

PCR and electrophoresis using *B. rapa* FPsc genetic markers to document segregation patterns of polymorphic molecular alleles

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All of the polymorphic markers that have been developed and placed on the *B. rapa* genetic map (by using a wt FPsc x R500 F2 population) amplify robustly through a simple and rapid thermocycling profile that we call “54 thirty thirty-five”: 54 degr annealing temp, 30 seconds for all cycling steps, thirty-five cycles total. The PCR products reliably discriminate among the three possible genotypes (the two parental homozygotes and the FPsc/R500 heterozygote) by fractionation on a 2% agarose gel and staining with ethidium bromide. There are two ‘tweaks’ that we exploited/developed and use routinely to speed things along. First, we use Cresol Red as a visual indicator to track migration of PCR products during electrophoresis; surprisingly enough, it can be added to the PCR reaction cocktail along with sucrose (the latter provides density to ensure that the samples later settle into the wells of the gel) with no ill effects on product yield. This saves a bit of time and manipulation between PCR and gel separation of products. Second, we use a simple Sodium borate (NaB) electrolyte solution in gels and running buffer (Brody and Kern (2004) *BioTechniques*, **36**:214-216). The NaB buffer allows you to run gels at relatively high (~250 V) current/voltage without overheating or distortion of banding patterns and thereby shortens considerably the time needed for product separation (just 45-75 min after loading in most cases).

We describe elsewhere a useful and non-toxic gDNA extraction protocol that your students can use to obtain template genomic DNAs (gDNAs) for use in PCRs. As indicated in that document (“gDNA Extraction Protocol”), ~0.1 cm³ of inflorescence or leaf tissue can yield sufficient gDNA to visualize on a gel and can be diluted >10-fold and still provide enough template DNA to support hundreds of PCRs. Alternative gDNA procedures can certainly be used, but the description provided here assumes that our “standard” extraction protocol was used to provide gDNA templates; your results may vary.

PCR: Reaction conditions for PCR are specified in the following but merit a bit of further comment/explanation before we get down to the details. First, we have used the cheapest commercial Taq polymerase we could find (GenScript brand, <http://www.genscript.com>) that provides robust amplification of PCR targets. Although we have not systematically surveyed the performance of alternatives, there is little reason to think that using polymerase and buffer obtained from different suppliers would yield different results. Second, we order our oligonucleotides from Integrated DNA Technologies (<http://www.idtdna.com>) in the form of de-salted and lyophilized pellets. Our students resuspend the oligos in TE (pH 7.9-8) to 100 uM final concentration to prepare archival stocks. Small aliquots of those stocks should be diluted 50-fold into H₂O to prepare working stocks (if used for genotyping, both the forward and reverse primers are included in the working stock solution at 2 uM final concentration of each oligo). The 100 uM archival stocks are stored at -80 degr, the working stocks in our -20 degr freezer. If you don’t have access to a -80 degr freezer, just use the coldest freezer you can find. As with the PCR enzyme and reagents, alternative oligo vendors will likely work just as well;

we're just describing how we do assays at the UW-Madison. Finally, we'd like to offer a few suggestions about how you might go about setting-up your PCRs:

1. Thaw all reaction components (except Taq polymerase) on ice; flick or vortex each reagent to distribute component compounds evenly before taking any aliquots.
2. Have the thermocycler reserved, programmed, and primed to start before you begin to assemble reactions so that it's a quick transition from reaction assembly to thermocycling.
3. Label and arrange thin-wall PCR tubes in a holder embedded in an ice bucket (*i.e.*, so that the tubes themselves are surrounded by ice but no crystals will be apt to accidentally drop into them). 200 ul micropipette tip holders in standard 8 x 12 96-cell format work well for this purpose. We use strip tubes (8x) to minimize the number of "reaches" that must be made to assemble reactions. Be sure to have the necessary number of caps at the ready before you begin.
4. In a typical experiment in which marker oligo primer pairs are used to interrogate the genotypes among gDNAs extracted individual F2 segregants, all reaction components are in common except the gDNA templates, and so we make a master mix (MM) cocktail containing all of the common reagents and add it to gDNAs previously distributed to tubes and held on ice. This minimizes the number of error-prone pipetting steps and speeds reaction assembly. The MM recipe is obtained simply by multiplying the volume of components contained in individual reactions by the total number of reactions to be assembled. It is useful to prepare a bit more of the MM than strictly required in order to avoid running short for those last few tubes.
5. Most thermocyclers provide a heated lid to prevent condensation/evaporation of PCR reaction components during the thermocycling run. If your instrument does not, you may need to overlay the assembled reactions with molecular biology-grade mineral oil to achieve the same ends.

Overview: Write the PCR protocol and recipes in advance, gather and thaw all components. Distribute gDNA template samples to PCR tubes; prepare MM, add to gDNAs, cap tubes and start the thermocycling run.

Thermocycling routine (programmed in advance):

- 2 min 94 degr, then 35 cycles consisting of
 - 30 sec 92 degr (denaturation)
 - 30 sec 54 degr (annealing)
 - 30 sec 72 degr (extension)
- Finish with 5 min, 72 degr, hold at 10 degr or in refrigerator thereafter.

