From Kyle Harris and Alison Nairn: 4/12/07

Protocol for cDNA synthesis and qRT-PCR

cDNA Synthesis

Superscript III 1st Strand Synthesis Kit (Invitrogen, Cat #18080-051)

We typically use 1μg of Total RNA per 20μl cDNA reaction, but you may be able to use less.
Initial Primer/RNA Mix:
0.5 μl random hexamer primers
0.5 μl oligo(dT) primers
1μl dNTP mix
8ul of RNA (1μg) + DEPC H_20
Heat to 95°C, 5 min

Buffer/Enzyme mix (assemble in this order):
2μl 10x cDNA synthesis buffer
4μl $MgCl_2$
2 μl 0.1 M DTT
1μ RNaseOut
1μl Superscript III
Mix Buffer/enzyme components and add to Primer/RNA mix in a 0.2 ml microfuge tube. Place the tube in the PCR machine programmed as follows:
25°C, 10 min, 50°C, 50 min, 85°C, 5 min.

Add: 1 ul RNase H, mix, incubate at 37°C, 20 min, 95°C, 10 min (an additional step to the Invitrogen protocol to remove any components that may interfere with the PCR (Ambion website). Store cDNA at -20ºC

Real Time-PCR

We typically dilute the cDNA reaction 1:10 with DEPC water and use 5μl as template for a 20μl RT-PCR reaction.

1. Pipette 5μl of diluted cDNA into all wells.
2. Pipette 5μl of each primer pair mixture into triplicate wells.
   Note: if the experiment requires multiple plates, a control primer (housekeeping gene) is run in triplicate on each plate to control for plate to plate or machine to machine variations.
3. Pipette 10μl of Bio-Rad iQ™ SYBR Green supermix (Cat. #170-1882) into all wells for a total reaction volume of 20μl.
4. Seal plate with optical film. Centrifuge PCR plate for 2 minutes at ~2500rpm to get all liquids to the bottom of the wells.

RT-PCR Amplification Protocol (Bio-Rad myIQ™ or iCycler iQ™ machines)**:**

1. Begin with a 3 min step at 95ºC

2. 40 cycles with a step of 10 sec at 95ºC, 45 sec at 65ºC, and 20 sec at 78ºC (Data acquisition step).
3. One step of 1 min at 95ºC.
4. One step of 1 min at 55ºC
5. 80 cycles of 10 sec each starting at 55ºC with a 0.5ºC increment at each step up to 95ºC (Melting point step to check for primer dimer formation).
6. For iCycler machine: use the FAM-490 filter for SYBR green detection.

Plate Setup (Bio-Rad myIQ™ or iCycler iQ™ software)**:**

1. Label triplicate wells starting with the first three wells being labeled 1, next three labeled 2, etc. up to 32 which is the maximum number of triplicate samples that can be tested on a single 96 well PCR plate.
2. Change standard quantity of all wells to 1.