General Ampli**Cot** Protocol 5 September 2006

Materials

Exonuclease I (NEB)
SYBR Green Real time PCR mastermix without ROX (MCLab)
ddH_2O
200 mM MOPS pH 7.5
500mM EDTA
5M NaCl
Brij-700 (5% solution in water)
SYBR Green dye (Invitrogen)
Oligonucleotides

Equipment

Real time PCR machine capable of multiple reads during annealing step
We recommend the MJ Research Opticon [Bio-Rad] but other machines such as the ABI7700 may be used as well.
Consumables for Opticon:
Optical caps
Strip tubes (white)
96 well plate (white)

Optional: Regular PCR machine for RT and non-real time amplification

Method

1. First round amplification*
   12.5 μl mastermix (MCLab)
   0.5 μl forward primer (15μM)
   0.5 μl reverse primer (15μM)
   x μl template
   water
   →25 μL total reaction
   Program:
   95°C 2 minutes
   35 cycles of
   95°C 15 seconds
   60°C 30 seconds
   final extension
   72°C 10 minutes
   Freeze leftover reactions at –20°C.

2. Second round amplification*
   - If using the same primers as in step 1:
     25 μl mastermix (MCLab)
     1 μl forward primer (15uM)
     1 μl reverse primer (15uM)

2.5 μl first round PCR reaction
20.5 μl water
→50 μL total reaction

Program:
95°C 5 minutes
3 cycles of
95°C 15 seconds
60°C 30 seconds
final extension
72°C 10 minutes

- If using one or two nested primers:
  25 μl mastermix (MCLab)
  1 μl forward primer (15uM)
  1 μl reverse primer (15uM)
  0.5 μl first round PCR reaction
  22.5 μl water
  →50 μL total reaction

  Program:
  95°C 5 minutes
  10 cycles of
  95°C 15 seconds
  60°C 30 seconds
  final extension
  72°C 10 minutes

  Follow fluorescence to make sure reactions did not reach saturation.
  If desired, clonal standards may also be amplified from plasmids, in order to create standards for the annealing reactions.

3. Remove single stranded DNA from reaction (excess primers, asymmetric PCR products, cDNA remnants):
   Add 1 μl Exonuclease I (NEB) to each tube.
   Incubate at 37°C for 60 minutes.

4. To each well add 12.5 μl of reannealing buffer. Mix well; spin plate if there are bubbles. Protect from light as possible.
   For 1 mL annealing buffer:
   552 μl 200mM MOPS pH 7.5
   110 μl 500mM EDTA
   276 μl 5M NaCl
   34 μl 5% Brij-700 (protect stock from light)

28 μl dye concentrate (10,000x SYBR stock from Molecular Probes/Invitrogen, diluted 1:10 in DMSO; protect from light and store at –20°C)

Final concentrations in annealing reaction:
20mM MOPS
10mM EDTA
250mM NaCl
0.03% Brij-700
5x SYBR

5. Check samples by doing 70-99°C melting curve on 10μl aliquots. Choose the annealing temperature based on the position and width off the peak on the negative first derivative plot of the melting curve. When formulating a new assay, it is helpful to repeat the melting curve a second time, to look at the temperature difference between homoduplexes and heteroduplexes.

6. Perform annealing reaction, choosing annealing temperature from the step 5 data). Suggested annealing temperature is Tm -3.5°C, but a lower annealing temperature may still be able to distinguish between heteroduplex and homoduplex formation if the sequences being measured are very different. Split reaction into 25 μl sample and 25 μl reference on either side of a 96 well plate. Up to 32 samples may be measured at once, using the left 4 columns and the right 4 columns of the plate. Fluorescence reads should be every 5 seconds to every 20 seconds, and annealing time can be 1-6 hours depending on expected diversity of sample.

a. Make sure that the gradient is set so the rightmost reference well will stay below the annealing temperature.
b. Make sure the machine's temperature regulation is set to block and not sample.
c. Do melting curves at conclusion of annealing step to double check annealing stringency.

*<u>Notes on PCR reactions</u>

- These amplifications may also be done as regular PCR reactions to save money. The advantage of using real-time amplifications is that you can monitor the relative amount of template in different samples.
- The volume of the first round reaction is determined by the amount of template you need to add—keep the template volume at 1/25 of the total reaction volume or less. The minimum volume of template required to avoid losing sample diversity will need to be determined empirically depending on your application.

- The second round reaction depends on whether the same primers are being used for both rounds of PCR, or if a nested amplification strategy is chosen.
- Annealing temperature, given as 60°C here, should be set to the lower Tm of the oligo pair. If regular amplification (rather than SYBR Green real time amplification) is chosen, then use an annealing temperature 5°C below the lower Tm of the oligo pair. This discrepancy is due to the duplex-stabilizing properties of SYBR Green.