Sequencing PCR products

PCR for amplification: Master Mix

	1x reaction	**10x**	**100x**
Genomic DNA (50-100 ng/ul)	2.0ul	20ul	200ul (only If using one genotype)
10X Taq polymerase buffer	2.0ul	120ul	200ul
Forward primer (20 pmole)	0.5ul	5ul	50ul (only If using one primer pair)
Reverse primer (20 pmole)	0.5ul	5ul	50ul (only If using one primer pair)
dNTPs (2 mM each)	2.0ul	20ul	200ul
Taq polymerase (1 unit)	0.2ul	2.0ul	20ul
Deionized water	12.8ul	128ul	1280ul

Total: 20 ul

PCR conditions:
5min - 94˚C
10 cycles of touchdown PCR with 0.5˚C decrease every cycle
20 sec - 94˚C
20 sec - 63˚C (decrease by 0.5C every cycle) (may vary according to primer)
2 min - 72 ˚C
35 cycles of
20 sec - 94˚C
20 sec - 58˚C (may vary according to primer)
2 min - 72˚C
72˚C - 10 min (to add A overhang for TA subcloning)
4˚C – standby
Take 3 ul of PCR product to check the quality of PCR products on 1% agarose gel.

Purification of amplicons: (*Note: Only strong single band PCR products could be purified by this method*)

We would run the gel to identify single band amplification and then treat the PCR product with with Exonuclease I (ExoI) and Shrimp Alkaline Phophotase (SAP) to remove excess of dNTPs and primers

Master Mix	1x reaction	**10x**	**100x**
PCR product	5 ul	5ul each well	5ul each well
SAP (2 units)	2 ul	20ul	200ul
ExoI (10 units)	1ul	10ul	100ul

Add the required amount of ExoI and SAP in a microfuge tube and mix well by vortex and spin. Then add 3ul of the mix to each of the 5ul PCR product and centrifuge. Incubate at 37C for 15 min, and then inactivate enzymes at 80C for 15 min. Freeze and store at –20 C or –70C until needed.

After treatment with ExoI and SAP add 8 ul of deionized water to reaction, and mix by pipetting up and down.

Sequencing reaction with Big Dye Terminator v 3.1 (ABI):

After treatment with ExoI and SAP add 8 ul of deionized water to each reaction, and mix by pipetting up and down. Transfer 3 ul aliquot of diluted reaction mix into a semi-skirted 96-well PCR plate.

Sequencing reaction mix	1x	10x	100x
Purified and diluted PCR product	3 ul	3ul each well	3ul each well
Sequencing primer (1.6 pmole/ul) using one primer)	2 ul	20ul (only If using one primer)	200ul (only If
5x sequencing buffer	1.5 ul	15ul	150ul
50% DMSO	1 ul	10ul	100ul
Big Dye v 3.1	1 ul	10ul	100ul
Deionized water	1.5 ul	15ul	150ul

Make master mix and add the required amount to purified PCR product and centrifuge.

Cycling conditions for sequencing reaction:
98 °C – 5 min
40 cycles of
96 °C – 10 sec
50 °C – 5 sec (may vary for your primer)
60 °C – 4 min

Purification of sequencing products:

Ethanol/Sodium Acetate mix.	1x	10x	100x
2.7M sodium acetate, pH 5.2	1.5 ul	15ul	150ul
100% ethanol	34.5 ul	345ul	3450ul

Add 5 ul of 25 mM EDTA to the each well and make sure that EDTA reach the bottom of the tube.

Then add 36 ul of ethanol/sodium acetate mix. Seal the plate and mix by inverting 4 – 5 times. Incubate at room temperature for 15 mins.

Spin at 2000-3000 x g for 30 min. Proceed to the next step immediately. If this is not possible, then spin the plate for 2 minutes immediately before performing the next step.

Invert the plate on paper towel and spin up to 185 × g, then remove from the centrifuge.

Add 60 µL of 70% ethanol to each well. Seal the plate and mix by inverting 4 – 5 times and centrifuge at 1650 × g for 15 min (set to 4 °C).

Invert the plate and spin up to 185 × g for 1 min, then remove from the centrifuge.

Resuspend the samples in 12 ul of deionized formamide and load the plate into ABI3730xl fragment analyzer.