NOTE: Tm values for PCR primers offered by Santa Cruz Biotechnology Inc. range between 55–60 C (19–21 nt, GC% ~55%). The A and B nested primer sets share similar base pair length, GC% and Tm values.

NOTE: Nested PCR utilizes two pairs of PCR primers for a single locus. The first primer pair A-set amplifies within the locus. The second primer pair B set (nested primers) then binds within the "A" amplicon to produce a second nested "B" amplicon.

1. cDNA Synthesis

- Prepare a solution containing:
  - 1 µl oligo $(dT)_{12-18}$ (500 µg/ml)
  - 1 ng-5 µg total RNA
  - 1 µl 10 mM dNTPs
  - and add RNase-free water to a final volume of 12 µl
- Incubate at 70° C for 5 minutes to minimize RNA secondary structure, quick chill on ice and then add:
  - 4 µl 5x reverse transcriptase buffer
  - 2 µl 0.1 M DTT
  - 1 µl RNase inhibitor
- Incubate at 42° C for 2 minutes to anneal primer and template.
- Add 1 µl reverse transcriptase (200 units) and incubate at 42° C for 50 minutes to extend the primer and then terminate the reaction by incubating at 70° C for 15 minutes.

NOTE: (As an optional step add 1 µl RNase H (2 unit/µl) and incubate at 37° C for 20 minutes)

2. First PCR Reaction

- Prepare a solution containing:
  - 5 µl 10x PCR buffer (with or without* $MgCl_2$)
  - *5 µl 25 mM $MgCl_2$ (It may be necessary to vary the $MgCl_2$ concentration, 2.5 mM final concentration recommended.)
  - 1 µl 10 mM dNTP
  - 1 µl primer pair A
  - 1 µl Taq DNA polymerase
  - 2 µl cDNA and add water to 50 µl
- Incubate at 94° C for 2 minutes to denature the cDNA.
- Perform 15–40 cycles of PCR. Annealing and extension conditions are primer and template dependent and must be determined empirically for each template-primer pair.

3. Second PCR Reaction

- Prepare a solution containing:
  - 5 µl 10x PCR buffer (with or without* $MgCl_2$)
  - *5 µl 25 mM $MgCl_2$ (It may be necessary to vary the $MgCl_2$ concentration, 2.5 mM final concentration recommended.)
  - 1 µl 10 mM dNTP1 µl primer pair B
  - 1 µl Taq DNA polymerase
  - 1-5 µl first PCR product and add water to 50 µl
- Incubate at 94° C for 2 minutes to denature the cDNA.
- Perform 15–40 PCR cycles. Annealing and extension conditions are primer and template dependent and must be determined empirically for each template-primer pair.
- PCR products are visualized on agarose gels stained with ethidium bromide.