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for functional analysis. Mutagenesis to the small promoter-only containing plasmid is preferred to that of the reporter gene construct, because amplification of a smaller plasmid is faster and more efficient and removes the slight possibility of introducing random mutations to the reporter gene. The transferred mutant region can be sequenced to confirm that no random mutations were introduced during amplification.

Each new plasmid is the same size as the parental plasmid and differs only in the sequential 25-nt replacement. Figure 2 details a restriction digest of the first six plasmids in the CMV-LSM series and illustrates the shifting mutation (XhoI cuts upstream of the mutations; MluI is contained in the mutations). In this example, linker length was specified to maintain the relative distances and topology between other sequences in the enhancer/promoter, but it is not necessary to do so. Thus, the power of this technique lies in the wide variety of mutants that can be generated. Using only one template, a relatively small series of primers and as many linkers as desired, literally hundreds of mutants, differing in size, location and content, can be generated with a single set of PCRs and ligations.

REFERENCES


Address correspondence to Dr. Kerry M. Barnhart, Vical, Inc., 9373 Towne Centre Drive, Suite 100, San Diego, CA 92121, USA. Internet: kbarnhart@vical.com

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Kerry M. Barnhart
Vical, Inc.
San Diego, CA, USA

Increased Efficiency of Arbitrarily Primed PCR by Prolonged Ramp Times

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Arbitrary primers are widely used in several applications of the polymerase chain reaction (PCR), including differential display PCR (6) and the random amplified polymorphic DNA (RAPD) (10). Whereas the first method has been demonstrated to be a powerful tool for the identification of differentially expressed genes, RAPD is often performed for analyzing genetic variabilities by generating a genomic fingerprint. However, due to the low annealing temperatures required for short, unoptimized primers, both resolution and reproducibility of arbitrarily primed PCRs are often low (2,3). To overcome this problem, several improvements have been suggested concerning (i) the temperature profile, (ii) the quality of the DNA template, (iii) the number and length of PCR cycles and (iv) the technical equipment (1,7).

With respect to the ramp time between the annealing and the extension temperature, different observations have been made. Lawrence et al. (5) reported a benefit for the reproducibility of RAPD by performing a 1-min ramp, whereas Benter et al. (1) observed no general influence on the reproducibility of RAPD by prolonged ramp times but an increase in number and yield of the DNA bands obtained. A similar effect on the band pattern obtained was found for RAPD by introducing a transition interval between melting and annealing temperatures (9). Small variations in ramp time (9–40 s) had little or no effect on the resolution and reproducibility of differential display PCR (8).

Here, we demonstrate that drastically prolonged ramp times between the annealing and the extension temperatures (up to several minutes) lead to a profound increase in number and yield of the DNA bands obtained in both methods. The benefit of our observation for differential display PCR lies in the improved detection of low-abundance mRNA species that could other-

Figure 2. Restriction digest of LSM plasmids detailing shifted location of mutant linker. The first six plasmids in the CMV-LSM series were digested with XhoI and MluI and electrophoresed on a 2% agarose TBE gel for 1.5 h at 100 V. Lane M, 100-bp marker (New England Biolabs); lanes 1–6, plasmids 1–6. Expected fragment sizes are 608, 633, 658, 683, 708 and 733 bp, respectively.
wise readily be overlooked. The more complex band pattern that is obtained in RAPD facilitates the identification of intra- and interspecific differences when used for genomic-fingerprint analyses.

In our laboratory, differential display PCR is used for the detection of differences in the hepatic gene expression patterns between SCP2/SCPx-deficient and wild-type mice (4). The isolation of total RNA from murine liver, its reverse transcription (RT) into cDNA and the exact PCR conditions have been described elsewhere (4). PCR amplification of the cDNAs generated with the degenerated anchor primer T\textsubscript{11}-GG was performed with three different arbitrary decamer primers (5\textsuperscript{\prime}-GATCAAGTCC-3\textsuperscript{\prime} and 3\textsuperscript{\prime}-GATCTCAGAC-3\textsuperscript{\prime}) that were obtained from the Differential Display\textsuperscript{\textregistered} Kit (Display Systems, Los Angeles, CA, USA). After an initial denaturation step at 80°C for 2 min, 40 cycles of annealing at 40°C for 1 min, extension at 72°C for 1 min, denaturation at 94°C for 30 s with a final elongation step at 72°C for 10 min were carried out in a thermal cycler with a heatable lid (GeneAmp® PCR System 9600; PE Biosystems, Foster City, CA, USA). PCR products were separated on a 6% denaturing polyacrylamide gel using a genomyxLR DNA Sequencer (Beckman Coulter, München, Germany). Autoradiography was performed for 4 days with Biomax\textsuperscript{\textregistered} MR-1 film (Eastman Kodak, Stuttgart, Germany).

