II. cDNA synthesis for PCR

*If RNA yield is below 30 ug, use all of it.

Using the **RevertAid™ First Strand cDNA Synthesis Kit**

Quantify –We need 0.1 - 1μg RNA for cDNA.

a) Use the Nanodrop in the science lab building to quantify RNA. Refer to Nanodrop protocol for instructions on use.

Materials –

1. Prepare an ice bath
2. Random hexamer
3. DEPC-treated Water
4. 5x rxn Buffer
5. RiboLock™ RNAse Inhibitor
6. 10mM dNTP mix
7. RevertAid™ M-MuLV Reverse Transcriptase

Methods –

Keep all reagents from kit on ice.

1. Do all procedures under UVP using DNA/RNA free tips, pipettes, and microcentrifuge tubes (also using RNAse free spray on gloves, pipettes and surfaces).
2. Use the Excel program to determine the ratio at which you use RNA to water.
    a. With > 30 ug of RNA _ 10 uL RNA with 2 uL Water (switch if <30 ug).
    b. M1V1=M2V2 is the long hand equation for determining the RNA: H_2O ratio.

****Volume of RNA and Water mixture should always equal 12uL total.**

3. Add RNA and Water to labeled 0.2 mL PCR tubes.
4. Add 1 uL of Random Hexamer (used to prime synthesis).
5. **SKIP THE FOLLOWING STEP** (this step is stated in the company directions but is **NOT** necessary)
    a. Mix, spin
    b. 70°C for 5 minutes
    c. Chill on ice
    d. Spin down
6. Add the next in order
    a. 4 uL 5x rxn buffer
    b. 1 uL RNAse inhibitor
    c. 1 uL dNTP 10mM
7. **SKIP THIS STEP** (stated in directions)
    a. 25°C for 5 minutes
8. Add 1 uL of Reverse Transcriptase (The total volume now is 20 uL)
9. Centrifuge for ~ 5 seconds.

Protocol on Thermocycler

1. Place samples in holes of thermal block, if possible, toward the center of the block avoiding the outer edges. Try to evenly space your sample tubes.
2. Close Thermocycler lid.
3. Run program- FERM-RT → Random Hexamer
    a. 25°C for 10 min
    b. 37°C for 60 min
    c. 70°C for 10 min
    d. 4°C ∞ hold

4. Take samples out and store in freezer until needed.

PCR Protocol

Materials –

1. cDNA (product of above protocol)
2. Forward and Reverse primers
3. Dreamtaq Mastermix
4. RNAse/DNAse free Water

Methods –

1. Create Mastermix and Water mixture.
    a. 7 uL RNAse/DNAse free water.
    b. 10uL Dreamtaq Mastermix.
2. Add 1 uL cDNA into each tube.
3. Vortex and Centrifuge ~ 5 seconds.
4. Add 1 uL Forward Primer.
5. Add 1 uL Reverse Primer.

***** Total volume should equal 20 uL.***

6. Vortex and Centrifuge ~ 5 seconds.
7. Place in Thermocycler, away from outer edges, and run PCR.

General Settings:

    a. 95°C for 3 min
    b. Set this step to repeat 34X: 94°C for 40 sec
                                   55°C for 30 sec
                                   72°C for 40 sec
    c. 72°C for 5 min
    d. 4°C ∞ hold

8. Store products of PCR in freezer until needed.

• Once reagents are used store on ice.
• Always try the house keeping gene first!
• Make sure you write down the program and PCR machine you are using.