# Real Time qRT-PCR using the Ambion Prism 7000

## Isolating RNA

**1.** Just after dissections are completed tissue is placed in 800ul of Trizol ® Reagent and then stored at -80C.

**2.** RNA is extracted following instructions for small RNA quantities and followed by lithium chloride precipitation using 7.5uM Lithium chloride solution (Ambion) and the manufacturer's protocols.

**3.** Extracted RNA is reverse transcribed into cDNA using the Sensiscript kit (Qiagen) at a concentration of 100ng of RNA /20ul rxn.

**4.** Three different amounts of RT product should be tested. I use 0.1ul, 0.5ul and 1ul of cDNA in a 25ul PCR rxn (this equals 0.5ng, 2.5ng and 5ng of equivalent RNA per rxn).

## Validating primers:

Primers were tested for use using standard curve method and performing tests for multiple products (multiple products decrease the ability to reliably calculate amount of product).

**1. Standard Curves**: The efficiency of the primer sets should be tested by creating standard curves. The cDNA template is diluted at 1:1, 1:5, 1:10; 1:25 and 1:50 with dH20. The CT value (calculated as described below) can then be plotted for each dilution on a log scale. Only primer sets that have a standard curve slope value higher than 0.85.

**2. Multiple Products:** only primer sets that create one product should be used in the analysis. The number of PCR products can be tested using two methods; **a)** Utilizing the Abi Prism software dissociation/melting curve function, multiple peaks in this dissociation curve indicate multiple products. **b)** The products were run on a 4% Nusieve agarose gel stained with Sybr Gold (Molecular Probes) and primer sets were eliminated when more than one band was present.

## PCR

Quantitative PCR was performed using the ABI Prism 7000.

**Reaction solution:**

-primers (0.8uM)

-Sybr green solution (Roche) containing AmpliTaq Gold® polymerase AmpErase® UNG dNTP mix and 25mM $MgCl_2$.

-cDNA (0.1-1ul)

Total volume =25ul

Use 96 well plates

**Cycle conditions**

2 minutes at 50C

10 minutes 95C,

35 cycles of:

95°C (30 sec),

60°C (45 sec),

74 °C (1min).

**Calculations**

The product is measured at the 74°C step (longest step) during each cycle and plotted in real time. The Abi Prism software calculates the CT value, the cycle number when the product crosses the threshold. Thresholds were set using whole embryo controls and RT- controls and kept constant for that primer set. The threshold should be set so that the CT value is very high in the RT- control. The relative amount of product is calculated using the delta-CT formula:

$2 \text{ ^ } CT_{\text{experimental. (geneX)}} - CT_{\text{control (EF1alpha)}}$ .

-scale to 100 for each primer set and plot using Microsoft Excel.