

## Analysis of data from qRT-PCR

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### I. Analyze the real time fluorescence data.

1. Open up the file for your experiment, click on **Analysis**, and on **Amplification Plot**.
2. On the left side of the screen you should see the plot pane (currently showing the amplification plot), and on the right side of the screen you should see the Plate Layout (or Well Table).
3. Take a look at the amplification plot. This shows you how much fluorescence was measured from each tube at each cycle. Samples containing more of the target mRNA will have fluorescence appearing after fewer cycles. A typical way to view the amplification plot is to use Plot Type =  $\Delta R_n$  vs Cycle and Graph Type = Linear, however you may also choose a log scale. Select the **Threshold** box to see the threshold that has been chosen by the OneStep software. Make sure that the threshold is in the exponential phase of the amplification.
4. While still in the amplification plot, change to Plot Type =  $C_T$  vs Well. This will tell you the threshold cycle ( $C_T$ ) value for each well of your reaction plate. If you have pipetted carefully, you should expect to see only very small differences in  $C_T$  between the two amplification replicates for each sample/target combination.
5. Save a pdf file (giving it a distinguishing name) of your  $\Delta R_n$  vs Cycle (linear) graph, your  $\Delta R_n$  vs Cycle (log) graph and of your  $C_T$  vs Well graph onto the desktop of the OneStep computer. Copy these three pdf files onto a memory stick.
6. You will also need to *record the average  $C_T$  value for each sample/target combination*. These values can be found in the Well Table. Now you are finished with the OneStep computer.
7. Take your memory stick to one of the Mac computers in the MoBio lab. Copy and paste each of your three graphs onto a Powerpoint slide. Adjust them to an appropriate size (that will fit well in your notebook) and print them on the color printer in Olin 236. *Include these three graphs in your lab notebook. Based on your  $\Delta R_n$  vs Cycle (log) graph, did you get a high ( $\approx 100\%$ ) efficiency of amplification? Based on your  $C_T$  vs Well graph, did you get good reproducibility between the two amplification replicates for each sample/target combination?*

8. Now it is time to figure out the amount of *RBCS* mRNA/*CBP20* mRNA for each sample. Using Excel, create a spreadsheet following the example below.

	A	B	C	D	E	F
1	SAMPLE	CT for RBCS	CT for CBP20	$\Delta$ CT	$2^{\Delta$ CT	rel. to ref.
2	reference	14.69	20.95	6.26	76.6	1.00
3	treatment 1	14.96	21.13	6.17	72.0	0.94
4	treatment 2	13.56	21.05	7.49	179.8	2.35
5	treatment 3	14.59	20.73	6.14	70.5	0.92
6	treatment 4	16.78	20.86	4.08	16.9	0.22
7	treatment 5	14.76	20.92	6.16	71.5	0.93

The values in columns B and C come from the Well Table. The values in column D are the differences between the  $C_T$  values for *RBCS* and *CBP20* for each sample. The values in column E ( $2^{\Delta$ CT) represent the actual ratios of *RBCS* mRNA/*RBP20* mRNA. Since a PCR reaction (assuming 100% efficiency) doubles the amount of product with each cycle, a  $\Delta$  $C_T$  of 2 cycles is equivalent to an *RBCS* mRNA/*RBP20* mRNA ratio of 4, a  $\Delta$  $C_T$  of 3 cycles is equivalent to a ratio of 8 etc. To make Excel calculate the values for column E, put the formula =POWER(2,D2) into cell E2. As you can see in the sample above,  $2^{6.26} = 76.6$ . The other values in column E can be obtained in the same way. In column F we are arbitrarily defining the amount of gene expression from the reference sample to be equal to 1.00. Cell F3 contains the formula =E3/76.6.

It is important to remember that the “mRNA amounts” given by this type of qRT-PCR are completely arbitrary units and do not represent anything about the actual number of mRNA molecules present. Thus the values are only quantitative in a relative sense. We can determine, for example, that treatment 2 caused the amount of *RBCS* mRNA to more than double (relative to the reference) and that treatment 4 caused a decrease in *RBCS* mRNA to about 22% of the original level. We cannot, however, get absolute quantitation (such as the actual number of *RBCS* mRNA molecules in each cell of our samples) from the data we have.

9. Make a bar graph to represent the gene expression from your Arabidopsis samples, and include the graph in your notebook.

## **II. Analyze the PCR products on a gel.**

- 1.** Label a 500  $\mu\text{L}$  tube for each of your (presumably 12) sample/target combinations. Add 1  $\mu\text{L}$  of 5x FG loading buffer to each tube. Based on your SYBR green fluorescence results, choose one representative well (on your 48 well plate) for each sample/target combination and mark it. Pipet 4  $\mu\text{L}$  from the marked well into the correct 500  $\mu\text{L}$  tube. Spin the tubes briefly to make sure all the loading dye/sample mixture is at the bottom.
- 2.** Remove a DNA FlashGel from the package and remove the white tapes. Fill each of the wells with water. Insert the gel into the FlashGel dock. Load your samples, and an additional well containing 5  $\mu\text{L}$  of DNA size standards. (The bands in the standards are 100, 200, 300, 500, 800, 1250, 2000, and 400 bp.)
- 3.** Run the gel at 275 V for about 7 minutes. Do not touch the FlashGel dock or gel while the voltage is turned on.
- 4.** Photograph the gel using the camera setup in Arey 204.
- 5.** Print out a photo of your gel and *include it (with lanes/bands etc. clearly labeled) in your notebook. Are PCR products of the expected size present?*