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PCR and analysis

In 1 collection

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ASAP Collaborative Rese… | Enrico Protocols

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Abstract

This protocol details the PCR analysis.

Materials

Materials:

- SybrGreen PowerUP
- Primers
- cDNA
- 384 well plates
- RT-PCR machine

PCR setup

6m 45s

1 Include at least two housekeeping control

Note

- The following steps refers to a 384 well-plates, but volumes can easily be adapted to 96 well plates. Perform calculation while waiting for primers to defrost. Always add a few spare wells to ensure enough master mix is made as Sybr Green is very viscous and difficult to pipette precisely.
- Use a dedicated set of pipettes and try to be as clean as possible. Ensure primers efficiency is satisfactory. Include wells with no cDNA as amplification control.
- Run samples in triplicates.
- Include at least two housekeeping control

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Note

If first time, prepare [M] 100 micromolar (µM) primer stock by diluting it with PCR-grade water.

Prepare a working solution at [M] 10 micromolar (µM) by diluting 1:10 the stock in PCR-grade water.

3 In each well of a 384 well-plate, 5 µL of samples + master mix have to be added, according to the following volumes:

- DNA: 2 µL
- MasterMix: 2.5 µL SyberGreen + 0.5 µL forward and reverse primers working stock

Prepare, for each gene used, a master mix, by adding 2.5 µL /well of SYBR green and 0.5 µL /well of primer mix in a clean PCR-grade tube.

4 Dilute stock of cDNA. A 1:5 or 1:10 dilution is normally sufficient to detect most genes and to have enough cDNA for all the samples.

5 Pipette 2 µL cDNA in each well assigned to the same samples.

6 Pipette 3 µL mastermix in each well assigned to the same primer.

7 When all plate is completed, seal the plate and quickly centrifuge it to prevent bubble interference.

8 Put the plate in a thermal cycler designed for quantitative measurements. 6m 45s

Note

- **Conditions of the qPCR:** (preamplification step) 95 °C for 00:05:00, (amplification step) 45 cycles of 95 °C for 00:00:10, 60 °C for 00:00:10 and 72 °C for 00:00:20.
- Finally, calculate a melting curve to confirm the presence of a single PCR product following these steps: 95 °C for 00:00:05, 66 °C for 00:01:00, and gradual increase in temperature up to 97 °C (fluorescence acquisition).

PCR analysis

9 The following sections outlined analysis of PCR data using the $\Delta\Delta C_t$ method.

10 For each sample and for each gene, calculate the average C_t value of the technical triplicate,, checking that intra-replicate variation is acceptable.

11 For each sample, calculate the geometric mean of the C_t value of the housekeeping genes ($C_{t\ HKG}$)

12 For each sample and for each gene of interest, subtract the average $C_{t\ HKG}$ value (from step 11) from the average C_t value calculated at step 10 to obtain the $\Delta C_{t\ GOI}$ for each sample and for each gene of interest

13 For each gene of interest, average the $\Delta C_{t\ GOI}$ for the control samples (untreated) across your experimental replicates to obtain the **control $\Delta C_{t\ GOI}$**

Note

Both arithmetic and geometric mean can be used, the latter is particularly appropriate when the ΔC_t values across experimental replicates are quite dispersed.
Normalising by averaging across experimental replicates allows to have an error bar also for the control group. If the normalization is performed intra-experiment, all control points will be at 1, without error bars and affecting statistical analysis.

14 For each gene of interest, calculate the $\Delta\Delta C_{t\ GOI}$ by subtracting the **control $\Delta C_{t\ GOI}$** from the $\Delta C_{t\ GOI}$ for each sample and for each experimental replicates

15 At this point, you should have, for each gene of interest and for each sample, n $\Delta\Delta C_{t\ GOI}$ where n is the number of experimental replicates.

Note

Perform the appropriate statistical analysis at this step, has the pints will most likely be normally distributed. In the last step, the normality will be possibly lost.

16 In order to plot the result, use the Fold change for each gene of interest by using the following formula for each data point:

$FC=2^{(-\Delta\Delta C_{t\ GOI})}$