

Universal method for producing ROX-labeled size standards suitable for automated genotyping

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Fluorescently labeled size standards are indispensable for genotyping via an automated DNA sequencer. Microsatellites, amplified fragment-length polymorphisms (AFLPs), single nucleotide polymorphisms (SNPs), and random amplified polymorphic DNA (RAPD) can all be assayed on platforms such as the ABI PRISM[®] 377 DNA Sequencer or the ABI PRISM 3100-Avant Genetic Analyzer (both from Applied Biosystems, Foster City, CA, USA), but each requires a fluorescent marker that typically is purchased from a commercial vendor. These markers are often prohibitively expensive. Indeed, the cost of size standards can exceed that of the genotyping assay itself. In response to

Table 1. Primer Sequences Complementary to pBS II SK (+) and pUC-19

pBS Primer ^a	pBS Primer Sequence ^b	pUC Primer Sequence ^b	pUC Primer ^a
ROXF1	5'-ROX-AAGGGCCGAGCGCAGAAGTGGTC-3'	5'-ROX-AAGGGCCGAGCGCAGAAGTGGTC-3'	ROXnaurox424L
ROX78	5'-ACTCTAGCTTCCCGGCAACAATTAATAGACTG-3'	5'-GCTCTAGCTTCCCGGCAACAATTAATAGACTG-3'	NAUROX79
ROX89	5'-GCGAACTACTTACTCTAGCTTCCCGGCAAC-3'	5'-GCGAACTACTTACTCTAGCTTCCCGGCAAC-3'	NAUROX90
ROX104	5'-GCAAACCTATTAAGTGGCGAACTAC-3'	5'-GCAAACCTATTAAGTGGCGAACTAC-3'	NAUROX105
ROX130	5'-GCCTGTAGCAATGGCAACAACGTTGC-3'	5'-GCCTGTAGCAATGGCAACAACGTTGC-3'	NAUROX131
ROX150	5'-GACGAGCGTGACACCACGATGCCT-3'	5'-GACGAGCGTGACACCACGATGCCT-3'	NAUROX151
ROX181	5'-GGAACCGGAGCTGAATGAAGCCATACC-3'	5'-GGAACCGGAGCTGAATGAAGCCATACC-3'	NAUROX182
ROX200	5'-GAACTCGCCTTGATCGTTGGGAACC-3'	5'-GAACTCGCCTTGATCGTTGGGAACC-3'	NAUROX201
ROX253	5'-GATCGGAGGACCGAAGGAGCTAACC-3'	5'-GATCGGAGGACCGAAGGAGCTAACC-3'	NAUROX254
ROX305	5'-GTGCTGCCATAACCATGAGTGATAACAC-3'	5'-GTGCTGCCATAACCATGAGTGATAACAC-3'	NAUROX306
ROX336	5'-ACGGATGGCATGACAGTAAGAGAA-3'	5'-ACGGATGGCATGACAGTAAGAGAA-3'	NAUROX337
ROX361	5'-CTCACCAGTCACAGAAAAGCATC-3'	5'-CTCACCAGTCACAGAAAAGCATC-3'	NAUROX362
ROX424	5'-TGACGCCGGCAAGAGCA-3'	5'-TGACGCCGGCAAGAGCA-3'	NAUROX425
ROX485	5'-AACGTTTTCCAATGATGAGC-3'	5'-AACGTTTTCCAATGATGAGC-3'	NAUROX486
ROX508	5'-CCTTGAGAGTTTTCGCCCCGAAGAAC-3'	5'-GCTTGAGAGTTTTCGCCCCGAAGAAC-3'	NAUROX509
ROX559	5'-GTTGGGTGCACGAGTGGTTACATCG-3'	5'-GTTGGGTGCACGAGTGGTTACATCG-3'	NAUROX560
ROX597	5'-CCAGAAACGCTGGTAAAAGTAAAAGATGCTG-3'	5'-GCAGAAACGCTGGTAAAAGTAAAAGATGCTG-3'	NAUROX598
ROX673	5'-GAGTATGAGTATTCAACATTTCCGTGTCGC-3'	5'-GAGTATGAGTATTCAACATTTCCGTGTCGC-3'	NAUROX674
ROX738	5'-TCAAATATGTATCCGCTCATGAGACAATAACC-3'	5'-GCAAATATGTATCCGCTCATGAGACAATAACC-3'	NAUROX739
ROX798	5'-GGTGGCACTTTTCGGGGAAATGTGC-3'	5'-GGTGGCACTTTTCGGGGAAATGTGC-3'	NAUROX799
		5'-GAGGTTTTACCGTCATCACCAGAAC-3'	NAUROX902
		5'-GCCCCGACACCCGCCAACAC-3'	NAUROX1007

^aPrimer names reflect the expected product size when used in conjunction with the ROXF1 primer (e.g., ROX305 produces a 305-bp fragment when used with ROXF1) or the ROX-naurox424L primer (e.g., NAUROX486 produces a 486-bp fragment when used with ROXnaurox424L).

^bDiscrepancies between pBluescript (pBS) and pUC primer sequences are shown in bold font.

BENCHMARKS

this dilemma, two recent reports have described novel approaches to the in-house production of fluorescently labeled size standards (1,2). Unfortunately, both of these methods require specific DNA templates that are uncommon in most laboratories, and one of the methods requires multiple labeled primers. Below, we describe a method for marker production that relies upon two of the most common laboratory cloning vectors, plasmids pUC and pBluescript® (pBS).

We used the sequences of pUC-19 and pBS II SK(+) to design PCR primers for the amplification of known-length fragments from plasmid minipreps. The primer sets (Table 1) each include a single labeled forward primer and multiple unlabeled reverse primers that are each used in conjunction with the labeled forward primer. Once the primer sequences were in hand, we used them to amplify DNA fragments from common laboratory plasmids.