Reaction recipe (enough, in principle, for 50 rxns but we're only aiming to assemble 48):

<u>Each 20 ul reaction will contain</u>	<u>Volume in 50x MM*</u>
• 2 ul gDNA template	--
• 2 ul 10x Taq buffer (with Mg ⁺⁺ at 15 mM)	100 ul (3)
• 1.6 ul 250 uM dNTPs	80 ul (5)
• 2 ul of 2 uM primer stock solution (containing both fwd and rev oligos)	100 ul (4)

- 0.25 ul Taq polymerase 12.5 ul (6)
- 2 ul of 10x Cresol Red/sucrose loading buffer** 100 ul (2)
- 10.15 ul H₂O. 507.5 ul (1)

* Numbers in parentheses indicate order of addition, from least to most expensive/labile.

** 10x Cresol Red loading buffer is 60% sucrose, ~1 mM dye (just enough to visualize the gel migration front).

Thaw and gently mix all reagents, including diluted gDNA extracts but excepting Taq polymerase), store on ice.

Dilute gDNAs prepared by the accompanying gDNA extraction protocol to 1/10 - 1/20x using H₂O and 0.2 ml strip tubes labeled accordingly. Because you may be using these samples repeatedly in assays with different markers, it is helpful to make a working stock in a volume in excess of that needed for a single experiment. Thus, transfer 5 ul of each gDNA prep to the corresponding 0.2 ml tube and then add 95 ul of deionized H₂O to each, expelling the water vigorously to thoroughly mix the working gDNA solution. Distribute 2 ul aliquots of diluted gDNAs to their respective PCR tubes (also on ice), return archival and working-stock gDNA preps to -20 degr freezer.

Assemble the MM as written and in the order indicated above. **LEAVE THE TAQ POLYMERASE IN THE -20 DEGR FREEZER until all other components of the MM have been combined and mixed.** Despite the fact that the enzyme will spend the next 2 hours or so between temps of 54 and 94 degr, it is best practice to assume that all biological reagents are fragile and thus perishable goods. Carry the MM to the freezer with P20 micropipette set at the appropriate volume in hand (with a clean tip, of course); take the volume of Taq polymerase needed and add immediately to the MM, return Taq to -20 degr storage.

Vortex or otherwise mix the MM to distribute the Taq enzyme uniformly throughout the MM. Use a P20 micropipette to distribute 18 ul aliquots to tubes containing gDNAs. Provided that it does not contact any gDNA sample, a single tip can be used to assemble all reactions and a vigorous 'expel' will ensure that all components are well mixed.

Cap tubes securely, immediately place in thermocycler, and start the run. Finished reactions can be stored in a refrigerator or -20 degr. Freezer until you're ready to run a gel, just be sure to spin the tubes or otherwise make sure all contents are at the bottom before loading samples.

Gel electrophoresis

-Prepare 1 liter of 20x NaB buffer solution:

- 8 gm NaOH
- 45 gm Boric acid
- 800 ml dH₂O

Stir to dissolve, adjust volume to 1 l and store in screw-cap bottle at room temperature.

-2% agarose gel: 2 gm electrophoresis-grade agarose/100 ml final volume, 1/20 final volume of 20x NaB buffer, H₂O as needed. Combine all in a flask with capacity 3-5x the final volume and add a magnetic stir bar. Stir on a plate at room temp for 5-10 minutes to allow agarose particles to hydrate. Place in microwave oven, heat at highest setting for 1-3 minutes, or until the gel solution has warmed to an uncomfortable (but not yet boiling) temperature (and yes, as long as it is submerged in liquid, the metal stir bar will get really hot, but it's not going to destroy your microwave oven). Return flask to the stir plate, mix ~5 minutes and then microwave until boiling for ~1 minute or until inspection indicates that the gel solution is entirely clear (*i.e.*, no undissolved agarose particles). Allow to cool with stirring until ~50 degr (cool enough that you can place your hands on the flask and hold them there for a 5-count). Add 10 mg/ml ethidium bromide solution (in water) to 0.5 ug/ml final concentration, continue stirring to disperse, and then cast the gel.

-Running buffer: Dilute 20x NaB stock to 1x concentration (using water) in a volume sufficient to submerge the solidified gel by 2-3 mm.

-Load PCR samples: Because the Cresol Red/sucrose loading buffer is included in the PCR MM recipe, PCRs (15-20 ul each) can be loaded directly into the wells of the agarose gel. Be sure to include a MW marker whose bands cover the 75-500 bp size range (our markers typically yield products 100-400 bp in length).

-Attach the electrodes of the gel apparatus to a continuous voltage power supply, set voltage to 250 V (or otherwise as high as your equipment allows). The Cresol Red dye migrates well in advance of any PCR or MW marker bands (comparable to the rate at which 50 bp DNA fragments might travel), so let the run continue until the dye front has moved ~5 cm towards the cathode (red) end of the gel. Alternatively, since ethidium bromide is included in the gel, you can monitor progress towards separation of polymorphic products by periodic viewing on a suitable UV transilluminator.

Special note: Agarose gels can be recycled to save time and money. Once you and your students have documented gel images, simply slice the gel into manageable pieces and store in a flask suitable for microwaving at a future time (include a magnetic stir bar and use, *e.g.*, parafilm to seal the flask). The gel can be re-melted by microwaving until thoroughly liquefied, cooled as above, and used to cast a new gel. We routinely re-use gels 3-4 times; it may be helpful to add back a little 1x NaB buffer to replace volume lost during repeated melting cycles.

PLEASE BE CAREFUL TO USE ALL PRECAUTIONS NECESSARY TO AVOID CONTACT WITH ETHIDIUM BROMIDE. This includes gloves when handling gels directly, a catch-tray vessel to collect drips of running buffer contaminated by ethidium that has leached out of the gel, and measures (a respirator mask and/or common sense) to avoid exposure to super-heated vapors that may carry the dye when melting agarose gel ingredients.

