Method to Improve Reliability of Random-Amplified Polymorphic DNA Markers

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Random-amplified polymorphic DNA (RAPD) markers (8) are a powerful tool for genome analysis (7). Nevertheless, the difficulties to achieve a high pattern repeatability represent a major drawback for the routine implementation of RAPD markers. Amplification products obtained depend on various factors, such as the concentration of the reaction mixture constituents, the source of *Taq* DNA polymerase, the thermal cycling program, the brand and model of thermal cycler and the target DNA used (2,5,6,9). A method, designated "touchdown" polymerase chain reaction (PCR) (3), has been proposed to circumvent spurious priming during specific PCR amplifications by decreasing the annealing temperature in every second cycle until the calculated T_m of the primers is reached. On the other hand, it seems logical to assume that the less reproducible the RAPD bands are, the lower the homology to the sequence of the primer will be. To improve

RAPD reliability, we have tested several programs with different touchdown annealing temperatures (46°–36°C, 50°–40°C, 55°–45°C and 57°–47°C). Amplifications were carried out using three different thermal cyclers (GeneAmp® PCR System 9600 from Perkin-Elmer, Norwalk, CT; PTC-100™ Programmable Thermal Controller with and without Hot Bonnet from MJ Research, Watertown, MA, USA). Sixty 10-mer primers (Kits A, C and S; Operon Technologies, Alameda, CA, USA) were used in this study. The PCR was done in 25 µL final volume containing 10–400 ng of template DNA, 100 µM of each dNTP (Perkin-Elmer), 200 µM primer, 3 mM MgCl₂ and 2 U of Stoffel fragment (Perkin-Elmer) in 1× reaction buffer (100 mM Tris-HCl, 100 mM KCl, pH 8.3). DNA was extracted from *Barbus sclateri*, *Mus musculus*, *Quercus suber*, *Rattus norvegicus* and *Saccharomyces cerevisiae* with two different extraction methods (1,4).

We found that the maximum temperature range that yields amplification is 55°–45°C. Furthermore, we tested several thermal cycling programs that combined different lengths of the amplification steps (denaturation step: 1 min, 30 s, 15 s; annealing step: 1 min, 30 s; extension step: 6 min, 4.5 min, 2 min). We have found that the denaturation step can be reduced from 1 min to 30 s without any changes in the profiles. Regarding the extension step, shorter extension steps than 4.5 min determine a very low amplification efficiency that mainly affects the longest

fragments. In light of these results, we finally decided to use the following thermal cycling program: a preliminary step of 2 min at 94°C; 10 cycles of 30 s at 94°C, a ramp of 1.5°/s to reach annealing temperature, 1 min at 55°C (decreasing 1° per cycle to 46°C), a ramp of 1.5 min to reach 72°C and 4.5 min at 72°C; 25 cycles of 30 s at 94°C, a ramp of 1.5°/s to reach annealing temperature, 1 min at 45°C, a ramp of 1.5 min to reach 72°C and 4.5 min at 72°C; a final extension step of 1 min at 72°C.

The key modification introduced by this cycling profile is the range of annealing temperatures used (55°–45°C), which are notably higher than the temperature commonly used in RAPD amplifications (36°C). Furthermore, we have found that the fixing of the time used in the transition ramps and particularly in the slow transition from the annealing to the extension steps is crucial to reproduce the amplification patterns in different thermal cyclers.

The influence of the target concentration on RAPD reactions (1) represents a drawback to the routine use of these molecular markers. Under the experimental conditions described in this work, this problem was overcome because we did not find any differences in the RAPD amplification patterns obtained when template concentrations ranging from 10–400 ng per amplification reaction were used.

We tested the reproducibility of the RAPD profiles obtained with different materials in three different thermal cyclers and in two laboratories that do not

Figure 1. Comparison of the RAPD patterns obtained in two different thermal cyclers (A: PTC-100 without Hot Bonnet and B: Perkin-Elmer 9600) located in different laboratories. Lanes 1 and 2, 3 and 4, 5 and 6, 7 and 8, and 9 and 10 contain the DNA profiles generated from two different strains of *S. cerevisiae* amplified with primers OPC-01, -02, -03, -04 and -05, respectively. Lane m is the size marker (*Hae*III digests of ϕ x174).

Figure 2. Consistency of the RAPD banding patterns obtained from different organisms in two different thermal cyclers (A: PTC-100 with Hot Bonnet and B: Perkin-Elmer 9600) located in different laboratories. DNA profiles generated from different organisms with primer OpS18 are shown on lanes 1–8 as follows: lanes 1–4: 4 different genotypes of *Q. suber*; lane 5: *M. musculus*; lane 6: *R. norvegicus*; lane 7: *B. sclateri*; lane 8: *S. cerevisiae*. Lane m is the size marker (*Hae*III digests of ϕ x174).

Benchmarks

share any components involved in the RAPD methodology. Figures 1 and 2 show the repeatability of the RAPD patterns obtained using several conditions that have been reported to be a source of artifactual variation on RAPD profiles. Our results indicate that the patterns obtained are nearly identical. Therefore, we propose the application of this protocol to greatly enhance the use of RAPD methodology in programs where the results from different laboratories have to be shared.

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Francisco J. Gallego and Isabel Martínez
Servicio de Investigación Agraria de la Comunidad Autónoma de Madrid
Madrid, Spain