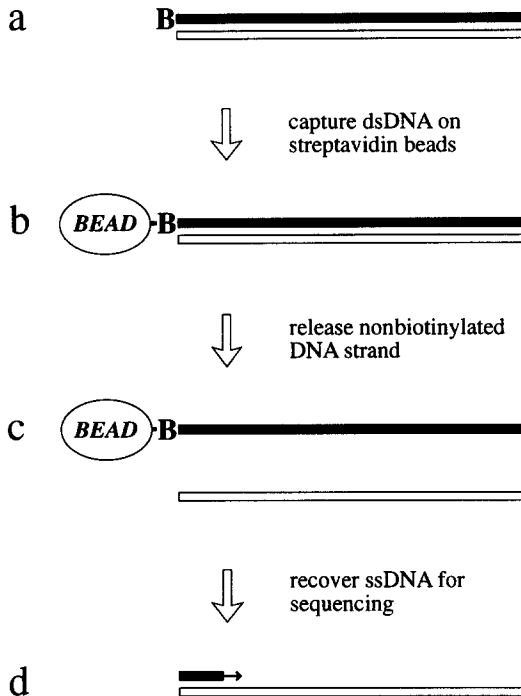


FIG. 1. Preparation of ssDNA from biotinylated PCR-amplified DNA. (a) DNA is amplified using one primer that has a biotin molecule (B) at the 5' end and one primer that lacks biotin. (b) Double-stranded amplified DNA is captured on a streptavidin-coated magnetic bead via the biotin molecule. (c) The dsDNA is denatured, with the biotinylated strand remaining attached to the streptavidin-coated magnetic bead and the nonbiotinylated strand being released into the supernatant. (d) The nonbiotinylated strand is used as an ssDNA sequencing template.

direct, dideoxy sequencing of an rRNA template using reverse transcriptase. Both DNA strands are available for sequencing, the rDNA will not contain new information incorporated by RNA editing, and considerably less starting material is required. If PCR is performed starting from a preparation of whole RNA⁵ instead of DNA, the present method shares with direct sequencing the advantage that only expressed genes are amplified and sequenced.

The streptavidin capture method is also of advantage to population biologists, because it provides for rapid, repeatable, and reliable sequences

⁵ T. W. Myers and D. H. Gelfand, *Biochemistry* **30**, 7661 (1991); E. S. Kawasaki, in "PCR Protocols" (M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds.), p. 21. Academic Press, San Diego, 1990.

from large numbers of individuals. When the same DNA region must be sequenced from hundreds or even thousands of individuals, this method provides the best compromise among accuracy, speed, and cost.

Procedure

Handling Magnetic Beads

For each step below, add liquid to the streptavidin paramagnetic beads in a microcentrifuge tube, agitate the mixture gently by hand or on a rotator, and then remove the supernatant. Before removing each supernatant, pulse-spin the tube in a microcentrifuge very gently (< 1000 rpm) and briefly, so as to remove bead slurry from the top of the tube without pelleting the magnetic beads. If necessary, use a micropipettor to dislodge beads from the bottom of the tube and resuspend them.

To concentrate the beads before removing the supernatant, lay the microcentrifuge tube on the laboratory bench and place a small magnet by its side. (Alternatively, tube and magnet can be placed into a holder that will keep them side by side.) When the beads have congregated on the side of the tube, lift the tube, holding the magnet in place, and pipette off the liquid. Summoning beads to the side rather than to the bottom of the tube makes it possible to remove all the liquid without concern that some magnetic beads will accidentally be pipetted into the supernatant.

Notes. The different brands of streptavidin magnetic beads have different molarities of attached streptavidin, different resuspension protocols, and different capture properties. Bead volumes given in this protocol are for Promega (Madison, WI) paramagnetic particles (No. Z5241) prepared as indicated below. If other beads are used, appropriate volumes may differ by a factor of 3 or more from those presented.

Bead Preparation

Remove the storage buffer from 0.6 ml magnetic bead slurry (one tube) and wash 3 times with bead buffer (0.2 M NaCl, 20 mM Tris-HCl, 1 mM EDTA, pH 7.6). Resuspend beads in 625 μ l of this buffer and store at 4°.

