Rapid Protocol for Template Preparation of Large Numbers of Clones


Sequencing large numbers of clones has been a time-consuming, costly and problematic task, requiring many manipulations to generate and purify template samples. Here we report a rapid and efficient protocol to generate template for a large number of clones. This procedure does not involve the preparation/purification of plasmid DNA and requires minimal hands-on work, thus realizing a considerable savings of material and time. Furthermore, template prepared according to the method described can be used for either manual or automated sequencing.

The protocol combines several previously reported technical tips with some novel shortcuts and is composed of three essential steps: (i) polymerase chain reaction (PCR) amplification of the insert region from a bacterial colony; (ii) rapid purification of the amplified material by gel filtration; and (iii) sequencing by the chain termination method. The following is a detailed description of the procedure tailored to sequencing clones from random peptide libraries displayed on phage (6). The same protocol, however, can be applied in determining the sequence of a DNA fragment from any plasmid or phage.

PCR Amplification

The clones used in this experiment are derivatives of pC89 phagemid (2) and obtained in the form of ampicillin (Ap)-resistant colonies on petri dishes (the same protocol can also be applied to phage plaques). The sequence to be determined for each clone is a 27-nucleotide-long insert that has been randomized. A sterile plastic pipet tip (4) is used to pick out a single colony (about 1 mm in diameter) and transfer it to a 0.2-mL MicroAmp® reaction tube (Perkin-Elmer, Norwalk, CT, USA) containing 20 μL of elution buffer (20 mM Tris-HCl pH 8.5, 2 mM EDTA, 1% Triton® X-100). The sample is heated 10 min at 95°C in a GeneAmp® PCR System 9600 thermal cycler (Perkin-Elmer). Clarification by centrifugation is thus avoided, since heating makes cells debris stick to the bottom of the tube. We prefer to keep the colony extract as master copy of the clone. Alternatively, the colony can be transferred directly to the PCR mixture and the heating step added to the PCR program. In this case, a master copy of the clone is made in the form of a stab in a well of a UV-sterilized microplate filled with agar containing 50 μg/mL Ap. The PCR is assembled by adding 3 μL of colony extract to a 0.2-mL MicroAmp tube in a final volume of 50 μL containing 150 nM forward (5'-AGAGATTACGCCAAGCC-3') and reverse (5'-TCCCATGTCAGACGT-3') primers, 50 μM dNTPs, 0.5% Nonidet® P-40 (NP40), 1× Taq buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl₂, 0.01% gelatin) and 1 unit AmpliTaq® DNA polymerase (Perkin-Elmer). The PCR process is run using the following program: 30 cycles of 94°C for 10 s, 60°C for 10 s and 72°C for 10 s.

Template Purification

Purification of the PCR-amplification product by gel filtration efficiently eliminates the problems derived from carryover of PCR primers and dNTPs in the subsequent sequencing steps. Purification is performed in a format that allows the parallel processing of 96 clones. Sephacryl® S-300 (Pharmacia Biotech, Uppsala, Sweden) aqueous slurry is deposited in the wells of a Silent Monitor™ membrane-bottomed microplate (Model SM120LP, 1.2 μm Loprodynê™; Pall BioSupport, Portsmouth, England, UK). Excess buffer is rapidly and easily drained by applying suction to the bottom of the plate using a vacuum filtration unit (Model EVENT 4160; Eppendorf, Hamburg, Germany), leaving a settled bed volume of about 400 μL of resin per well. The PCR-amplified material from each clone is pipetted onto the drained Sephacryl-containing wells. Flow-through is collected using vacuum aspiration in a 96-well microplate. The length of the PCR-amplified template dictates the choice of the gel-filtration medium; we found that Sephacryl S-300 gives the best results for our 330-bp PCR-amplified fragment. Using the above procedure, both handling and assembly of

Figure 1. Manual sequencing of PCR-generated templates. Panel A: Cycle-sequencing using 32P-labeled primer A. Panel B: T7 polymerase sequencing using primer B and [α-32P]dATP.
Benchmarks

the samples is easier and faster than using commercially available, prepacked spin columns, and also saves on material costs.

Sequencing the PCR-Amplification Product

The purified PCR product can be used directly for sequencing according to one of the following protocols:

