Protocol

Part 1: cDNA synthesis

With these reactions you will convert your RNA to single stranded DNA, using a kit supplied by Epicenter (Tuesday/Thursday lab section) or Invitrogen (Wednesday/Friday lab section) for the conversion. Regardless of supplier, the protocol requires that you anneal random primers (9mers) to the RNA then add nucleotides and enzyme (reverse transcriptase) to extend the annealed primers into complementary DNA ("cDNA"). A reaction without reverse transcriptase will serve as a control. In part II of today's experiment you will use the cDNA from both reactions as template for q-PCR.

Protocol for Epicenter kit

1. Clean your bench in preparation for RNA work. If you are unsure what this involves, review the protocol from last time.
2. In an RNase-free PCR tube mix
    - ___ µl Rnase free H2O
    - ___ µl RNA (ideally 1µg but 0.5 µg for each sample is OK if volume would be >26 µl)
    - 4 µl Random 9-mer primers (50 µM)
    - to a final volume of 30 µl
3. Incubate 65°C 1 minute in the thermal cycler and then allow the tubes to cool (4°C hold).
4. Move 15 µl of each rxn to new PCR tubes and add add 4 µl MonsterScript 5X cDNA premix and 1µl reverse transcriptase to each new tube. Flick to mix.
5. To what remained in the original annealing tubes, add 4 µl MonsterScript 5X cDNA premix and 1µl RNAse free H_2O. Flick to mix.
6. Incubate in thermal cycler (program "cDNA")
    - 37°C 5 minutes
    - 42°C 5 minutes
    - 60°C 40 minutes
    - 90°C 5 minutes
    - 4°C hold
7. These samples can be used directly for q-PCR, as you will do today. Alternatively, they can be stored at –20°C for later use.

Protocol for Invitrogen kit

1. Clean your bench in preparation for RNA work. If you are unsure what this involves, review the protocol from last time.
2. In an RNase-free PCR tube mix
    - ___ µl Rnase free H2O
    - ___ µl RNA (ideally 1µg but 0.5 µg for each sample is OK if volume would be >16 µl)
    - 2 µl random hexamers (50 ng/µl)

- 2 µl 10 mM dNTP mix
- to a final volume of 20 µl

3. Incubate 65°C 5 minute in the thermal cycler and then allow the tubes to cool (4°C hold).
4. Add 20 µl cDNA synthesis cocktail to each tube then move 20 µl of each rxn to new PCR tubes. To one of each pair, add 1 µl reverse transcriptase. Flick to mix.
5. To what remained in the original annealing tubes, add 1µl RNAse free H_2O. Flick to mix.
6. Incubate in thermal cycler (program "cDNA")
   - 25°C 10 minutes
   - 50°C 50 minutes
   - 85°C 5 minutes
   - 4°C hold
7. Add 1 ul RNaseH to each tube and incubate at 37°C for 20 minutes.
8. These samples can be used directly for q-PCR, as you will do today. Alternatively, they can be stored at –20°C for later use.

Part 2: q-PCR

You will set up reactions to compare the amount of *LacZ* RNA (now conveniently cDNA) in your two samples. Primers specific for *LacZ* will be used for the reactions. For better confidence in your data, you will make each measurement in triplicate.

The remaining samples you will prepare today are controls, broadly addressing the questions of **product specificity** and **concentration**.

Specificity is an issue because the machine will measure fluorescence in your reactions (arising from the fluorescent dye, SYBR Green, in the reaction mix), and you'd like to assume that the fluorescence arises from the number of *LacZ* sequences in the tube. The fluorescence of SYBR Green increases more than 300 fold when it binds to double-stranded DNA, but in solution it has some natural fluorescence you must correct for. Double-stranded DNA could also arise from contaminating *LacZ* DNA that is being amplified (DNA carried over from the cells themselves, or brought in with the Taq polymerase which in many kits was purified from bacteria). Fluorescence could arise from amplification of sequences that are not *LacZ.* You'd see these as extra bands on a gel if you were running the products out but since you're not doing that, you'll have to detect other products in other ways. Finally, some of the controls you'll set up are identical to ones you ran when you performed "end point" PCR in the DNA engineering module, looking for contamination of the PCR tubes or reaction mixes with template.

You will also make serial dilutions of DNA template to generate a fluorescence vs concentration standard curve. The source of DNA for these reactions will be the lysed cells you prepared last time. From the number of cells, you'll know the number of *LacZ* templates you're adding to each tube. Next time you will assess the accumulation of *LacZ* products as a function of cell number, then use this standard curve to determine the number of copies in each of your cDNA reactions, expressing the result and # of *LacZ* mRNA/cell.

Use the following pattern to help you plan your reactions. You will need two strips of 8 PCR tubes, two strips of caps, and a cold block to use as you assemble the reactions. **Each of your reactions today should have a final volume of 25 µl.**

1st strip of 8 tubes

1	2	3	4	5	6	7	8
No RT	cDNA	cDNA	cDNA	No RT	cDNA	cDNA	cDNA

1. You should prepare a "master mix cocktail" sufficient for four reactions. Each reaction will have cDNA (1 µl/reaction), primers (0.5 µl of the forward primer and 0.5 µl of the reverse primer/reaction), reaction mix (12.5 µl of 2X mix), and water. Since you have two samples of cDNA you will prepare two such master mixes. Aliquot the reactions into the PCR tubes using the pattern above.
2. You should prepare one complete reaction (no RT cDNA, primers, reaction mix and water) for each of your "no reverse transcriptase" controls.

2nd strip of 8 tubes

1	2	3	4	5	6	7	8
DNA standard 1	DNA standard 1:10	DNA standard 1:100	DNA standard 1:1000	DNA standard 1:10000	DNA standard 1:100000	primers only	blank

1. You should prepare a master mix cocktail sufficient for 8 reactions, each with 12.5 µl of 2X reaction mix, 0.5 µl forward primer, 0.5 µl reverse primer, and enough water to bring the volume to 24 µl. Aliquot these into the first 7 tubes of the second strip.
2. Serially dilute the DNA you isolated from cells with the Lyse-N-Go protocol, making 1:10 dilutions in water.
3. Add 1 µl of undiluted DNA to first tube in the second strip.
4. Add 1 µl of each dilution to the following 5 tubes in the second strip.
5. The 7th tube of the second strip should be your "primers only" reaction. There should be enough volume in the cocktail you prepared for this reaction.

6. The 8th tube of the second strip should be a "blank" reaction which leaves out both template and primers. Specifically, this reaction should have only 2X reaction mix and water.

When everyone is ready we will walk our samples to the BioMicroCenter in Building 68 and begin the q-PCR cycles.

1.
    1. 94° 3 minutes
    2. 94° 15 seconds
    3. 53° 30 seconds
    4. 72° 30 seconds
    5. Plate read
    6. repeat steps 2-5, 35 times
    7. melting curve 65°-95°C, read every 0.5°C
    8. end