Molecular Biology Lab

Spring 2016

Lab 8 Part 2

qPCR analysis

 Please take a moment to review Lab 8, qPCR background information to refresh your memory of the qPCR experiment. Our template was the pGLO plasmid containing 3 genes, one of which was GFP. We decided to use Emeka’s primers as they showed successful priming in the endpoint PCR product and had an annealing temperature close to 56 degrees C. I had a programmed qPCR protocol that works well at that annealing temperature.

We set the following objectives.

 1. Understand the theory of qPCR ( e.g. SYBR green, CT)

 2. Understand the use of standards in optimizing qPCR

 3. Prepare serial dilutions—use dilution series to calculate concentrations or absolute quantities of DNA.

 \*\*4. Interpret the qPCR results after a run.

You did a terrific job setting up, but the computer froze and basically interrupted/stopped the thermal cycler program early in the process…not data was collected. ☹ Let’s proceed to analyze a previous year’s data. The data are real and show a first-time attempt at qPCR. I did not alter them to make them look perfect!

**A**. Part of the experiment involved diluting your pGLO template serially. The template we used was 98.9 ng/l ) *Be sure to know the exact concentration of each dilution sample you used in qPCR.* ***Discuss with your partners now.***

**B.** Review your handouts from last time, which discuss 10-fold dilutions for standard curves. The handout discusses the exponential nature of PCR, as each template copy doubles with each cycle. *If we started out with 200 copies of our template, how many copies were there after 40 cycles of PCR?* ***Discuss with your partners now.***

Can we predict the difference in CTs for our dilutions? Yes!

We use the formula:

 2n=dilution factor. For our experiment, our dilution factor was 10 (each sample was diluted 10-fold). So for 10-fold dilutions, n=3.32. Therefore we expect our CT values to be offset by ~3.32 cycles.

*What if we had chosen 4-fold dilutions? What would you expect for CT differences in dilutions?*

**C.**  Begin to analyze the qPCR data. I’ve created a power point using the main pages one would normally review after a qPCR experiment. You should review them yourself, and with lab partners and determine how well our data would fit the expectations for this experiment. I’ve included raw data for all groups. Use the questions to start analyzing your experiment.

**Raw data spreadsheet and corresponding graph of fluorescence vs. cycle number**.

 *What does the spreadsheet include in terms of data? Discuss with your partner(s) what each column represents and how well your data fits your expectations.*

*The graph is unlabelled, but the computer assigns similarly colored lines for replicates?( Each line corresponds to data in your spreadsheet.) Find the threshold line and predict the CT for each group of lines. How tight are your replicates (confirm with spreadsheet).*

*NA can mean the values are outside the limits for the machine. Why are the undiluted and 1:10 values NA?*

*For dilutions higher than 1:10, what is the CT for your group for each dilution? What is the mean CT for each dilution? How far apart are the average means? What would you expect?*

*Is the no template control (NTC) data what you might have expected? Is there cause for concern? Explain.*

Page 3---**Graphing of CT vs. Log of the starting quantity.** (typical standard curve)

Look at the graphs for your standard curves (done for you). This graph summarizes our dilution scheme and the corresponding CT .

*Is it clear that PCR gives an exponential increase in DNA copies? Explain.*

This analysis is essential to determine the **validity** of the PCR run and could be used to **analyze an unknown sample**. *Explain.* Would you change anything if you ran qPCR again?

**Page 4—Melt curve for the products made during the qPCR amplification.**  A very important final check on your qPCR run is analysis of the endpoint product. Since SYBR green will show amplification of any double-stranded product, it is essential that you check to see that a single, expected product is produced. To check this, at the end of the run, the thermal cycler goes through continuous series of temperature changes (from the annealing temp up to about 95 degrees C).

*As the temperature increases what will happen to the PCR products?*

*How does this influence fluorescence ?(think SYBR green)*

The melt curve plots the change in fluorescence (negative first derivative of fluorescence vs. temperature). A sharp peak will form at the temperature at which fluorescence changes rapidly. This corresponds to the melting temperature (Tm) for an amplification product in the tube.

*What determines Tm for a DNA duplex?*

A common problem with PCR is the interaction of primers that allow small amplification products to form (called primer-dimers). They will cause SYBR green to fluoresce brightly too.

*Why is this a problem in SYBR green qPCR? How does the melt curve help sort this out? (Be sure to look at the SYBR5 group data).*

*How does the melt curve look for our experiment?*

*How could you analyze the PCR products using a different method?*