An efficient method for purification of PCR products for sequencing

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A high-throughput DNA sequencing method that generated high quality data was developed. A frame fashioned from a standard agarose gel combined with 0.1%–0.2% low-melting point (LMP) agarose gel was used to isolate the PCR product of interest. Collected PCR products were centrifuged without any reagents and the supernatants were directly used for a sequencing reaction. This method is simple and labor efficient, provides high quality sequences at a low cost, and bypasses problems with impure PCR products. This technique has been used for single nucleotide polymorphism (SNP) discovery in Populus angustifolia trees.

Quality DNA sequencing is one of the most important tools in molecular biology research. Because the genomes of more than 300 organisms have been sequenced and another 750 are in process (1), there is increased demand for high-throughput and low-cost methods for resequencing genomes in order to identify genetic variants and make inferences about gene function (2). Because the Sanger sequencing method provides relatively long read lengths and low error rates (3,4), direct sequencing of PCR products with the Sanger-based method is one of the most widely used procedures for resequencing (4–7).

PCR products can be either cloned or directly used for Sanger sequencing. The former procedure involves cloning, transformation, bacterial incubation, and DNA isolation steps to prepare template DNA for sequencing. Direct DNA sequencing of PCR products is a much more efficient approach. If a single fragment is amplified, PCR products can be directly sequenced after removal of PCR primers and/or inactivation of dNTPs with phosphatase (8). However, PCR products are often comprised of a mixture of nonspecific products in addition to the target product. In this case, the band of interest must be isolated, usually from an agarose gel, followed by costly and time-consuming clean-up procedures prior to sequencing (9,10).

We have developed a procedure using a combination of standard agarose and low-melting point (LMP) agarose to purify and recover PCR products, which can then be sequenced directly with little additional purification. The agarose gel is initially prepared using a standard gel mold [Model A2 (28 cm W × 37.5 cm L × 11 cm H) or B2 (18 cm W × 24.5 cm L × 9.5 cm H); Owl Separation Systems, Portsmouth, NH, USA], after which a rectangle is cut from the interior portion, where the LMP agarose is subsequently poured (Figure 1). Size separation takes place primarily in the standard agarose

portion of the gel, which can be varied in length and concentration to increase the resolution of the bands. The products then enter the LMP portion of the gel, from which they can be easily pipetted and sequenced directly. We initially tested five different concentrations of LMP agarose gel (i.e., 0.1%, 0.2%, 0.4%, 0.6%, and 0.8%). Concentrations >0.2% LMP agarose interfered with pipetting and prevented the formation of firm pellets in 96-well plates, often resulting in loss of the pellet upon inversion of the plate. Templates pipetted from 0.2% and 0.1% LMP gel consistently generated strong sequencing signals.

Previous studies have shown that agarose gel concentrations >0.2% inhibit sequencing reactions (5). Our method ensured that the agarose concentration was <0.2% in the sequencing reaction, since we began with a 0.1–0.2% LMP gel, and the LMP agarose was then further diluted in the sequencing reaction. Consequently, the remaining agarose should not affect the sequencing reaction. On the other hand, the very low concentration of agarose in the sequencing reaction might even improve the sequence length and quality, as has been shown previously (11).

We tested our method using eight loci from the *Populus angustifolia* genome using 30 to 32 trees collected from wild populations in Utah (12).

Figure 1. PCR reactions electrophoresed in a 2.0% agarose gel frame with an embedded LMP agarose gel reservoir. Composition of each lane (product/template): 1, 1 Kb DNA Ladder; 2, HSP1_0834_2477/Sample 15; 3, HSP1_2121_3424/Sample 15; 4, Brun1_2002_3355/Sample 15; 5, Brun2_2175_3188/Sample 15; 6, Brun24470_5959/Sample 15; 7, PTLF_1094_2078/Sample 15; 8, PTLF_2059_3046/Sample 15; 9, PTLF_2059_3046/Sample 19; 10, TFL_2019_2976/Sample 15. White arrows indicate PCR products for which sequences are shown in Figure 2.