We use the RAPD technique to investigate the genetic variabilities between different isolates of the yeast species Candida glabrata. Therefore, yeast cells of an 18-h culture were incubated with 200 U Lyticase (Sigma-Aldrich GmbH, Deisenhofen, Germany), and genomic DNA of Candida spheroblasts was prepared using the QIAamp\textsuperscript{\textregistered} Tissue Kit (Qiagen GmbH, Hilden, Germany). The PCR amplification of 100 ng genomic DNA was performed in an OmniGene\textsuperscript{\textregistered} Thermal Cycler with a heatable lid (Hybaid, Teddington, England, UK) using the arbitrary decamer primer 5\textsuperscript{\prime}-GATTCAGACC-3\textsuperscript{\prime} and 2.5 U AmpliTaq\textsuperscript{\textregistered} DNA Polymerase (PE Biosystems). PCR conditions were the following: initial denaturation at 94°C for 5 min, annealing at 35°C for 2 min, extension at 72°C for 2 min and denaturation at 94°C for 1 min. After 35 cycles, a final extension step at 72°C for 10 min was performed, and PCR products were separated on a 2% agarose gel containing 1 µg/mL ethidium bromide (EtBr). The standard maximum heating rates of the thermal cyclers used (GeneAmp 9600, 1°C per s; OmniGene, 0.5°C per s) were compared with longer ramp times between the annealing and extension temperatures. Figure 1 shows that the number and yields of cDNA bands obtained by differential display PCR increased remarkably by introducing a 5-min ramp. Whereas a ramp of 2.5 min gave slightly better results (Figure 1), a further increase to 7.5 min had no additional benefit for the band pattern obtained (data not shown). A comparable effect of prolonging ramp times on number and yields of the amplified DNA bands was observed in RAPD except that the best results were obtained with a longer ramp time of 7 min (Figure 2). A further increase in this ramp time to 9 min improved yields of smaller PCR products, whereas yields of longer PCR products were diminished. In accordance with the observation by Lawrence et al. (5), we found that the reproducibility of both methods was improved by prolonging ramp times. As a potential mechanism, we presume that the lower heating rates stabilize the primer/template complexes by avoiding premature detachment of the primer from the template (1). Taken together, we conclude that increasing the ramp time between the annealing and extension temperatures is not a loss of time but rather a simple option for improving the efficiency of arbitrarily primed PCR.

REFERENCES

3. Debouck, C. 1995. Differential display or dif-

![Figure 1. The number and yields of cDNA bands obtained by differential display PCR increase after prolonging ramp times. The cDNAs generated by RT of total RNA from murine liver using the anchor primer T\textsubscript{11}-GG were amplified with the same primer and three different arbitrary decamer primers. Lane 1, 5\textsuperscript{\prime}-GATCAAGTCC-3\textsuperscript{\prime}; lane 2, 5\textsuperscript{\prime}-GATCTCAGAC-3\textsuperscript{\prime} and lane 3, 5\textsuperscript{\prime}-GATCTAAGG-3\textsuperscript{\prime}. The PCR was carried out 3× under identical conditions, except that the ramp times between the annealing and extension temperatures were modified (no ramp, 2.5-min ramp, 5-min ramp).

![Figure 2. Increased efficiency of RAPD by prolonging ramp times. 100 ng of genomic DNA from Candida glabrata were amplified with the arbitrary decamer primer 5\textsuperscript{\prime}-GATTCAGACC-3\textsuperscript{\prime} and PCR products were separated on a 2% agarose gel containing 1 µg/mL EtBr. The PCR products were performed under identical conditions with four different ramp times. Lane 1, DNA size marker; lane 2, no ramp; lane 3, 5-min ramp; lane 4, 7-min ramp; lane 5, 9-min ramp.](image-url)
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Address correspondence to Dr. Udo Seedorf, Institut für Arterioskleroseforschung, Domagkstr. 3, 48149 Münster, Germany. Internet: seedorfu@uni-muenster.de

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P Ellinghaus, D. Badehorn, R. Blümer, K. Becker and U. Seedorf
Westfälische Wilhelms-Universität Münster
Münster, Germany

Retrieval of Flanking DNA Using a PCR-Based Approach with Restriction Enzyme-Digested Genomic DNA Template

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Molecular analyses are often facilitated by efficient retrieval of DNA fragments immediately adjacent to known sequence. An early breakthrough for amplification of such DNA was the development of single-specific-primer polymerase chain reaction (SSP-PCR) (5), in which total genomic DNA is ligated to a vector, and a vector-specific primer is used together with a gene-specific primer to amplify a fragment contiguous to known sequence. A modification of this procedure involves