RNA Extraction & cDNA Synthesis

Measuring gene expression relies upon the extraction of RNA molecules from cells, removal of genomic DNA, conversion of RNA into cDNA, and quantification of cDNA during PCR. This procedure details the steps of the process prior to quantitative real-time PCR (qRT-PCR).

RNA Extraction

Worm lysis and RNA extraction are based on a published protocol utilizing lysis buffer, heat, and proteinase K to degrade samples and release nucleic acids (Kien Ly, Reid, and Snell, *MethodsX*, 2015). This method allows for the extraction of usable RNA from single worms or small populations, and does not require phenol-chloroform or other hazardous chemicals. Proteinase K (1 mg/mL) is added to the lysis buffer (Promega Cell Lysis solution) to facilitate the breakdown of the worm cuticle.

1. Make sure you have a stock of **Lysis Buffer with Proteinase K** added to a concentration of 1 mg/mL.
2. Pipette **8 uL** of Lysis Buffer into the cap of a small PCR tube.
3. Pick **1 - 10 worms** (usually 5 Adults) off of a plate while minimizing the amount of bacteria that is transferred.
4. Using a dissecting microscope, place the pick into the Lysis Buffer in the cap and swirl to allow the worms to be released. Visually ensure that the worms are now swimming in the liquid.
5. Carefully put the cap on the tube and repeat the steps above for any additional samples. Keep the samples on ice.
6. Once all of the worms have been placed in the Lysis Buffer, spin the tubes briefly to bring the worms to the bottom of the tube.
7. Put the tube(s) into a thermocycler and incubate at **65° C for 10 min, followed by 85° C for 5 min**.

   ***Note: there is a pre-programmed protocol on the main thermocycler in SCA 209.*
8. After incubation the tube may be used immediately for downstream applications, or it can be stored at -80° C.

Genomic DNA Degradation

In order to maximize the efficiency of the reverse-transcription reaction and subsequent PCR, we will degrade the remaining genomic DNA present in the lysate with DNase enzyme (Thermo Scientific).

1. Add **1 uL of 10X DNase I reaction buffer with $MgCl_2$** to each of the worm lysis tubes.
2. Add **1 uL of DNase I (RNase-free) enzyme** to each of the lysis tubes.
3. Incubate the tubes at **37° C for 30 min** to degrade genomic DNA.
4. Add **1 uL of 50 mM EDTA** to each lysis tube and incubate at **65° C for 10 min** to inactivate the DNase I enzyme. Each lysis tube should now contain approximately 11 uL of liquid.

cDNA Synthesis

Once the worms have been lysed to release RNA and the genomic DNA has been degraded, the sample is ready for **first-strand cDNA synthesis** (Maxima H Minus First Strand cDNA Synthesis Kit). This process introduces a reverse-transcriptase (RT) enzyme that will synthesize a complementary DNA sequence to each mRNA in the lysis reaction. The enzyme requires a primer, which is called Oligo(dT) - a short nucleotide sequence consisting of a series of thymine residues. This primer will bind to the 3' UTR of mRNAs and initiate the RT reaction. The resulting RNA:DNA duplex molecule will be the substrate for downstream PCR and qPCR experiments.

1. Add the following reagents to each lysis tube:
    a. 0.25 uL Oligo(dT)
    b. 1.0 uL dNTP mix
    c. 2.75 uL nuclease-free water
    d. 4.0 uL 5X RT Buffer
    e. 1.0 uL Maxima H Minus Enzyme Mix
2. Incubate the tubes at **25° C for 10 min** followed by **55° C for 30 min**. Then denature the RT enzyme by heating at **85° C for 5 min.**

***Note: there is a pre-programmed protocol on the main thermocycler in SCA 209.*

3. Store at **-20° C for up to 1 week**, or at **-80° C for longer storage**.