Amplification

Prepare a 100- μ l PCR mix of the target to be sequenced, using one primer that has been biotinylated² and one that has not. The strand

initiated with the nonbiotinylated primer will become the single-stranded sequencing template. Because the yield of dsDNA directly determines the amount of ssDNA recovered, procedures that maximize the efficiency of the PCR reaction, such as the addition of empirically determined concentrations of glycerol or dimethyl sulfoxide (DMSO),⁶ are recommended. Determine the yield of amplified DNA by evaluating 3–5 μl of the dsPCR product on an agarose gel stained with ethidium bromide. Another 3–5 μl may be saved for a later gel to evaluate the recovery of ssDNA.

All of the following procedures are done at room temperature.

Optional Reduction of Primer Concentration

1. Reduce the concentration of excess primers relative to product by prespinning the remaining product plus 1.9 ml sterile water in a Centricon-100 tube (Amicon, Beverly, MA, 4211 or 4212). Centrifuge for 25 min in a fixed-angle rotor (e.g., Sorvall, Norwalk, CT, SA600 or SS34) at the highest speed recommended by the manufacturer for the Centricon tubes.

2. Cap the tube with the Centricon retentate cup, then backspin the retentate (about 40 μl) into the cup in a swinging-bucket rotor by bringing the speed to 1000 rpm and then braking immediately. The retentate contains the biotinylated dsDNA for use in the capture step below.

3. If desired, 100–250 μl of the liquid in the Centricon filtrate cup may be concentrated by evaporation, and selective elimination of primers can be evaluated on an agarose gel.

4. Centricon tubes may be rinsed and reused for the same preparations later in the procedure. Fill the top of the tube (Centricon concentrator) with sterile water, cap with the retentate cup, and shake gently. Repeat a total of 3 times. The tube need not be dried, but the retentate cup must be pipetted dry before reusing it to capture the final ssDNA, so that it will not be diluted. Rinsing can conveniently be done during incubation in the capture step, below.

Notes. A Centricon-30 tube may be substituted for use with very small DNAs that may not be retained by the Centricon-100 tube. Millipore (Bedford, MA) MC 100,000 MWL filter units (UFC3 THK 00) may be substituted if desired. Because of their smaller volume, additional dilution steps may be required to desalt the ssDNA before sequencing. For brands of streptavidin magnetic beads whose binding efficiency for biotinylated DNA may decrease as the length of the DNA increases, it may be essential to reduce the primer concentration via this prespin step.

⁶ K. T. Smith, C. M. Long, B. H. Bowman, and M. M. Manos, *Amplifications* 5, 16 (1990).

Capture of Double-Stranded Amplification Product

In a 2.0- or 1.5-ml microcentrifuge tube, combine the amplified DNA, or prespun product from the optional step, with 100 μ l of well-suspended magnetic bead slurry. Place on a rotator or agitate gently by hand for 15–30 min. Remove the liquid. Save at least a 10- μ l sample for later analysis of capture efficiency on an agarose gel. (Typically, some dsDNA is visible in this supernatant fraction. Only a very bright band, combined with low yield of ssDNA, should be of concern.)

Washing

Wash with 500 μ l TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) or bead buffer by shaking gently or placing on a rotor for about 1 min. Remove and discard liquid. Repeat.

Denaturation of Double-Stranded DNA, Recovery of Single-Stranded DNA, and Neutralization

1. Dilute 5 M NaOH to 0.2 M just before use. Add 150 μ l of 0.2 M NaOH to streptavidin magnetic beads to which dsDNA is bound. Agitate or rotate for 6 min.

2. During the incubation, add 100 μ l of 5 M ammonium acetate, pH 6.8, to a Centricon-100 tube.

3. After the 6-min incubation, remove the NaOH solution (which contains the released, nonbiotinylated ssDNA strand) from the magnetic beads and add it to the ammonium acetate in the Centricon-100 tube. It is very important not to pipette any magnetic beads into the NaOH fraction. Most ssDNA will be recovered in this single NaOH wash. If desired, Steps 2 and 3 can be repeated.

Desalting and Concentration of Single-Stranded DNA

1. Add sterile water to the neutralized ssDNA solution in the Centricon-100 tube to a total volume of 2.0 ml. Spin for 25 min in a fixed-angle rotor at the highest speed recommended for the Centricon tubes. Add 2.0 ml of sterile water and repeat the spin.

2. Discard the water in the filtrate cup of the Centricon-100, add 2.0 ml more of sterile water, and spin a third time. This spin may be extended to 45 min, if this is necessary to bring the volume of the retentate down to 40–60 μ l.