Briefly, plasmid DNA is prepared using conventional miniprep techniques (3), and this DNA is used as a template for subsequent PCR. Plasmid DNA is low complexity and thus makes an excellent template for PCR. We amplified fragments individually as opposed to experimenting with multiplex reactions. Am-

plifications all utilized the same thermal profile, namely 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. A final extension reaction of 10 min at 72°C ensures that all PCR products are full length. For the pBS size standard, we used *Pfu* DNA polymerase to avoid the A-tailing inher-

ent to *Taq* DNA polymerase (4). For the pUC size standard, a naturally occurring or artificial 5' guanine residue was included in all but four of the unlabeled primer sequences to promote the A-tailing. The pUC fragment sizes reflect this added 5' guanine and the A-tailing. In our hands, only the 90-bp pUC fragment

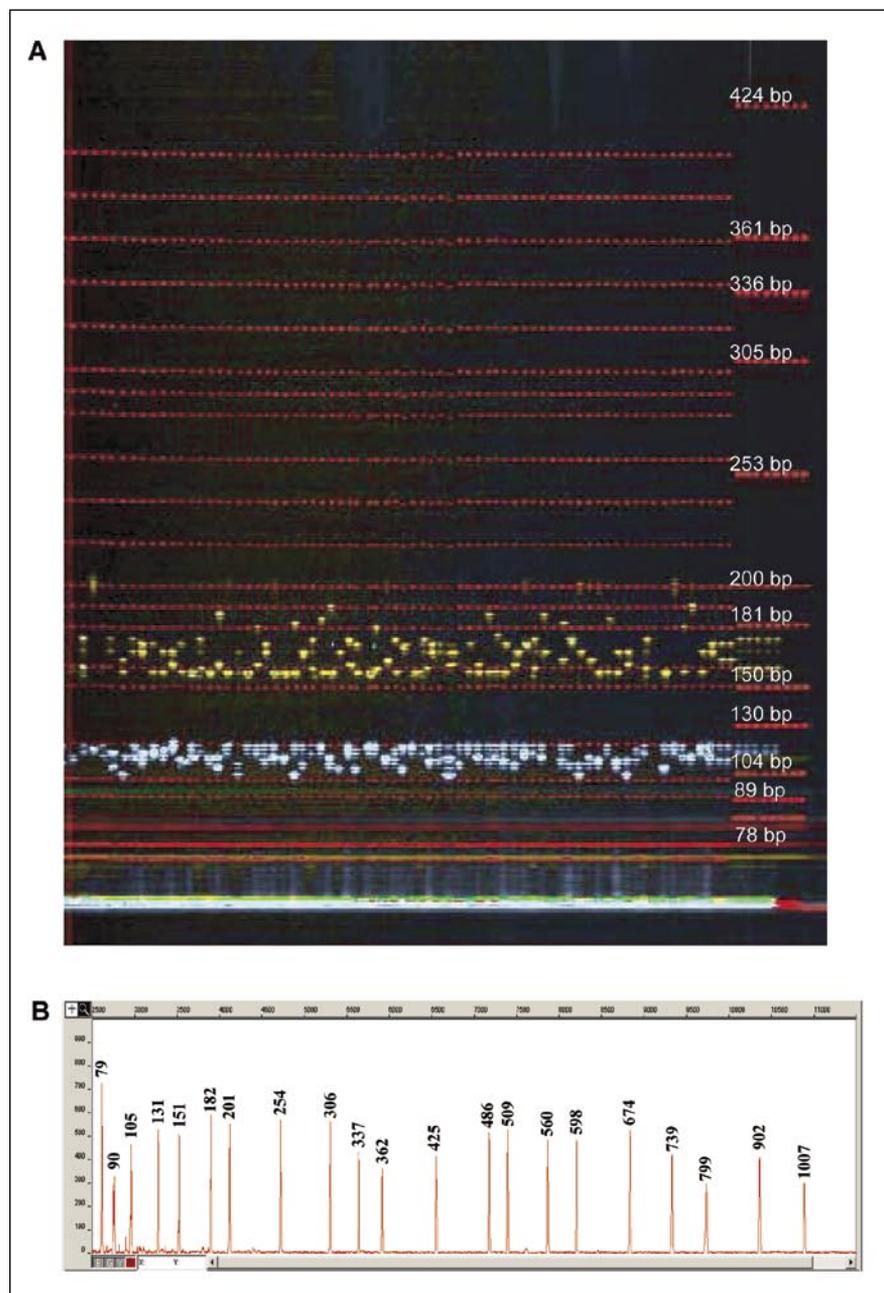


Figure 1. ROX-labeled standards. (A) Gel image from an ABI PRISM 377 DNA Sequencer showing ROX-labeled commercial size standards (from Applied Biosystems) and our ROX-labeled pBluescript (pBS) standard. The pBS size standard in the rightmost lanes appears brighter on this gel, but can easily be diluted to the same concentration as the commercial size standard. (B) Chromatogram of pUC size standard run on an ABI PRISM 3100-Avant Genetic Analyzer with fragment sizes in bases indicated above each peak.

gave a significant doublet, but fragment analysis parameters can be set such that only the larger molecular weight peak is used for size calculation.

Individual amplifications were tested via agarose gel electrophoresis, and PCR products were subsequently combined into a single tube. An aliquot of this mixture was then run on an automated DNA sequencer alongside a known (i.e., commercial) size standard as described earlier (1,2). Our results indicate that plasmid-based size standards are comparable to commercial size standards in their reproducibility, stability, and accuracy (Figure 1). Plasmid-based size standards are also easily customizable for specialized sizing requirements. Furthermore, we estimate their cost to be less than 10% of commercial products.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing interests.

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