E. GENERAL PCR PROTOCOL

A PCR reaction is assembled on ice in a volume of 25 µl using the following reagents:

- DNA sample (2.5 µl of 1:20 dilution of the original tail fin sample)
- forward and reverse primers (the final concentration of each primer in the PCR reaction is 0.5µM)
- 2'-deoxynucleoside triphosphates (dNTPs) including dATP, dGTP, dCTP and dTTP (the final concentration of each dNTP in the PCR reaction is 0.2 mM)
- Taq DNA polymerase buffer (2.5 µl of 10 X buffer)
- Taq DNA polymerase (0.9 units in a 25-µl reaction)
- water

Mg^{2+} in the PCR reaction comes from the Taq DNA polymerase buffer. The standard Mg^{2+} concentration is 1.5 mM. The Mg^{2+} concentration is occasionally increased (up to 3.0 mM) when primers with mismatches are used (e.g. dCAPS protocols). This ensures better primer annealing and results in more reproducible PCR reactions.

PCR reactions are assembled and performed either in 96-well plates or eppendorf tubes. 96-well plates are typically used when a large number of PCR reactions are carried out. To assemble PCR reactions, the forward and reverse primers are first pre-mixed with water to generate **a primer mix.** The primer mix is then combined with the dNTP stock solution, Taq buffer, water and Taq DNA polymerase to form **a master mix**. The master mix is added to the DNA samples and the entire solution is mixed thoroughly. PCR reactions are then covered with 7 µl of mineral oil and placed in the thermal cycler. To decrease non-specific amplification and therefore to increase sensitivity of the assay, PCR reactions are assembled on ice.

At ZIRC, PCR reactions are performed in PTC-100 and PTC-200 Peltier Thermal Cyclers (Bio-Rad). A typical PCR program entered into the thermal cycler is shown below:

1. 94ºC for 3 min
2. 94ºC for 30 sec
3. **55**ºC for **40** sec
4. 72ºC for **40** sec
5. Go to step 2 (above) for 34 cycles
6. 72ºC for 5 min
7. 8.0ºC hold
8. END

In this program, steps 1 and 2 are conducted to denature the DNA sample. In step 3, primers anneal specifically to the DNA template. The annealing temperature depends on

the primer set and can vary for different primer sets. In step 4, the primers are extended by Taq DNA polymerase and new complementary DNA strands are synthesized. This process takes place at 72^{O}C. The timing of step 4 depends on the size of the synthesized product. Typically, 60 seconds are required to generate 1-kb-long products. To obtain a significant amount of a product, PCR cycles are reiterated a number of times. PCR products that are subjected to restriction enzyme digestion (e.g. RFLP or dCAPS genotyping assays) are typically synthesized in 40 cycles. For products that are not digested by restriction enzymes, 35 PCR cycles are used. PCR is finalized in step 6 by a 5-minute long primer extension. After this step, PCR reactions are cooled down and stored at 8.0ºC

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