

OBTAINING REPRODUCIBLE PATTERNS FOR RAPD DNA FINGERPRINTING AS AN AID TO GERMPLASM EVALUATION

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SUMMARY

The use of single random primers for DNA fingerprinting is very sensitive to variation in procedures. Several factors that affect reproducibility are examined. The stability of a RAPDs stock (TAQ, MgCl₂, 10X buffer, dNTPs) was found to be very good (4 d, 60 d, @ 22°C). Interim storage of DNA at 4°C was found to be a source of variability. Variability due to varying amounts of polysaccharide inhibitors in the DNA were minimized by diluting the DNA to about 0.25 ng/rxn (12.5 ml). The effects of glove powder, corn starch, and talcum powder were examined but only glove powder was found to inhibit RAPDs PCR. Inadequate mixing of TAQ and other components is the largest single factor causing PCR failures. Other sources of variability are discussed and remedies suggested.

INTRODUCTION

RAPDs (Random Amplified Polymorphic DNAs) is a PCR technique that generates DNA fingerprints using a single oligonucleotide primer. The polymorphisms observed may result from point mutations, insertions, deletions, and inversions (Williams et al., 1990). RAPDs are usually dominant markers and are inherited in a simple Mendelian fashion. In comparison with RFLP, the procedure is less expensive, faster, requires a smaller amount of DNA (0.1–0.5 ng), does not involve the use of radioisotopes, and requires less skill to perform. Because of these advantages, RAPDs have proven useful in genotype identification and gene mapping as well as evolutionary studies.

However, obtaining reproducible RAPD patterns does present a problem in many labs. In fact, Penner et al. (1993) have investigated reproducibility in RAPDs using the same target DNA and primers in different laboratories. They found most RAPD markers to be reproducible with differences between PCR machines accounting for most of the differences.

Yu and Pauls (1992) explored various PCR programming details to optimize the reactions for RAPDs production. Levi, Rowland, and Hartung (1993) evaluated a range

of concentrations of Triton X-100, gelatin, dNTPs, primer, template DNA, TAQ, $MgCl_2$, as well as various times for annealing, elongation, and denaturation. More recently, Bielawski, Noack, and Pumo (1995) examined changes in protocols to obtain reproducible RAPD markers in vertebrate DNA (striped bass, plus a few other vertebrate species). In general, they found that using 30-sec denaturing and 30-sec annealing times, coupled with the addition of a single-strand binding protein, Gp32, to the reaction mixture, prevented nonspecific primer annealing during preparation of the reaction.

Our experiences indicate that one may have difficulty obtaining reproducible RAPDs, even when using a single PCR machine. In this paper we examine several factors that cause difficulty and present some solutions to these problems.

MATERIALS AND METHODS

Leaves were obtained from native plants (species, collection number): *Juniperus ashei* Buch. (Adams 7433, 7473), *J. flaccida* Schlecht. var. *flaccida* (Adams 6892), and *Prosopis glandulosa* Torr. (Adams 7375, 7401). Vouchers are on deposit at the Baylor Herbarium (SCRG). DNA was extracted using the hot CTAB protocol (Doyle and Doyle, 1987).

PCR was performed in a volume of 12.5 μ l containing 50 mM KCl, 10 mM Tris-HCl (pH 9), 2 mM $MgCl_2$, 0.01% gelatin and 0.1% Triton x-100, 0.2 mM of each dNTPs, 0.36 μ M primer, 0.25 ng of DNA (except as noted below), and 0.5 unit of Promega TAQ DNA polymerase. The primers used in this study were (5'-3'): #237 CGA CCA GAG C; #250 CGA CAG TCC C; #327 ATA CGG CGT C, from the University of British Columbia. Amplification was performed in a MJ Research Programmable Thermal Cycler. The thermal cycle used was: 94°C (1.5 min) for initial strand separation, then 40 cycles at 38°C (2 min) for annealing, 72°C (2 min) for extension, 91°C (1 min) for subsequent strand separation. Two additional steps were used: 38°C (2 min) and 72°C (5 min) for final extension. Amplification products were analyzed by electrophoresis in 1.5% agarose (Sigma) gels and detected by staining with ethidium bromide.

