Streamlined method for purifying single-stranded DNA from PCR products for frequent or high-throughput needs

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Single-stranded DNA (ssDNA) is very useful in diagnosis, molecular therapy, and basic research in molecular biology. When high-throughput or frequent preparation of ssDNA is needed, it is highly desirable to accomplish the task by a procedure that is rapid, efficient, cost-effective, and automation-compatible. Although many methods of preparing ssDNA exist (1–5), they are not satisfactory. Here I present a modified protocol based on the popular method of alkaline treatment of PCR products, which are biotinylated and immobilized on streptavidin (SA)-coated substrate (polyacrylamide or superparamagnetic beads) (1–3). Despite its popularity in research laboratories, the original protocol is neither ideal for automation nor for frequent preparation of ssDNA. The major dilemma is in the choice of SA-coated substrates. Because fewer than 15% of primers are utilized in a typical PCR, it is not economical to use SA-coated superparamagnetic beads to capture all biotinylated double-stranded DNA (dsDNA) products directly, along with the unused biotinylated primer. On the other hand, the polyacrylamide substrate is much cheaper than the superparamagnetic one in terms of unit biotin binding capacity but is bulkier and inconvenient for robotic manipulations. In addition, the traditional methods (1–5) become laborious when the volume of the PCR is large.

The main advance of this modified protocol is the use of an inexpensive primer-removing substrate to eliminate the unused biotinylated PCR primer, so that the consumption of expensive SA-coated superparamagnetic beads can be reduced dramatically. The primer-removing substrate was prepared by coupling 10 nmol biotinylated antisense oligonucleotide [in 200 μL phosphate-buffered saline, pH 7.4 (PBS)], whose sequence was complementary to that of the biotinylated PCR primer, on 100 μL bed volume of SA-coated polyacrylamide gel (UltraLink® immobilized streptavidin; Pierce Biotechnology, Rockford, IL, USA) and then blocking the vacant SA sites with 100 nmol biotin (dissolved in 100 μL PBS; Sigma-Aldrich, St. Louis, MO, USA), followed by washing the gel with PBS. The second improvement is the use of silica adsorption to concentrate and recover ssDNA instead of the ethanol precipitation, which is time-consuming and difficult for automation.

Figure 1 shows an example of applying the new protocol to purify an amplified 149-nucleotide ssDNA pool during an in vitro selection experiment. PCR was performed with a biotinylated primer (btnP5), a regular primer (P6), and template derived from previous screening. After the amplification, the PCR mixture was combined with a one-tenth volume of 5 M NaCl and then incubated with a one-twentieth volume (bed volume) of primer-removing polyacrylamide beads at room temperature for 10 min with gentle mixing. The unused biotinylated PCR primer annealed with the antisense oligonucleotide of primer-removing substrate and was removed from the PCR mixture by filtering through a MULTI-SPIN separation column (lane 3; Axygen Scientific, Union City, CA, USA). The filtrate was then incubated with 5 mg Dynabeads® MyOne streptavidin (Dynal Biotech ASA, Oslo, Norway) for 10 min, and the majority (>90%) of dsDNA of the reaction mixture (lane 2, All) included biotinylated primer (btnP5), a regular primer (P6), dsDNA product (open triangle), a small amount of self-dimer of P6 (P6′2), and an uncharacterized species (U). The unused biotinylated primer was removed from the mixture by the primer-removing substrate (lane 3, -btnP). The majority of dsDNA was immobilized on streptavidin (SA)-coated superparamagnetic beads and disappeared from the solution (lane 4, -dsDNA). The ssDNA (filled triangle) was released into the solution after alkaline treatment and neutralization (lane 5, alkaline). The ssDNA was concentrated and recovered by using a silica-based membrane (lane 6, silica). To visualize the nucleic acid species, a small amount of btnP5 was labeled with [α-32P]dATP at the 3′ end with terminal transferase (New England Biolabs, Beverly, MA, USA) and added into the mixture after PCR. In addition, a small amount of dsDNA along with co-purified nucleic acids (by the MiniElute PCR Purification Kit) were labeled with 32P at the 5′ end with T4 polynucleotide kinase (New England Biolabs), and added back into the reaction mixture. A 100-bp DNA ladder (New England Biolabs) was dephosphorylated and labeled with 32P at the 5′ end (lane 1, M). The nucleic acids were separated on a 10% polyacrylamide gel with 1 mM magnesium acetate and revealed by a phosphorimager (FLA-3000R; Fuji Photo Film Co., Ltd., Tokyo, Japan). Notice that a portion of the ssDNA was stuck at the top of the native gel. The recovery yield was determined by measuring the phosphorimager signals of dsDNA and ssDNA with quantification software Image Gauge V3.12 (Fuji Photo Film Co., Ltd.). The details are available as Supplementary Material on the BioTechniques’ web site at www.BioTechniques.com. dsDNA, double-stranded DNA; ssDNA, single-stranded DNA.
dsDNA was removed from the supernatant (lane 4), which was discarded. The bead-bound dsDNA was denatured in alkaline solution (0.1 M NaOH and 1 mM EDTA) at room temperature for 15 min, and the ssDNA without biotin groups was released in the alkaline solution (lane 5). After neutralization, the ssDNA solution was combined with five volumes of Buffer PB from the MiniElute™ PCR Purification Kit (Qiagen, Valencia, CA, USA), and passed through MiniElute columns. The ssDNA was then eluted from the columns with a small volume of 10 mM Tris-HCl, pH 7.4 (lane 6). The recovery yield was 85% for this 149-nucleotide ssDNA. The whole procedure could be easily performed in 1 h either manually or by an automation workstation (e.g., Biomek® 2000; Beckman Coulter, Fullerton, CA, USA) with a robotic liquid handler, vacuum and manifold, washing unit, and a magnetic plate.