Benchmarks

Primer pairs that gave single or multiple PCR products were tested using the BigDye Terminator v3.1 Cycle Sequencing kit on a 3130x1 DNA Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). With this method, the pass rate (>100 bp with Phred >Q20) ranged from 92% to 100%. The average read length ranged from 549 to 744 Q20 bases using a 50 cm capillary on the ABI 3130x1 DNA Genetic Analyzer.

The average read length ranged from 483 to 526 Q20 bases with a 35 cm capillary (Table 1). Direct sequencing of the PCR products generated strong signals and low background intensities, such that the SNPs were readily identified in the sequence profiles (Figure 2). The quality of the sequence is demonstrated by high Phred quality scores, which were >47 on average (Table 1). These high quality sequences were

obtained even with a 1:8 dilution of the recommended quantity of BigDye Terminator v3.1 kit and a total sequencing reaction volume of only 5 µL. Finally, a high quality sequence was obtained even when multiple products were amplified by a given primer set (Table 1; Figure 2).

This method provides many advantages over traditional procedures for direct sequencing of PCR products. First, pipeting the PCR product from the LMP gel to a new tube is simpler and easier than excising the gel slice. Second, the procedure was successful even for impure PCR products with nonspecific amplification. The concentration of standard agarose and the position of the LMP agarose can be altered according to the number of bands and proximity of nonspecific bands to the target band because enhanced separation can be achieved with longer runs through standard agarose. The disadvantages of isolating DNA from LMP gels, including poor resolution of closely migrating bands, can be overcome by this method (9). Third, the method is highly scalable, and could be automated using robotics, particularly for unique PCR products. Finally, this method greatly decreases the cost of direct sequencing. The 1.0%–2.0% agarose gel frame is reusable and the concentration of LMP agarose is 0.1%–0.2%, so little agarose

Figure 2. Sequencing results obtained from single and multiple PCR products. Equally high quality sequences were obtained from products with clear single bands as from products with multiple bands. Images were captured from Consed and scored with PolyPhred (14,15). The SNPs are clearly scorable from these sequences (indicated by boxes color-coded by PolyPhred). (A) Product Brun1_2002_3355 with single PCR product, forward primer, samples 15 and 19. (B) Product PTLF_2059_3046 with multiple PCR products, forward primer, samples 15 and 19.

Table 1. Summary of Sequencing Quality for Selected *Populus trichocarpa* Loci Resequenced in *Populus angustifolia*

Product	Number of Samples ^a	Pass Rate ^b	Average Read Length ^c	Average Phred Quality ^d
HSP1_0834_2477	61	1.00	626.15 ± 131.01	52.73 ± 8.60
HSP1_2121_3424	61	0.98	743.53 ± 101.80	47.68 ± 11.42
Brun1_2002_3355	64	0.92	515.21 ± 144.33	50.40 ± 7.19
Brun2_2175_3188	64	1.00	602.28 ± 161.82	48.60 ± 10.05
Brun2_4470_5959	62	0.97	548.75 ± 233.15	51.87 ± 7.58
PTLF_1094_2078 ^e	64	1.00	483.13 ± 108.61	50.04 ± 10.06
PTLF_2059_3046	64	0.91	599.07 ± 218.00	48.65 ± 13.47
TFL_2019_2976 ^e	64	0.98	526.11 ± 25.36	57.51 ± 5.63

^aSome genomic DNA samples were not amplified, so there were <64 forward and reverse sequenced samples for some primers.
^bPass rates are based on lanes producing >100 bases scoring Phred 20 or better.
^cRead lengths are the average of the Phred 20-qualified bases in the passed lanes ± standard deviation (sd).
^dDNA sequencing quality was determined using the base caller Phred (13) ± sd.
^eThese PCR products were sequenced with 35 cm capillary in our ABI DNA Genetic Analyzer, while the others were sequenced with 50 cm capillary.

is consumed. In fact, we calculate that the total cost of LMP agarose is approximately \$0.02 per sample, compared with up to \$1.80 per sample for commercially available gel purification kits such as the QIAquick Gel Extraction kit (Qiagen, Germantown, MD, USA). Therefore, this approach is one of the most efficient methods currently available for direct sequencing, and should be a boon for labs on a tight sequencing budget.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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