Stability of RAPD stock

In order to determine how stable the RAPD stock was, the RAPD stock was stored at 22°C and used after 4 days and again after 60 days. In addition, a complete PCR RAPD mixture (stock, DNA, primer) was stored at 22°C for 4 days before running the PCR.

Stability of DNA stored at 4°C

We usually make up a dilute stock of DNA (0.1 ng/ml). Because we are running so many PCR reactions, we began to keep the DNA stocks in the refrigerator. After a few months, we noticed the loss of many bands. When we made up a new dilute stock (from DNA stored at -20°C), the banding pattern was restored. To investigate the stability of dilute DNA, samples were stored at 4°C and -20°C, and samples run after 5 to 8 months. In addition, DNA samples were thawed every day, PCR was run, and the sample re-frozen each day for 60 days, to investigate the effects of freeze/thaw on PCR.

Dilution of inhibitors by diluting genomic DNA

Many plant species produce considerable amounts of polysaccharides that are inhibitory to RAPDs PCR (Pandey, et al. 1996). *Juniperus flaccida* var. *flaccida* is such a species. PCR was performed using primer 237 with various amounts of this DNA: 1 ng/rxn, 0.5 ng/ rxn, 0.25 ng/rxn, and 0.125 ng/rxn to examine these effects.

Effects of glove powder, corn starch, and talcum powder on PCR-RAPDs

We had experienced some PCR failures and wondered if the powder on the gloves might be inhibitory to PCR. So, we washed 20 small gloves (Fisher) with 200 ml of sterile distilled water (only the outer surface of the gloves) and created a suspension with a stirrer. The suspension was centrifuged and yielded 49.12 mg/glove. The dried powder from the gloves was mixed with sterile water at 30°C. Because corn starch and talcum powder are often used on gloves, commercial sources of these were mixed with sterile water in concentrations of 800, 400, 200, and 100 ng/ml. Then 1 ml was added to the PCR rxn containing 0.25 ng of *P. glandulosa* DNA (7375) and primer 327.

RESULTS AND DISCUSSION

Because errors in pipetting are frequent, it was felt that these could be minimized by preparing a large quantity of RAPD stock (ddwater, MgCl₂, 10x buffer, dNTPs, and TAQ). However, to our knowledge, no one has examined the stability of this kind of mixture. To examine the stability of a RAPD stock, we stored a stock at 22°C to quicken the rate of chemical reactions (we had not seen any changes in PCR-RAPDs using RAPD stock stored at 4°C for several weeks). Figure 1 shows that the RAPD stock is unaffected after 4 days at 22°C. It was also unaffected after 2 weeks

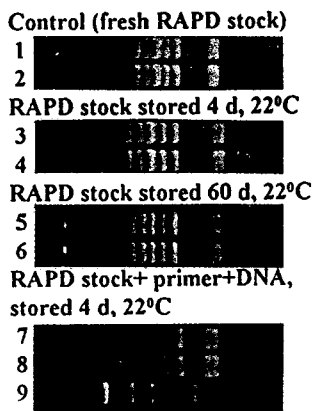


Figure 1. Effects of storage on RAPD stock stability. PCR using primer 250 and *Prosopis glandulosa* (mesquite) DNA: Lanes 1,2: fresh RAPD stock; Lanes 3,4: RAPD stock stored 4 days at 22°C; Lanes 5,6: RAPD stock stored 60 days at 22°C; Lanes 7,8: RAPD stock + primer 250 + mesquite DNA stored for 4 days at 22°C; Lane 9: pGEM DNA size markers.

(data not shown). However, after 60 days at 22°C, there is a noticeable decrease in band brightness, but the pattern and relative intensity of the bands is very similar to the control. Apparently the RAPD stock is very stable (particularly if stored in a refrigerator). However, when the primer and target DNA were included in the mixture, it was not stable after 4 days at 22°C (Figure 1).

