7.003 Spring 2022
Day 18 qPCR Analysis Worksheet

This worksheet will guide you through the analysis of your *FUS1* and *ACT1* qPCR results for your WT and mutant strains. Follow all instructions and answer all questions in your Day 18 Post-Lab, which is due on LabArchives as usual by 1:00 PM on Thursday, Apr 21st.

Determine *FUS1* and *ACT1* primer efficiencies:

Before you can accurately compare different qPCR samples, you first need to verify the qPCR primer pairs you used have a high PCR efficiency (and are similar in efficiency to each other) during the exponential phase of PCR.

Determining primer efficiency requires first performing a standard curve using serial dilutions of a set DNA template (e.g. yeast genomic DNA). A standard curve using diluted gDNA as a template was previously done by the instructors with both the *FUS1* primers and the *ACT1* primers. The average C_t value measured for each gDNA dilution in the standard curve for each set of primers is given below.

***FUS1* primers standard curve**			***ACT1* primers standard curve**	
Template	C_t value		Template	C_t value
gDNA 1:10	15.43		gDNA 1:10	16.17
gDNA 1:100	18.77		gDNA 1:100	19.52
gDNA 1:1000	22.21		gDNA 1:1000	22.89

The efficiency (E) of a PCR primer pair can be calculated with the following formula:

$$E = 10^{-(1/m)}$$

In the above equation, *m* represents the slope from graphing the C_t value vs the log of the serial dilution factor.

1) Use Excel (or other similar program) to create two standard curve graphs, one for *FUS1* and one for *ACT1*. For each graph, C_t values should be plotted on the y-axis and the log of the serial dilution factor should be plotted on the x-axis. Add a trendline or "best-fit" line to each graph. Make sure the graphs each have a title and the axes are properly labeled. Insert an image of each standard curve graph into your Day 18 Post-Lab on LabArchives.

2) What is the slope of the trendline for each of your standard curve graphs?

3) Calculate E (PCR efficiency) for the *FUS1* primers and for the *ACT1* primers.

4) A maximum PCR efficiency (100%) would mean E = 2 (the amount of amplification product doubles with each cycle during exponential phase). Calculate the % efficiency (E / 2) for the *FUS1* primers and for the *ACT1* primers.

5) Do the *FUS1* primers and *ACT1* primers have similar PCR efficiencies? Are they each close to maximum PCR efficiency? Are you confident in the accuracy of any comparative analysis using these primers?

Check consistency of *ACT1* reference gene expression:

For *ACT1* to serve as an effective reference gene for normalizing your samples, you first have to confirm that *ACT1* expression stays relatively consistent in the different strains/conditions tested. To directly compare the expression levels of a gene between two samples (without any normalization), you can calculate and compare 2^{-Ct} values.

6) Calculate the average C_t value for the triplicate reactions performed for each of your four *ACT1* cDNA sample conditions (WT UT, WT αF, Mut UT, and Mut αF).

7) Calculate the 2^{-Ct} value for *ACT1* for each of your four conditions.

8) Calculate the fold-change difference in 2^{-Ct} values for *ACT1* for each of the following three comparisons:

Mut UT vs WT UT
WT αF vs WT UT
Mut αF vs Mut UT

9) Based on your calculations, what would you conclude about *ACT1* expression in the various strains/conditions tested? Is *ACT1* a valid reference gene to use for your experiments?

Use $-\Delta C_t$ and $-\Delta\Delta C_t$ methods to compare *FUS1* expression:

To directly compare the expression levels of a target gene (e.g. *FUS1*) between multiple samples, you can calculate and compare $2^{-\Delta Ct}$ values. The $2^{-\Delta Ct}$ value uses a reference gene (e.g. *ACT1*) to normalize your different samples.

For a given sample's $2^{-\Delta Ct}$ value, ΔC_t = C_t(Target gene) – C_t(Reference gene)

10) Calculate the average C_t value for the triplicate reactions performed for each of your four *FUS1* cDNA sample conditions (WT UT, WT αF, Mut UT, and Mut αF

11) Calculate the $2^{-\Delta Ct}$ value for *FUS1* expression for each of your four sample conditions (WT UT, WT αF, Mut UT, and Mut αF), using *ACT1* as the reference gene.

