cDNA Synthesis from Total RNA

In this experiment we will produce cDNA from *B. malayi* adult male and adult female total RNA (available free of charge through the FR3 molecular division, www.filariasiscenter.org). Each RNA aliquot is at a concentration of 100 ng/ul. The cDNA will later be used as template for quantitative PCR.

The setup for this exercise takes approximately 30 minutes. Incubation in the thermalcycler takes 45 minutes.

• Each group will set up two cDNA synthesis reactions. Label one 1.5 mL as your Master mix. To this tube carefully add:

8 ul Nuclease Free H_2O
20 ul RT buffer
2 ul Random Primers
4 ul Reverse Transcriptase Enzyme
34 ul Total Volume

• Mix your master mix gently by inversion and spin briefly in your nanofuge. Then aliquot 17 ul of Master mix to your tube labeled Adult Male cDNA and 17 ul of your master mix to your tube labeled Adult Female cDNA.

• Add 3 ul each of your Adult Male RNA and Adult Female RNA to the appropriate tube. Stir gently with your pipette tip to mix and spin briefly in nanofuge.

• Place your cDNA synthesis reactions in the thermocycler. The thermocycler will be set as follows:

25 C for 10 minutes to anneal primers
37 C for 30 minutes for reverse transcription
85 C for 5 minutes to terminate the reaction
8 C hold

• Your cDNA synthesis Reaction can then be stored at 4 C for a short period or at -20 C for long term storage. You may now proceed to quantitative real-time RT-PCR (next page).

Quantitative Real-Time PCR using SYBR Green

The SYBR Green System uses real-time quantitative PCR to accurately analyze levels of gene expression. For this assay we will be doing relative quantitation of two genes expressed in *B. malaya*i adult males and adult females: a gene that codes for a High Mobility Protein (HMP, GenBank: EF418609.1) and a gene that codes for a Major Sperm Protein (MSP, NCBI Reference Sequence: XM_001894150.1). We will also be using NADH I as a control (GenBank: AF538716.1). NADH I should be equally expressed in both adult males and adult females. Our template for the reaction will be adult male cDNA and adult female cDNA.

The primer sequences are as follows (from Li et al. 2004. Quantitative analysis of gender-regulated transcripts in the filarial nematode *Brugia malayi* by qPCR, PMID: 15383303).

HMP F: CAAGCGGAGCATCAACATCA
HMP R: CGGTGCATTCGGATCTTTG

MSP F: CCACCGGGTGATATCCATACC
MSP R: CGACCACCGGCATTAGTAATCTTAAT

NADH I F: GGGTGGCACTCAGTGTCGTA
NADH I R: ACAACGCCTGAAAAATACCAGAGTA

The setup for this exercise takes approximately 30 minutes. Real-time PCR cycling takes 2.5 hours.

- You will receive a total of 3 tubes of Master: HMP Master Mix, MSP Master Mix, and NADH Master Mix. Each tube of Master Mix contains:

25.5 ul Nuclease Free H_2O
37.5 ul 2x Master Mix
1.5 ul ROX
3.75 ul Forward Primer (10 uM)
3.75 ul Reverse Primer (10 uM)
72 ul Total Volume (This is enough for three 25 ul reactions)

• Each group will receive an 8 strip of PCR tubes. Aliquot 24 ul of the appropriate master mix to each tube. Going from left to right the master mix should be aliquotted as follows:

HMP, HMP, MSP, MSP, NADH I, NADH I, Empty, Empty

• Then add 1 ul of the appropriate template (your cDNA) to each tube). Please add your template as follows:

(HMP) Female, (HMP) Male, (MSP) Female, (MSP) Male, (NADH I) Female, (NADH I) Male, (Empty), (Empty)

• Briefly centrifuge your strips and transfer them to real-time machine. The program will be set as follows:

50 C for 2 minutes
95 C for 10 minutes

Then 40 cycles of:
95 C for 15 seconds
60 C for 1 minute

• Each group will then receive their results in lecture!

In class, the instructor will go over the mechanics of real-time PCR and analysis of each student's results. Additional activities: use the primer sequences to BLAST against the *Brugia malayi* database in NCBI to find the mRNA sequences, map the primer sequences onto the mRNA sequences, use bioinformatics resources to find the genes that correspond to the mRNAs, use Wormbase, Nematode.net and Nematodes.org to interpret the expression of these genes in the context of the genome.