Due to the reproducibility problems we encountered when storing DNA in the refrigerator (for ease of access), we did a systematic investigation of DNA storage temperature vs. PCR-RAPDs. Figure 2 shows the triplicate analyses of DNAs from *P. glandulosa* and *J. ashei* stored frozen (-20°C) vs. refrigerated (4°C). Notice that in every case, the frozen DNA yielded reproducible bands, whereas the refrigerated DNAs (from 5 to 8 mos.) showed considerable variability among the bands and a general loss of the higher molecular weight bands. Clearly, if one is using template DNA for several months, one should keep it frozen. However, we wondered if several freeze-thaw cycles might also effect the PCR-RAPDs. But after 60 days of freeze-thawing, we found no effects on the RAPD pattern (data not shown).

We have previously shown that polysaccharides can be inhibitory to PCR and RAPD banding (Pandey et al., 1996). It is likely that other inhibitors such as proteins may be in the DNA extract. Although several methods have been proposed to eliminate inhibitors, a general method is unlikely to be found that will work on all plants (or organisms). *Juniperus flaccida* var. *flaccida* is a species that produces PCR inhibitors. As a practical matter, we examined the potential of using dilution of the DNA to dilute the inhibitor's effects. It is clear (Figure 3) that 1 ng of DNA/rxn is completely inhibitory for primer 237. At 0.5 ng/ rxn the banding appears, but the highest

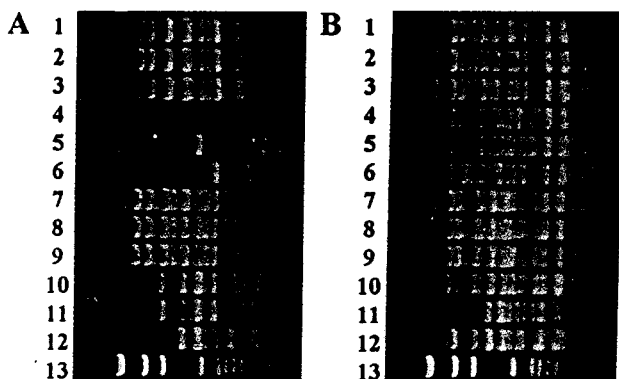


Figure 2. Comparisons of RAPDs (primer 327) obtained from frozen DNA vs. refrigerated DNA (4°C). Panel A. Lanes 1-6: *P. glandulosa* (7401) DNA; Lanes 1-3: frozen DNA; Lanes 4-6: DNA stored for 8 mos. at 4°C; Lanes 7-12: *P. glandulosa* (7375) DNA; Lanes 7-9: frozen DNA; Lanes 10-12: DNA stored for 8 mos. at 4°C. Panel B. Lanes 1-6: *J. ashei* (7473) DNA; Lanes 1-3: frozen DNA; Lanes 4-6: DNA stored for 5 mos. at 4°C; Lanes 7-12: *J. ashei* (7433) DNA; Lanes 7-9: frozen DNA; Lanes 10-12: DNA stored for 8 mos. at 4°C; Lane 13: pGEM markers.

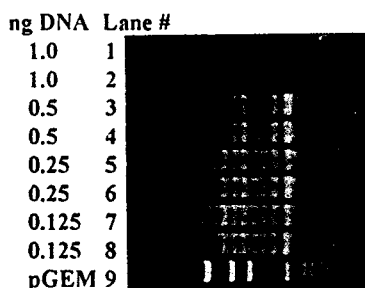


Figure 3. Effects of the dilution of DNA from *Juniperus flaccida* (6892) on the inhibition of PCR (for primer 237). Lanes 1,2: 1 ng/rxn; Lanes 3,4: 0.5 ng/rxn; Lanes 5,6: 0.25 ng/rxn; Lanes 7,8: 0.125 ng/rxn; Lane 9: pGEM DNA size markers.

molecular weight band is missing (we have found that the loss of HMW bands is an excellent indicator of the presence of inhibitors!). The banding is fully restored at 0.25 ng/ rxn and 0.125 ng/ rxn (Figure 3).

