

Gene Expression Comparison using the $\Delta\Delta$ CT Method

Notes: The following protocol describes how to use qRT-PCR to compare RNA levels between two samples of extracted pure RNA. The $\Delta\Delta$ CT method is effective for qPCR reactions which have already been calibrated i.e. the efficiency test protocol was followed and yielded an efficiency between 90-110%.

Set-up:

Before starting, it is advisable to plan out how many reactions will be performed to ensure enough cDNA and master mix is generated. See the example below for a reference plate set-up.

Always perform qPCR on the housekeeping gene for each biological replicate measured. Always run no template control qPCRs for each gene measured.

		1	2	3	4	5	6	7	8	9	10	11	12
WT/Control	A	Gene 1B1	Gene 1B1	Gene 1B1	Gene 1B1	Gene 2 B1	Gene 2 B1	Gene 2 B1	Gene 2 B1	Gene 3 B1	Gene 3 B1	Gene 3 B1	Gene 3 B1
	B	Rpl13 B1	Rpl13 B1	Rpl13 B1	Rpl13 B1	Gene 1B2	Gene 1B2	Gene 1B2	Gene 1B2	Gene 2B2	Gene 2B2	Gene 2B2	Gene 2B2
	C	Gene 3B2	Gene 3B2	Gene 3B2	Gene 3B2	Rpl13 B2	Rpl13 B2	Rpl13 B2	Rpl13 B2	NTC Gene1	NTC Gene2	NTC Gene 3	NTC Rpl
Mutant/Treated	D	Gene 1B1	Gene 1B1	Gene 1B1	Gene 1B1	Gene 1B1	Gene 1B1	Gene 1B1	Gene 1B1	Gene 3 B1	Gene 3 B1	Gene 3 B1	Gene 3 B1
	E	Rpl13 B1	Rpl13 B1	Rpl13 B1	Rpl13 B1	Gene 1B2	Gene 1B2	Gene 1B2	Gene 1B2	Gene 2B2	Gene 2B2	Gene 2B2	Gene 2B2
	F	Gene 3B2	Gene 3B2	Gene 3B2	Gene 3B2	Rpl13 B2	Rpl13 B2	Rpl13 B2	Rpl13 B2				
	G												
	H												

On this example plate, 3 genes of interest (1, 2, and 3) and housekeeping gene (rpl) are measured over 2 biological replicates (B1 and B2). No template control qPCR reactions are performed for each gene.

Part A:

Prepare cDNA at a similar concentration as was effective for calibrating the qPCR reaction. Typically, selecting an input RNA template concentration which matches the middle of the previously generated calibration curve is effective.

For example, if for the middle of the calibration, 10 μ L of 2.5 ng/ μ L RNA was used to generate 20 μ L of cDNA which was then diluted, and 2 μ L was used as template for the calibration, follow the same steps for template set-up to perform qPCR.

Use an RNA to cDNA kit (the one for this protocol was a Thermofisher high-capacity reverse transcriptase kit) to synthesize cDNA:

1. Dilute RNA to desired concentration in fridge-cold RNase-free water. Room temp RNase-free water is fine too. Keep RNA samples on ice.
2. Thaw kit buffer on bench top or over ice.
3. Pulse spin buffer and enzyme.
4. Combine the following per sample in a PCR strip tube.
 - Always perform a negative control where 9 μ L water is added in place of diluted RNA:

10 μ L 2x cDNA buffer

9 μ L diluted RNA

1 μ L RT Enzyme Mix

5. Place strip tube in thermocycler and run the cDNA synthesis saved protocol (cDNA_synth).

- This saved protocol is effective for the kit used when creating this protocol; ensure the steps used are compatible with the kit used during operation.

1 hr 37 C

5 min 85 C

Hold 4 C

6. Dilute cDNA as necessary to provide template for all wells. E.g. 35 μ L of nuclease free water was typically added to 20 μ L cDNA before performing qPCR for both calibration and $\Delta\Delta$ CT.

Part B:

7. Prepare the following master mix, ensuring to create at least 1 extra reaction to sufficiently cover all wells including the no template control:

PowerUp SYBR (2x) 5 ul

Primer F (10 uM) 0.8 ul

Primer R (10 uM) 0.8 ul

Nuclease-free water 1.4 ul

8. Dispense 8 μL of master mix per well corresponding to the plate set-up.
9. Add 2 μL of diluted cDNA to wells, again depending on the set-up.
10. Seal plate and spin down 1-2 minutes. Look carefully at the plate to make sure all liquids are adequately spun down/mixed.
11. Load plate in quantstudio3 and select the following options:

Start a new experiment and at the landing screen, select comparative CT/ (ΔCT) for the method to be used. Select the reagent system as powerup SYBR Green.

At the second (Method) screen, make sure to tell the machine what volume of reaction was used in the top left corner. Additionally, select a PCR melt temperature compatible with the reactions.

- Most qPCR primers are in the 55-60 C melt temp range, but ideally the same temperature as used to generate standard curves should be used here.

Leave all other parameters the same on the methods page.

At the third (Plate) page, label the wells to correspond to the plate set-up. This step can be done after performing the qPCR reactions.

12. Start the reaction.

Part C:

13. Save data including the date the experiment was performed.
14. Export data.
15. Perform $\Delta\Delta\text{CT}$ calculations using the template excel sheet or other.