3. Remove the filtrate cup and backspin the retentate into the retentate cup in a swinging-bucket rotor by raising the speed to 1000 rpm and braking immediately.

Evaluation of Yield

Test the ssDNA yield (as well as the effectiveness of intermediate steps in the procedure, if desired) by agarose gel electrophoresis of the following samples: (1) retained PCR product, (2) concentrated filtrate from the prespin, (3) supernatant containing the primers and dsDNA that were not captured on the magnetic beads, and (4) 3–5 μl of ssDNA. (The ssDNA band will not run at the same speed as dsDNA.) A larger volume of ssDNA may be necessary to visualize very short products on an agarose gel.

Sequencing

Standard sequencing reactions can be performed on 7.5 μl of the resulting ssDNA. Possible sequencing primers include the nonbiotinylated version of the biotinylated primer or any primer that anneals to the same strand. If the ssDNA yield is low, the ssDNA can be concentrated by evaporation under vacuum before sequencing.

Sequencing reactions have been performed using as a template a 350-base biotinylated ssDNA attached to Dynabeads M-280 (Dynal, Great Neck, NY).⁷ The beads must be washed with TE or bead buffer to remove residual NaOH and then resuspended in low-EDTA TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). The bead slurry then can be used in a sequencing reaction just as other ssDNAs. Primers may include the original, nonbiotinylated primer or other primers that anneal to the attached strand. Beads must be resuspended during sequencing reactions, but after final denaturation they must be excluded from the fraction loaded on the sequencing gel. Sequences using the template attached to the beads have not been as clean as those using the released strand as a template; optimization of this step will greatly increase the desirability of the procedure.

Alternative Low-Cost Procedure

The following procedure reduces the cost and time requirements for ssDNA preparation and yields enough template for one or two sequencing reactions. It is designed to speed analysis of a short DNA segment from many different individuals in a population. The PCR reaction is performed in a smaller volume, the Centricon prespin is omitted, and concentration steps are replaced by 2-propanol precipitation.

1. Amplify the DNA to be sequenced in a 50- μl volume. After evaluating yield on an agarose gel, add 45 μl of the PCR reaction to 50 μl of paramagnetic bead slurry and agitate for 15–30 min.

⁷ T. Hultman, S. Bergh, T. Moks, and M. Uhlen, *BioTechniques* **10**, 84 (1991).

2. Remove the supernatant and wash with 200 μ l bead buffer or TE.
3. Add 60 μ l of 0.2 M NaOH and agitate 6 min. Remove the NaOH, which contains the released ssDNA, to a fresh microcentrifuge tube.
4. To the NaOH fraction, add: 70 μ l of 5 M ammonium acetate (pH 6.8), 140 μ l of 2-propanol, and 1 μ l of 10 μ g/ μ l tRNA of 1 μ l of 1.25% linear polyacrylamide. (The tRNA or linear polyacrylamide is necessary to achieve sufficient yield for sequencing.)
5. Incubate at -20° for at least 1 hr.
6. Spin at high speed in a microcentrifuge for 10 min at 4° .
7. Pipette or decant off the supernatant, taking care not to dislodge the pellet.
8. Add 100 μ l of 70% (v/v) ethanol. Tap the tube lightly to wash the pellet. Pipette or decant off the ethanol.
9. Air- or vacuum-dry the pellet completely. Resuspend in 7–14 μ l TE.

Notes. Initial experiments show that Steps 3–9 of the above procedure can be eliminated in some cases. Washed beads from Step 2 can be resuspended in an annealing reaction designed for sequencing double-stranded templates.⁴ Following boiling, instead of the usual rapid-freezing step, the beads are concentrated immediately and the supernatant is removed. The remainder of the sequencing procedure is conducted on the supernatant as described.⁴ Our experiments show good sequencing yields from templates less than 500 bp in length. Longer templates have not yet been tested.

Acknowledgments

We thank P. Clyne, S.-Y. Chang, K. S. Greisen, and T. J. White for helpful comments on the manuscript and W. E. Rainey, K. S. Greisen, and C. Orrego for feedback and suggestions on the method described. B.H.B. is supported by a grant from the National Institutes of Health (RO1-A128545). S.R.P. holds National Science Foundation Grant BSR-9000006.

[30] DNA Sequences from Old Tissue Remains

By W. KELLEY THOMAS and SVANTE PÄÄBO

Introduction

One aspect of molecular evolution concerns the structural change of macromolecules over time. Traditionally, inferences about such changes are made from information about the differences within and between