Recently, we experienced considerable problems in obtaining reproducible RAPD bands that seemed to be associated with a shipment of new gloves. Just dusting a glove over a PCR resulted in inhibition. So we did a systematic examination of this effect. Figure 4 shows that the addition of 800 ng of glove powder to the PCR-RAPD rxn (with primer 327) completely inhibits the reaction. One can see a progressive increase in PCR amplification as the glove powder is decreased to 400, 200, and 100 ng/rxn (Fig. 4). We now routinely wash our gloves after we put them on. After several calls to the supplier, we were assured that the powder was corn starch. We suspected it might be talcum powder. Analyses using up to 1500 ng/rxn showed both corn starch and talcum powder to not be inhibitory. The active agent in the glove powder is still unknown.

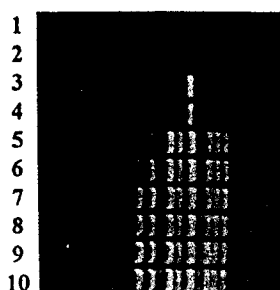


Figure 4. Inhibition of PCR and RAPD profiles by glove powder using *P. glandulosa* DNA (7375) and primer 237. Lanes 1-2: 800 ng of powder/rxn; Lanes 3,4: 400 ng of powder/rxn; Lanes 5,6: 200 ng of powder/rxn; Lanes 7,8: Control, no powder; Lane 9: pGEM DNA size markers.

Lab techniques for obtaining reproducible RAPD amplifications

1. Vortex all reagents and DNA that has been frozen. This is critical for the Mg stock, as it tends to precipitate upon freezing/thawing. It is also critical for DNA and TAQ. The TAQ is normally shipped in 40% glycerol and is very difficult to mix uniformly. Mixing is a problem with all components (e.g., primers, DNA, dNTPs, TAQ, Mg, 10X buffer, etc.). We now vortex all the components and then go back and vortex all the PCR tubes again before adding oil. This additional vortexing (mixing) step has virtually eliminated PCR failures.
2. If you use powdered gloves, wash the powder from the outside of your gloves with DI water.
3. Prepare an entire tube of TAQ for your RAPD stock (don't add a primer or DNA). Storing the RAPD stock at 4°C is okay for several weeks. Freeze the RAPD stock if it is to be left for more than one month. Centrifuge the TAQ tube before adding any components to get the TAQ near the bottom of the tube. Centrifuge the tube again after all components (ddwater, MgCl₂, 10X buffer, dNTPS) have been added. Vortex to get the components well mixed, then centrifuge (but only for a pulse). Using a large RAPD stock solution will reduce errors and decrease variability in your RAPD analyses.
4. Make up working stocks of your DNAs (ex. 0.1 ng/ml). Store both the original DNAs and your working stocks in the freezer (-20°C). Thaw the working DNA stocks prior to making up the PCR reactions and then return the working DNA stocks back to the freezer.
5. When extracting DNA and making dilutions, bring the DNA into solution in sterile, ddwater not TE buffer. The TE in the EDTA will chelate with Mg and lower the concentration of Mg in the PCR reaction. This lower concentration of Mg will cause either a failure to obtain any bands, or the production of only a few, faint bands.
6. Perform a concentration test on your DNA with a good primer. Run your DNA at the lowest concentration possible where you still get good bright bands. For our system, 0.25 ng of DNA/12.5 ml PCR reaction (0.3 ng/15 ml) has effectively eliminated problems with indigenous inhibitors.
7. Although Proteinase K is listed for many protocols, we have found that one can substitute Pronase E and it costs only about 5% the cost of Proteinase K. Proteinase is not often included in most plant DNA extraction protocols, but we have found it to be essential for the extraction of alcohol-preserved specimens (Flournoy, et al. 1996).
8. Try different methods for grinding materials. We have found, for most plants, that grinding fresh leaves in hot CTAB (60°C) resulted in higher yields and higher molecular weight DNA than grinding the leaves in liquid nitrogen and then placing the ground material in hot CTAB. However, recently, we found that for *Vetiveria*, a tropical grass, the DNA yield was very small, and it was almost completely degraded when either fresh or dried leaves were ground in hot CTAB. In contrast, the DNA was of good quantity and quality when we ground either fresh or dried leaves in liquid nitrogen, then added the hot CTAB and vortexed (to get a good mixing of the CTAB and plant materials).

