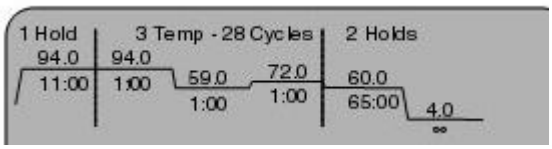
Molecular Markers in Chestnut

Introduction: we are using bits of DNA as the markers to say whether two types of chestnut tree are the same. Individual trees of the same species will be unique in some locations, so the type of marker we select depends on the question being asked. Here, we want to have lots of markers, so that some will be the same and some will be different, giving a way to estimate the relationship. We will use a Randomly Amplified Polymorphic DNA (RAPD) procedure that adds a short piece of DNA (only 10 bases long) as a primer to the PCR reaction. Because it is short, we won't use a very high temperature for the annealing reaction, then very gradually increase it to the temperature that the Taq polymerase enzyme prefers, 72C. For the restriction digestions we used a regular heating block that kept the reaction at one temperature. Because PCR requires that we melt the two strands of DNA apart with high heat and then quickly cool the mixture to the temperature where the 10-mer can bind the DNA and then slowly heat ('ramp') to the temperature that Taq polymerase likes best, we use a specialized heat block called a thermocycler. The pattern shown in the picture is the cycling profile – we program this into the instrument before we put in the samples. Note that if we do 30 cycles this will take ~3 hours. Since we don't want to wait to take the samples out, the instrument can cool to 4C and hold that temperature indefinitely, so the samples will be safe until the following morning.



The RAPD primers serve as both forward and reverse primers, so you only need to add one primer per reaction.

Each team will set up 7 PCR reactions, using the chestnut DNA and 6 different RAPD primers, along with one control. The RAPD primers should each give 8-10 bands on an agarose gel . You can run them separately, but you can also combine them into one lane on the gel, since you do not really care which primer gives which pattern so long as the total is informative.

PCR reactions are usually run in small (200ul) thin-walled tubes – thin so that the heat transfers quickly. Normally you do not put more that 50ul into one of these tubes (we are using 25ul). Since you are heating the reaction almost to boiling, the solution should evaporate to the top of the tube. However the lid is heated to just a little more than the base, which forces anything that evaporates to move even faster in the opposite direction (down to the bottom of the tube). This way you don't have to add a layer of oil or wax, which was really inconvenient.

RAPD markers

Each individual reaction has a final volume of 25 ul. Since you are going to run reactions with 6 primers , you need enough of the different solutions to make 6*25 = 150 ul. We should always include a negative control when doing PCR – this means adding no DNA or no primer, to make sure they don't give bands all by themselves. This means there actually 7 reactions we need to do, for a total volume of 7 * 25 = 175ul.

The primers are made by a company called Operon, and they have simple labels (you can look up the sequences on the Web). The ones we will use have been shown to work in other trees, and include OPA#2,3,4,7,9,10.

Components of the PCR reaction

V_t = 25 ul
1uM primer
0.2mM of each dNTP
1X PCR buffer.
50 ng DNA
1.5Unit Taq DNA polymerase

The ***Master Mix*** idea: if you are adding almost the same thing to every reaction, you can make one solution and distribute it equally across the tubes. Then you add the one thing that is different (here, the primer).

Why do this? It limits the pipetting you have to do, which is good but it also reduces the number of places where you can make errors.

Note on Master Mixes: Since there are errors associated with pipetting small volumes, the rule is to always make at least 10% (multiply the volume by 0.1) more volume than you think you need. If we need 150ul of solution, we multiple 175ul * 0.1 = 7ul more, so we make 175ul + 17 ul = 194 ul. Since I like round numbers for doing the calculations, I will round this up to 200ul, which is what I would need to do 8 reactions.

Calculations for Master Mix.

We need to leave some volume for the primer, let us say that we will add 2.5ul primer to each tube, since the Stocks are at 10uM (25ul*1uM/10uM = 2.5 ul).

25ul – 3ul = 22ul . For 8 reactions we need to make 8 * 22.5 = 180 ul (if we are going to check the final volume) - but we do the calculations for everything but the water for 200ul.

1. Buffer: 200ul (1X) / 10X = 20ul of 10X buffer
2. DNA: 50ng/reaction (8 reactions) = 400 ng needed. The DNA stock is at 100ng/ul.
   400 ng/100ng/ul = 4 ul of DNA
3. dNTP: 0.2mM (200ul)/ = 2.5mM = 16ul
4. Taq DNA polymerase: 1.5 U/reaction * 8 reactions = 12 U.
5. Water: 180-20-4-16 = 140ul

Component	Stock Concentration in tube in ice bucket	Vol to use (ul) for Master Mix (MM)
Water	NA	140
10X PCR buffer	10X	20
dNTP mix	10mM (2.5mM each)	16
DNA	100ng/ul	4
Taq pol.	5U/ul	2.4

All solutions should be on ice. You should have in your ice bucket

1.5 ml tube of 10X PCR buffer	(1)	Labeled 10X
1.5 ml tube of Water	(1)	Labeled H2O
0.5ml tube of dNTP	(1)	Labeled dNTP
0.5 ml tube of Taq polymerase	(1)	Labeled Taq
0.5 ml tube of DNA	(1)	Labeled DNA
0.5 ml colored tubes of Primers	(6)	Labeled P2, P3, P4, P7, P9, P10
0.5ml tube to make Master Mix	(1)	Labeled MM (empty)
0.2 ml PCR reaction tubes	(7)	No labels

To the MM tube, add the volume of each solution in the given order shown in the table. Mix carefully, spin briefly if necessary. Then pipette 22.5 ul of the MM into each of the 7 PCR tubes. Label them. They should be kept on ice the whole time.

To six of the tubes add 2.5ul of each primer (one primer per tube – label them!). TO the 7^{th} tube add 2.5 ul of water. This is your PCR Control.
Leave the PCR tubes on ice while you set up the thermocycler with the following profile:

PCR ThermoCycling Profile

Melt
- 94C for 10 min

Cycle 45 times
- 94C 30 sec (fast ramp)
- 36C 1 min (slow ramp)
- 72C 1 min (fast ramp)

Finishing
- 72C 60 min

4C hold

Place the tubes in the thermocycler and start the program.

To analyze the samples, remove 5ul of each PCR reaction from the tube, add 1.5ul of 4X loading dye. Visualize on 2.0% agarose gels run in 1X TBE buffer.