Cost-Effective Method to Synthesize Fluorescently Labeled DNA Size Standards Using Cloned AFLP Fragments

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Fluorescent detection of PCR products is an established method in genome mapping, population genetics, and the individual identification of many organisms. The growing use of fluorescent detection and analysis techniques has facilitated high-throughput genotyping procedures, but running costs have been raised by the need to include a fluorescently labeled size marker for each sample. Recently, a method has been described for cost-effective labeling of size standards derived from a conserved region of plant chloroplast DNA (1). However, this technique requires as many as seven fluorescently labeled primers to generate 12 fragments of standard sizes. Here, we applied a different approach and propose a more cost-effective method to synthesize fluorescently labeled size standards. We are now constructing linkage maps of a herbaceous plant, *Primula sieboldii*, using AFLP (2), microsatellite markers, and fluorescently labeled size standards derived from *P. sieboldii* DNA.

Genomic DNA was extracted from a leaf of *P. sieboldii* with a DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany). An AFLP® Core Reagent Kit (Invitrogen, Carlsbad, CA, USA) was then used for DNA digestion and adapter ligation, following the protocol provided by the manufacturer (except that we reduced the reaction volume to reduce costs). Briefly, genomic DNA (20–100 ng) was double-digested with *EcoRI* and *MseI* in a 5- μ L reaction mixture. After inactivation of the restriction enzymes at 70°C, adapters complementary to the *EcoRI* (E) and *MseI* (M) restriction sites were ligated. The ligation products were diluted 10-fold and used as templates for preselective amplification, which was carried out by primers complementary to the adapter sequences with a 3' extension of two nucleotides (E-AA and M-CA).

The amplification was performed in a 50- μ L reaction mixture containing 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 0.2 mM each dNTP, 1.5 mM MgCl₂, 1.5 U *Taq* DNA polymerase, and 7.5 μ L diluted template DNA with the following thermal conditions: 3 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 56°C, 30 s at 72°C, and finally 30 min at 72°C. The amplification products created smeared banding patterns when separated on a 2% agarose gel. The products were cloned into pCR®-TOPO® with a TOPO TA Cloning® Kit (Invitrogen). The presence/absence and the size of the inserts were analyzed by colony PCR, and appropriate clones were selected. Plasmid DNA was extracted from these clones, and the inserts were amplified by M13 pUC Sequencing Primers (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The products were diluted 100-fold and used as templates for subsequent PCR. A TAMRA-labeled primer complementary to the *EcoRI* adapter sequence and a primer complementary to the *MseI* adapter sequence were used for fluorescent labeling of the DNA fragments. The labeling reactions for each fragment were performed separately in a 50- μ L reaction mixture comprising 1 $\times$ PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 100 mM each dNTP, 0.02% Triton® X-100, and 0.01% gelatin), 1.5 mM MgCl₂, 1.25 U *Taq* DNA polymerase, 0.25 μ M each primer, and 5 μ L template DNA with the following conditions: 3 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 56°C, and 30 s at 72°C, concluding with 10 min at 72°C. The size of each labeled fragment was estimated by co-electrophoresis with a commercial set of size standards (GeneScan® TAM-RA500; Applied Biosystems, Foster City, CA, USA) and least square sizing

analysis by GeneScan software (Applied Biosystems). The resulting data were rounded to the nearest integers, which were used to define the size of the standard. The actual size of the fragments was not determined by sequencing because differences between actual and designated sizes do not matter for genotyping. Finally, equal volumes of each product were mixed, diluted to an appropriate fluorescent intensity, and used as standards. We randomly selected 34 recombinant clones, 14 of which were used as size standards, and Figure 1 shows an electropherogram of this set of standards.