For rice leaves, we have found that SDS worked well, but CTAB did not work well. So try different surfactants if necessary.

9. Check the temperature in each well at your maximum temperature (94°C) and the lowest temperature (37–38°C). You may find some cool spots around the margin of the heating block. If so, don't use these areas.

10. Get a linear chart recorder and monitor the temperature pattern for each run. This will enable you to have a record of the performance of your temperature cycler every day. This saves lots of second guessing. We place a copy of the temperature profile in the lab book with each PCR analysis.

11. Make up the RAPD stock and do the DNA transfers in a laminar flow hood. This reduces contamination.

12. Make up enough RAPD stock + the primer for the entire set of samples to be run in the current study. Then pipette the necessary amount of this 'master mix' into each PCR tube, then add the different DNAs used in the study. This will ensure very uniform application the TAQ and primers to each PCR tube as one usually uses from 6 to 9 ml from the 'master mix'; this volume is easy to pipette accurately.

13. After loading all components into PCR tube for a RAPD run, it is important to make sure all the mixture is in the bottom of the tube (pulse centrifuge in a mini-centrifuge), then vortex 10 sec to give a uniform mixture. The TAQ tends to settle on the bottom and it is critical that the TAQ be well dispersed in the solution. Then pulse centrifuge the mixture to get all the solution to the bottom of the tube (check to see if any air bubbles are in the tube. Tap tube on a table until all the bubbles are gone, then centrifuge).

14. Add a drop of oil to the holes in the PCR machine. When you place the PCR tube in a hole, a little oil should come out around the tube.

15. Be sure tubes fit down securely in the holes in the PCR machine. Press each tube firmly into the well.

16. There seems to be no great hurry to run the PCR, leaving the completed RAPD tube at RT for a few hours has no effect. However, it should be noted that there are major effects after 4 days (see Figure 1).

17. Double-check the PCR tube caps to be sure that the lids are completely closed.

18. Evaluate PCR tubes from several suppliers, using the same DNA, primer(s), TAQ, etc. Use the PCR tubes that work best for your PCR machine and lab conditions.

19. Screen lots of primers. Using a Mg concentration of 2mM, we have found about 25% of random sequence primers give no bands, about 50% give a few bands, and about 25% produce 5 or more bands. Of these best 25%, about 1/4 will be really good. So out of 100 primers we expect to find 5 to 10 really good primers. Interestingly, we have

generally found these primers to be useful for all species we have studied (ranging from gymnosperms to monocots and dicots). We don't find it worth our time to try to optimize the PCR (Mg concentration particularly) for every possible primer.

20. Some primers are more useful at the specific level, whereas other primers are more useful to characterize differences between individuals. Screen until you find primers that will do what you need to do.

21. Place a piece of plastic (1/16" thick) on top of a horizontal gel to help keep the bands running straighter. The bands run a bit farther and are better resolved.

22. We have found no difference in the luminance when loading 5 ml vs. 10 ml of DNA (vol. includes loading buffer). In our gels, 10 ml gives a vertical band height of about 5 mm, whereas 5 ml gives a vertical band height of about 2.5 mm. We have found the luminance to be almost the same. So we now make our 1.5% gels, 4.33 mm thick and load 4 ml of PCR product plus 1 ml of loading buffer. This saves on agarose and the gels seem to run straighter. With these smaller sample sizes, we can get 3 gel runs from one 15 ml PCR reaction. This helps when we have a failure in one of the tubes, as we can make a new run on that DNA, then use it with the current tubes to make a new gel the next day.

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