Protocol To Test Polymorphism of Primers

We have used the following protocols to test for polymorphisms within our testcross family as well as one unrelated Nigerian and one unrelated Ivory Coast frog.

1. Preparation PCR reaction mix

Reagent	1X	final concentration
10X Buffer/15mM $MgCl_2$	1.0 µl	1x buffer/1.5mM $MgCl_2$
2mM DNTPs	1.0 µl	0.2mM
2mM dCTP	0.1 µl	0.02mM
5 units/µl Taq	0.1 µl	0.05 units/µl
10 µM Forward Primer	0.5 µl	0.5 µM
10 µM Reverse Primer	0.5 µl	0.5 µM
10 ng/µl DNA	1.0 µl	1.0 ng/µ
10µCi/µl α-^{32}P-dCTP or α-^{35}S-dCTP	0.07 µl*	0.07 µCi/µ
H_2O	5.73 µl	
	10.0 µ	

* Depends on concentration, we have used both 10µCi/µl α-^{32}P-dCTP or 12µCi/µl α-^{35}S-dCTP. The α-^{35}S-dCTP give sharper bands that are easier to size accurately, however the exposure times and the cost of using α-^{32}P-dCTP are both less than α-^{35}S-dCTP.

2. PCR amplification protocol

The same PCR amplification protocol is used for both the initial primer testing (on agarose gels) and the polymorphic testing described here.

Step	Temperature	Time	Cycles
Initial Denaturing	94^0 C	4 min.	1
Denaturing	94^0 C	1 min.	30
Annealing	58^0 C	1 min	30
Elongation	72^0 C	1 min.	30
Final elongation	72^0 C	5 min.	1

3. Preparation for DNA ladder

We run a dideoxy DNA sequencing reaction of a known plasmid DNA and include it on the acrylamide gel in order to size the amplified fragments. For this we just follow DNA sequencing kit protocols.

4. Setting up the acrylamide gel plates/apparatus

We use Sequi-Gen GT systems from Bio-Rad, but any sequencing gel system will work. Clean plates and set up as per manufacturers specifications. In our experience, proper cleaning of the gel plates is critical.

5. **Preparation of 6% acrylamide gel:**

We purchase a 40% stock acrylamide solution from Amresco (19:1 acrylamide and bis-acrylamide), which we use to prepare our "insta-gel" mixture. However the stock solution can also be easily prepared in house.

We run 40x21 cm gels using 0.4 mm spacers. For each gel, the acrylamide gel mix is prepared combining the following: 40 ml Insta-gel solution, 54 *μ*l 25% ammonium persulfate, 46 *μ*l TEMED.

1. Insta-Gel Solution

Urea	210 grams
10X TBE	50 ml
40% Acrylamide stock	75 ml
dH_2O	to 500 ml

Filter through a 0.45 μm filter and store at 4^0C.

6. **Gel electrophoresis**

After pouring the gel, let it stand for at least an hour to polymerize, prior to setting up apparatus. After setting up the gel apparatus, pre-run the gel for about 30-60 minutes using 1X TBE buffer as running solution. Using our system, we pre-run at 25-30 mAmps until the gel temperature reaches about 50^0C. Once the gel is at temperature, add 3μl of sequencing gel Stop solution to the 10μl of PCR product and place in the heat block and heat at 65^0C for 10 min. We generally load about 3-5 μl of each sample. We generally run the gel for 1-2 hours at constant amperage making sure the temperature does not go above 50^0C to avoid cracking the glass plates.