12) Using Excel (or other similar program), create a bar graph comparing the $2^{-\Delta Ct}$ *FUS1* values for your four sample conditions. The bar graph should have a title, and the axes (and any bar categories) should be properly labeled. Insert an image of your bar graph into your Day 18 Post-Lab on LabArchives.

To look at fold-changes in target gene expression between two different conditions (e.g. treated vs untreated for a particular strain, or mutant vs WT, etc), you can use $2^{-\Delta\Delta Ct}$ comparative analysis. The second "Δ" in $2^{-\Delta\Delta Ct}$ comparative analysis comes from calculating the change in ΔC_t values between the two different conditions.

When calculating the $2^{-\Delta\Delta Ct}$ value,

$$\Delta\Delta C_t = [C_t(\text{Target gene, Condition 1}) - C_t(\text{Reference gene, Condition 1})] - [C_t(\text{Target gene, Condition 2}) - C_t(\text{Reference gene, Condition 2})]$$

Typically, "Condition 1" is the experimental condition (e.g. the treated condition or mutant condition, etc), while "Condition 2" is the control condition (e.g. untreated condition or WT condition, etc). Using this convention, the $2^{-\Delta\Delta Ct}$ value will give you the fold-change in target gene expression in the experimental condition, relative to the control condition. For example, if $2^{-\Delta\Delta Ct}$ = 3.5, then that target gene is expressed 3.5-times more in the experimental condition than in the control condition.

13) Calculate the $2^{-\Delta\Delta Ct}$ value to find the fold-change expression in *FUS1* mRNA for the following three different comparisons:

Untreated mutant vs untreated WT
αF-treated WT vs untreated WT
αF-treated mutant vs untreated mutant

14) Based on your $2^{-\Delta Ct}$ and $2^{-\Delta\Delta Ct}$ analysis, what would you conclude about *FUS1* expression in the four different strains/conditions you tested? Discuss the observed vs. expected results of *FUS1* expression for each sample.

Check "-RT" qPCR control samples:

15) Calculate the average C_t value for the duplicate reactions performed for each of your four *FUS1* –RT sample conditions (WT UT, WT αF, Mut UT, and Mut αF) and for each of your four *ACT1* –RT sample conditions (WT UT, WT αF, Mut UT, and Mut αF)

16) What would the "ideal" expected result be for the C_t values for each of your –RT qPCR samples? How do the observed C_t values compare with the expected ideal results (and with the C_t values of the cDNA qPCR samples)? What might be a potential explanation for any unexpected results in your –RT qPCR samples, and how might that affect your overall qPCR analysis?

Check No DNA qPCR control samples:

17) Calculate the average C_t value for the duplicate reactions performed for the *FUS1* No DNA sample and the *ACT1* No DNA sample. (Note: If no C_t value is given for a particular sample in the Excel file, that indicates the SYBR Green fluorescence for that sample never rose above the threshold cutoff and no C_t value can be determined.)

18) What would the "ideal" expected result be for each of your No DNA qPCR samples? How do the observed C_t values compare with the expected ideal results (and with the C_t values of the cDNA qPCR samples)? What might be a potential explanation for any unexpected results in your No DNA qPCR samples, and how might that affect your overall qPCR analysis?

Check melting curve analysis:

A melting curve analysis was done at the end of the entire qPCR program to look at the SYBR Green fluorescence of the sample as temperature increases.

A melting peak analysis (change in slope of the melting curve vs. temperature) was also graphed for each qPCR sample well of your plate.

Note: Information about the *FUS1* qPCR primers and their binding locations in the *FUS1* gene can be found in the lab manual (Day 16). The *ACT1* qPCR primers are as follows:

ACT1-For primer (binds +319 to +341 of *ACT1* gene) =
5' - GAG GTT GCT GCT TTG GTT ATT GA - 3'

ACT1-Rev primer (binds +369 to +347 of *ACT1* gene) =
5' - ACC GGC TTT ACA CAT ACC AGA AC - 3'

19) Paste the JPG of the melting peak analysis graph in your Day 18 Post-Lab on LabArchives. How many prominent large peaks are there? What do each of these large peaks most likely represent? Explain your reasoning.

20) Are there any other smaller peaks present in the melting peak analysis graph? If so, explain what they may represent. If there are not any other smaller peaks in the graph, explain the significance of that.

7.003 Applied Molecular Biology Lab
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