

## **REAL TIME QUANTITATIVE PCR**

### **A. cDNA Synthesis**

Protocol is written for a 15µl reaction but may be doubled in volume if using over 4µg Total RNA. This procedure is very similar to the RT labeling reaction. The protocol is written for quantitation of gene expression but the template may be varied for other uses. The protocol is described for use with the Light Cycler (Roche Diagnostics) but may be used with other machines as well and the reaction volumes may be adjusted accordingly. Primers are designed using the Light Cycler Probe Design Software, Version 1.0 (Idaho Technology Inc.) and purchased from IDT. We have had good luck with these but other programs and primer vendors may be used.

1. Combine DNase treated total RNA (3-4µg), mRNA or aRNA (300-500ng) in a 2µL volume or less, 1µg Oligo-dT (2µL at 0.5µg/µL) and 1µl 10x Random Decamers (AMBION #5720G2) to an eppendorf tube.  
Bring volume to 5µl with DEPC treated water.
2. Heat tube at 70C 10 min, chill on ice.
3. Add in order :
  - 3µl 5x first strand buffer (Invitrogen comes with enzyme)
  - 0.5 µl DTT (0.1M Invitrogen comes with enzyme)
  - 0.75 µL dNTPs (20mM Promega Corporation)
  - 5 µL DEPC treated water
  - 0.75 µL reverse transcriptase (200u/µl Superscript II RT Invitrogen)\***ADD LAST!!!**
4. Incubate at 37C 90 min. Bring the reaction volume to 100ul with cold DEPC water. (Keep on ice to continue; store -20C). This 100ul is the stock template for the Real-time protocol described in B>

**NOTE:** The amount of RNA used for the RT reaction may be varied. 0.5ug aRNA produces enough template for approximately 500 Real-time reactions, 4 ug of total RNA is sufficient for approximately 50 reactions.

### **B. Quantitative Real Time PCR (QPCR)**

Assemble the following reaction on ice. An aliquot of the aRNA stock template is further diluted 1/10 and the total RNA is used without further dilution. As a rule of thumb we try to add 1 nanogram of stock template to the reaction.

1. Add 2 ul of template and 8ul DEPC H<sub>2</sub>O to each tube
2. Prepare a mastermix from the following reagents (values are per reaction):

1µl	Primers Forward/Reverse (each; 10µM)
2µl	Buffer (10x; Invitrogen comes with enzyme)
1µl	Mg (50mM; Invitrogen comes with enzyme)
0.4µl	dNTP (10mM; Promega Corporation)
1.5µl	BSA (10mg/mL; Sigma A-2153)** <b>IMPORTANT</b>
1µl	SYBR Green I (1:1000; Biowhittaker Molecular Applications)
2µl	H2O

Mix well and add 1 unit of Platinum Taq (Invitrogen) per reaction and mix again (do not vortex with enzyme!).

1. Add 10µl of mastermix to each capillary/tube containing the cDNA template (total 20ul).
2. Cover the capillaries/tubes with caps and spin them for 1min at 3000 RPM (Be careful pushing caps into place – capillaries are fragile – push caps on straight)
3. Start reaction in the Light Cycler instrument immediately. Read Light Cycler manual for instructions on running a Real time cycle.

\*\* The BSA addition is very important. Not all BSA works and we have listed the catalogue number we are presently using. We usually compare a new batch of BSA to the old one before it runs out.