Manual sequencing. (i) Cycle-sequencing. This sequencing protocol involves a primer-labeling reaction that allows the priming sequence to be very close to the region of interest, thus reducing the time required for gel electrophoresis. In addition, all sequencing reactions are performed on a DNA thermal cycler, which requires minimal hands-on work. The primer labeling protocol is as follows: Sequencing primer A (5'-GCTACCCTCGTTCCGATGCTGTCT-3'; 12 nucleotides from the insert sequence and about 200 nucleotides to the end of the PCR-amplified fragment) is 5'-labeled with [γ-32P]ATP using T4 polynucleotide kinase according to standard protocol (5). Unincorporated, labeled nucleotides are removed by gel filtration. Peak fractions are pooled and primer concentration is estimated, assuming losses are insignificant. Labeled primer can be stored at -20°C up to two weeks. The sequencing reaction is performed as follows: For each clone a sequencing mixture is made up containing 2 pmol of 32P-labeled sequencing primer A, 1× Tag buffer, 1% NP40, 5 U AmpliTaq DNA polymerase and 10 µL of purified template, in a total volume of 20 µL. Four microliters of the sequencing mixture are distributed to each of the four tubes, each containing 2 µL of dNTP/ddNTP mixture (30 µM dNTPs and either 1200 µM ddTTP, 600 µM ddCTP, 120 µM ddGTP or 1200 µM ddATP). The PCR program is run as follows: 25 cycles of 94°C for 10 s and 72°C for 10 s. Four microliters of sequencing dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanole FF) are added to each reaction tube. Samples are heated at 95°C for 90 s and then chilled on ice. Two to three microliters are loaded onto a pre-run 6% acrylamide gel. The sequencing gel is cast between two 40-cm-long, 34-cm-wide glass plates, assembled with a "sharktooth" comb to create fifty-six 5-mm wells. (ii) T7 polymerase sequencing. Using this protocol, no primer labeling is required; however, the position of the sequencing primer has to allow sufficient labeling of the elongating chain prior to the insert region. An annealing mixture for each clone is made up containing 7 µL of purified template, 10 pmol of sequencing primer B (5'-GCTTGATCATCGATAGTTG-3'; 90 nucleotides from insert sequence and about 250 nucleotides to the end of the PCR-amplified fragment) in 1× Sequenase® buffer (United States Biochemical, Cleveland, OH, USA), in a total volume of 10 µL. PCR template is denatured by heating 3 min at 95°C, after which it is snap-cooled on ice (3). The sample is left on ice for 5 min, briefly centrifuged to collect drops from the

Figure 2. Automated fluorescence plot of a PCR-generated template. Automatic base calling is performed by the PE/ABI Version 1.2.1 analysis program.
walls and then returned to ice. The above mixture is then processed essentially according to the Sequenase protocol, with the addition of 0.5% NP40 to the labeling reaction (1).

Automated sequencing. The same purified PCR product can be used directly for automated DNA sequencing using the cycle sequencing protocol with dye-labeled dideoxynucleotides. With this method, the cost and time required for preparing and purifying the template are significantly lower than with many of the current protocols. We adopted the protocol recommended by Perkin-Elmer/Applied Biosystems Division (PE/ABI, Foster City, CA, USA), assembling for each clone a cycle-sequencing reaction containing sequencing primer B and 10 μL of purified template. Excess dideoxynucleotide terminators not incorporated are removed by gel filtration. Sephacryl S-200 aqueous slurry is deposited in the wells of a Silent Monitor membrane-bottomed microplate and compacted by centrifugation in an Omnifuge 2.0RS (Heraeus Sepatech GmbH, Osterode, Germany) using a Model 2250 rotor with buckets for Model 2359 microplates at approximately 1800 rpm (ca. 735×g) for 60 s to give a settled bed volume of about 400 μL of resin per well. The cycle-sequencing reaction is diluted up to 50 μL with H2O and pipetted onto the drained Sephacryl-containing wells. Flow-through is then collected in a 96-well microplate by centrifugation at 735×g for 120 s, transferred to a microcentrifuge tube, dried, dissolved in 4 μL of formamide, heated at 95°C for 2 min and cooled on ice. Sequence reaction products are analyzed on a PE/ABI Model 373A DNA sequencer according to the manufacturer’s instructions.

REFERENCES


Address correspondence to Paolo Monaci, Biotechnology Department, I.R.B.M. P. Angeletti, Via Pontina km 30.600, 00040 Pomezia, Rome, Italy. Internet: monaci@irbm.it

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F. Bartoli, M. Nuzzo, M. Pezzanera, A. Nicosia and P. Monaci I.R.B.M. P. Angeletti Pomezia, Rome, Italy

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Creation of a Novel, Versatile Multiple Cloning Site Cut by Four Rare-Cutting Homing Endonucleases


Homing endonucleases, or meganucleases, are a new addition to the repertoire of site-specific endonucleases. The recognition sites for these enzymes are much longer than those of conventional restriction enzymes, encompassing 15–25 nucleotides; and, although within the recognized DNA sequence some sequence variation is tolerated, cleavage points in natural DNA are extremely rare. These endonucleases are involved in the insertion of mobile genetic elements, a process termed “homing”. Some are encoded in mobile group I introns and play a role in the insertion of intron elements in intronless alleles. Others are excised post-translationally by “protein-splicing” (16). The novel polynolinker includes sites for four of these endonucleases. PI-SceI, or VDE (14), is spliced out of the VMA ATPase of Saccharomyces cerevisiae (3,16) and has been expressed as a separate enzyme in E. coli (6,7). I-Ppol (14,18) stems from the intron of the extrachromosomal nuclear rDNA of Physarum (10,13). I-CeuI (14) is the homing endonuclease encoded by the fifth intron in the chloroplast large subunit rRNA gene of Chlamydomonas eugametos (11), and PI-TiiI (14) is excised by protein splicing from the Vent DNA polymerase of the Archaea Thermococcus litoralis (15,16). PI-PspI (14) and I-SceI (omega nuclease) (2) are two additional commercially available homing nucleases not yet included in our polynolinker. The reader is referred to recent reviews for the cleavage specificities, the biological role and the nomenclature of homing nucleases (9,12,16).

Because of the rarity of meganuclease cleavage sites in naturally occurring DNA, these enzymes are ideal tools to engineer large segments of DNA of (yet) unknown sequences in vitro. They can, for example, be used to create