DNA Extraction – Qiagen DNeasy kit.

Note the number of spin columns you use.
The centrifuge can hold up to 30 samples at once.

Forceps are to be sterilized in Fine Science Tools heat block at ≥200 C for ≥20 seconds.

1. Isolate a suitable piece of tissue and place in a UV-crosslinked 1.5mL tube.
2. Add 180□L Buffer ATL and 20□L Proteinase K and vortex.
3. Place in the 55 C incubator for 3 hours or overnight.
4. Remove from incubator, vortex, add 200□L Buffer AL and vortex.
5. Place in heat block at 70 C for 10 minutes.
6. Add 200□L 100% Ethanol and transfer entire volume onto spin column.
7. Centrifuge at 8000 rpm for 1 minute; discard flow-through.
8. Add 500□LBuffer AW1 and centrifuge at 8000 rpm for 1 minute; discard flow-through.
9. Add 500□L Buffer AW2 and centrifuge at 13000 rpm for 3 minutes; discard flow through.
10. Place spin column on UV-crosslinked 1.5mL tube, add 200□L buffer AE. Let sit for 1 minute, then centrifuge at 8000 rpm for 1 minute. Repeat and then combine flow-throughs for a total volume of 400□L.

PCR – Ready-to-go Beads.

Recipe: 2μL DNA
21μL H_2O
1μL each direction of primer (10□M)

Seal with caps or wax.

Thermal Profiles:

Basic program	
94 C 5:00	
94 C :30	
50 C :30	can increase the length of annealing up to 1 minute
72 C :45	can increase the length of extension up to 1:30 (for longer genes).
35 cycles	
72 C 7:00	
hold at 10 C	

Rock and Roll	
94 C 5:00	
94 C :15	
42 C :05	can increase the length of annealing up to :30
68 C :15	can increase the length of annealing up to :30
40 cycles	
72 C for 7:00	
hold at 10 C	

Down/Up			
94 C 5:00			
94 C :30	94 C :30	94 C :30	94 C :30
54 C :45	50 C :45	52 C :45	54 C :45
72 C 1:30	72 C 1:30	72 C 1:30	72 C 1:30
10 cycles	10 cycles	10 cycles	10 cycles
72 C 7:00			
hold at 10 C			
This can be useful for double bands, and you can use any range of annealing temperatures.			

Recommendations:
12s: basic program, 50 C or 48 C, go as low as 46 C
16s: basic program, 50 C or 48 C

18s: basic program, 50 C or 48 C
28s: basic program, 50 C or 48 C
COI: Rock and roll, start at 48 C, go as low as 42 C
ExtA/B: Rock and roll with longer annealing/extension, start at 48 C, usually best at 42 C
Histone3: basic program, 50 C or 52 C
EF-1 alpha: basic program with longer annealing and extension, 54 C or 56 C or Down/Up.

Agarose gel

1. To make a gel, add 8-10g agarose to 400mL 1XTBE buffer.

2. Microwave for 5 minutes, pour into gel tray, let cool 30 min.

3. Add SYBRsafe stain (0.1µL per lane) to loading dye (3µL per lane). Remember to add SYBRsafe stain to ladder.

4. Use 2µL of your PCR product and 3µL loading dye to check amplifications on the gel.

5. If you get double bands consistently, do a gel extraction (see below).

6. If you get very faint bands, clean as usual using AMPure, then concentrate product (see below).

Gel Extraction (for double bands): Promega Wizard Kit

1. Using the small gel rig, pour a 1% low melting point agarose gel (0.5 g in 50mL 1XTBE, 1μL ethidium bromide).
2. Add 5μL of loading dye to your PCR and load the entire volume onto the gel.
3. Run at 70 volts for 1-1.5 hours.
4. Protecting yourself from the UV, and using a razor blade or spatula, excise the band you want and put it in a 1.5mL tube.
5. Weigh each tube and add 10μL of Membrane Binding Solution per 10mg or gel.
6. Place in the 55 C incubator for 10 minutes, or until the gel is dissolved.
7. Transfer entire volume onto minicolumn; let sit 1 minute.
8. Centrifuge at 13000 rpm for 1 minute; discard flow-through.
9. Add 700μL Membrane Wash Solution.
10. Centrifuge at 13000 rpm for 1 minute; discard flow-through.
11. Add 500μL Membrane Wash Solution.
12. Centrifuge at 13000 rpm for 5 minutes; discard flow-through.
13. Re-centrifuge for 1 minute with lid open.
14. Transfer minicolumn to a 1.5mL tube.
15. Add 50μL nuclease-free water (included in kit); let sit 1 minute.
16. Centrifuge at 13000 rpm for 1 minute.
17. Go directly to sequencing or re-amplify using a regular PCR protocol to concentrate product.

AMPure Cleanup: Robot or by hand.

1. Transfer 18μL AMPure to an Eppendorf plate.
2. Add 10μL PCR product and mix.
3. Let sit 5 minutes.
4. Place on magnet, let sit 5-10 minutes.
5. Remove entire volume, leaving beads, discard liquid.
6. Add 200μL 70% Ethanol, let sit 30 seconds, flip while still on magnet to remove ethanol.
7. Repeat Ethanol wash.
8. Take plate off magnet; let air dry for 12-25 minutes.
9. Add 40μL H_2O, mixing well, to re-suspend the DNA.
10. When you are ready to set up your sequencing reactions,
11. Place plate on magnet for 10 minutes.

***Faint band concentration*:**
After AMPure cleanup, place plate on magnet for 10 minutes and transfer product to a new tube or plate. Evaporate to dryness on DNA speedvac. Re-suspend in 10μL H_2O and proceed to sequencing reaction.

Sequencing

Set up in Eppendorf plate.

Recipe: 5µL cleaned PCR product
1µL Big Dye Extender Buffer
1µL 3.2 □M primer
1µL Big Dye
Seal with wax or heat seal.

Thermal profile:

Basic program
94 C :30
50 C 1:00
60 C 4:00
35 cycles
hold at 4 C

CleanSeq Cleanup: Robot or by hand.

1. Add 10µL CleanSeq directly to sequencing reaction product.
2. Add 37.5µL 85% Ethanol and mix.
3. Place plate on magnet for 5 minutes.
4. Flip plate while still on magnet and discard ethanol.
5. Add 100µL 85% ethanol and let sit for 30 seconds
6. Flip plate while still on magnet and discard ethanol.
7. Take plate off magnet and let air dry for 10 minutes.
8. Add 40µL 0.5mM EDTA and let sit for 5 minutes.
9. Place plate back on magnet for 5 minutes.
10. Transfer 33µL finished product to Optical or Eppendorf plate; briefly spin down to get rid of any bubbles and heat seal.
11. Run on ABI 3730.