Compared to the original protocols (1–3) or other standard methods involving gel purification and ethanol precipitation (4,5), this renovated protocol saves time and effort and is cost-effective (by a dramatic reduction in the use of expensive SA-coated superparamagnetic beads), although additional materials are used. The recovery yield of this protocol is a little lower than that (approximately 95%) of the original ones (1–3) because the binding affinity of silica membrane is weak for short DNA (approximately 75% for an 88-nucleotide ssDNA; data not shown) while alcohol precipitation can effectively recover short nucleic acids from solution. When short ssDNA (60–75 nucleotides) is to be prepared with high recovery yield, instead of using silica membrane, this protocol can be modified by using a molecular sieve column (Sephadex® G-25; Amersham Biosciences, Piscataway, NJ, USA) to desalt. However, the volume of prior alkaline and acid solutions must be reduced to increase the ssDNA concentration.

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COMPETING INTERESTS

STATEMENT

The author declares no competing interests.

REFERENCES


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Simplex PCR assay for sex determination in mice

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Some biomedical research procedures using laboratory mice, such as the preparation of sex-specific fetal cell cultures, require the sex of fetuses and newborn pups to be determined. Although neonate mice can be sexed anatomically on the basis of the anogenital distance (AGD), 48% of newborn pups are reported to be unclassifiable using this method (1). The existing molecular methods for sexing are PCR-based assays that combine two pairs of primers together in a multiplex reaction to amplify the Y-chromosome-specific gene Sry and an autosomal gene—either Il3 (chromosome 11) or Tshb (chromosome 3)—that serves as an internal control of PCR amplification (2,3). Here we present a novel PCR assay that uses only one pair of primers in a single reaction tube to simultaneously amplify DNA fragments from both the X- and Y-chromosomes.

Using the Sequencher™ 4.1 software (Gene Codes Corporation, Ann Arbor, MI, USA), we aligned DNA sequences of the X-chromosome-specific gene Jarid1c (ENSMUST00000082177) and the Y-chromosome-specific gene Jarid1d (ENSMUST0000055032) that we had obtained from Ensembl Mouse Genome Release 25 (www.ensembl.org/mus_musculus), knowing these genes to be homologous (4). The alignment showed that Exons 9 and 10 of Jarid1c have a high degree of sequence similarity with Exons 9 and 10 of Jarid1d (90% and 76%, respectively), with the corresponding exons of both genes having the same length (120 and 159 bp, respectively). In contrast, the intron between the two