To evaluate the reliability of the size standards, three microsatellites in a *P. sieboldii* plant (3) were amplified and electrophoretically separated with the developed size standard on 7% denaturing acrylamide gels (12 cm well-to-read) in 0.5 $\times$ TBE buffer using an ABI PRISM® 377 DNA sequencer equipped with GeneScan software. Data from three gels, each containing triplicate lanes from the same PCR amplification reaction were analyzed by the local Southern algorithm (4). Amplification of the three microsatellites yielded six fragments, resulting in a total of 54 observations. Standard deviations were calculated from estimated size differences within and between gels, ranging from 0.02 to 0.04 and from 0.03 to 0.05 nucleotides, respectively (Table 1). These results indicate the reliability of the synthesized size standard. The low standard deviation values reflect the fact that single alleles were analyzed and sized.

Cost analysis for routine production of the synthesized size standard showed that it is more economical, by a factor of about 128, than a commercial size standard (Table 2). The costs for extracting DNA from the plant materi-

Figure 1. Electropherogram of the synthesized size standard. Sizes of the fragments (in base pairs) are indicated above each peak.

Benchmarks

Table 1. Observed Differences in the Estimated Fragment Sizes for Microsatellites in *P. sieboldii*

Locus	Allele	Within-Gel Difference (nt ^a)			Between-Gel Difference (nt)		
		Max. ^b	$\bar{x}$	SD ^c	Max.	$\bar{x}$	SD ^d
ga0235	335	0.18	0.08	0.05	0.15	0.07	0.04
	343	0.09	0.05	0.03	0.15	0.05	0.04
ga0668	118	0.07	0.04	0.02	0.11	0.03	0.03
	124	0.12	0.04	0.04	0.12	0.07	0.04
ga1277	229	0.07	0.02	0.02	0.13	0.07	0.04
	231	0.12	0.06	0.04	0.17	0.08	0.05

^aNucleotides
^bMaximum differences observed
^cStandard deviation of within-gel differences across three gels ($n = 9/\text{allele}$)
^dStandard deviation of between-gel differences across three gels ($n = 27/\text{allele}$)

Table 2. Cost Analysis for Routine Production of the Synthesized Size Standard and Comparison with a Commercial Size Standard

	Synthesized Size Standard			Commercial Size Standard
	Expendables	Labor	Total	
Unit ^a cost	\$9.30 ^b	\$13.60 ^c	\$22.90	\$450
Cost/96 gel lanes	\$0.17	\$0.25	\$0.42	\$54

^aOne unit for the synthesized size standard is defined as one synthesis of the 14 standard fragments by PCR with the fluorescently labeled primer in 50 μL each reaction mixture, and one unit for the commercial size standard is defined as one package.
^bOnly costs unique to the production of the standard (synthesis of the fluorescently labeled primer and PCRs) are included. Costs of extracting DNA from the plant material and amplified fragment library construction are excluded.
^cAssuming \$6.8/h for a laboratory technician and 2 h for one unit of the synthesized standard.
 Note that the cost analysis is based on a 2003 exchange rate of 120 yen/dollar.

al and constructing the library of amplified fragments were estimated to be \$140, including all reagents and labor. However, the initial investment will be paid off because, once the fragment library is constructed, it can be routinely used to produce infinite amounts of size standards at low cost.

The biggest advantage of this method is that only one fluorescent primer is needed to label the sizing standard. Moreover, fragments of appropriate size are chosen from the recombinant clones so that many sets of sizing standards can be constructed, according to specific needs. Modification of this simple PCR approach to generate size standards may include the use of any recombinants of suitable size as template DNA. Another advantage of our method is that consis-

tent nontemplated addition of adenine (+A) to the fluorescently labeled strand by *Taq* DNA polymerase is expected, so batch-to-batch variations in the size of standard fragments due to variations in the degree of adenylation may be avoided. Since the 5' end of the primer complementary to the *Mse*I adapter ends with guanine, *Taq* DNA polymerase promotes additions of +A to the corresponding fluorescently labeled strand (5).

In summary, we have developed a cost-effective method to generate fluorescently labeled size standards using cloned AFLP fragments. This enabled us to amplify many fragments of different sizes using a single primer combination. Amplified fragments were cloned and several clones with appropriate size were selected. The inserts

were amplified and used with a fluorescently labeled primer for the production of fluorescently labeled size standards by PCR. The protocol described here can be easily followed by laboratories equipped to perform standard molecular techniques and will substantially reduce costs of genotyping projects.

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