qPCR TOOL

(by TIGEM Bioinformatics Core)

Table of Contents

1. Introduction

qPCR Tool provides a simple interface to help users in performing qPCR data analysis, with the possibility of selecting the method to analyze data, and the hypothesis test to highlight the presence of statistical significance for the differences among the conditions. qPCR Tool is implemented in **PHP/R** languages and it is available at the following link:

http://bioinformatics.tigem.it/qpcr.php

2. Brief theory

Relative quantification uses an internal control (reference gene) and/or a control group (reference group) to quantify the mRNA of interest relative to these references, which is suited to provide results from qPCR experiment.

The proposed **comparative method** works under the following hypotheses:

 a) the cDNA templates of the genes (genes of interest and reference genes)should have similar (almost perfect) amplification efficiency;
 b) the differences between two genes and/or two conditions should be captured by subtraction;
 c) the reference genes do not change with the experimental treatment.

We assume:

- C_T as the number of cycles required for the fluorescent signal to cross the threshold;

- $\Delta C_T = C_{T,goi} - C_{T,ref\_gene}$ as the difference of the number of cycles between the gene of interest and the reference gene;

- $\Delta\Delta C_T = \Delta C_{T,treat} - \Delta C_{T,ctrl}$ as the difference between differences for the treatment condition and the control condition (group of interest vs reference group);

- $2^{\Delta\Delta C_T}$ as the relative expression,

and we provide two methods of analysis: $\Delta\Delta \boldsymbol{C_T}$ **analysis** or $\Delta \boldsymbol{C_T}$ **analysis**.

For the statistical analysis, we can compare conditions pairwise with the **unpaired t-test** for normally distributed and homoscedastic data, or with **Wilcoxon test** if the two previous hypotheses are not verified. Moreover, the **linear regression** can be used considering the null hypothesis as $\Delta\Delta C_T = 0$, and the alternative hypothesis as $\Delta\Delta C_T \neq 0$.

For further theoretical details, please refer to [1]. If you are interested in R pipeline, please refer to [2].

3. How to use

The **file to upload** for the analysis should be in XLS or XLSX format. Please, avoid special character in the filename (e.g., whitespace). The first column should have the name 'Condition', and reporting the conditions/groups to compare (e.g. treatments, amino acids substitution, …). Other columns should have the name of the genes to compare, with the reference gene in the second column. The first condition should be the reference condition (e.g., WT). An example of dataset is in Tab. 1.

Condition	actin	gbx2	th
WT	19.65	36.58	22.51
WT	19.25	35.49	23.00
WT	18.65	35.94	23.85
WT	18.56	36.93	24.06
WT	18.24	36.08	23.50
WT	18.57	37.37	23.12
KO	18.7	36.9	23.05
KO	18.4	36.52	23.81
KO	18.94	37.72	23.37
KO	18.83	37.81	23.28
KO	18.24	36.65	23.23
KO	18.72	36.46	23.06
KO	18.15	37.11	23.33
KO	18.81	37.44	23.52
KO	19.79	37.53	24.42

Table 1. **Example of dataset to upload.** Conditions are in the first columns, genes number of cycles in the other columns.

The **Upload screen** is in Fig. 1. The XLS/XLSX file should be selected from your folder, by clicking on 'Scegli file' button, then uploaded for the analysis by clicking on 'Upload' button. After uploading the XLS/XLSX file, you should select in the **Selection screen**: the reference condition (e.g. WT), the reference gene (e.g. actin), the analysis method (ΔC_T or $\Delta\Delta C_T$), and the statistical method (unpaired t-test, Wilcoxon test, or Linear Model).
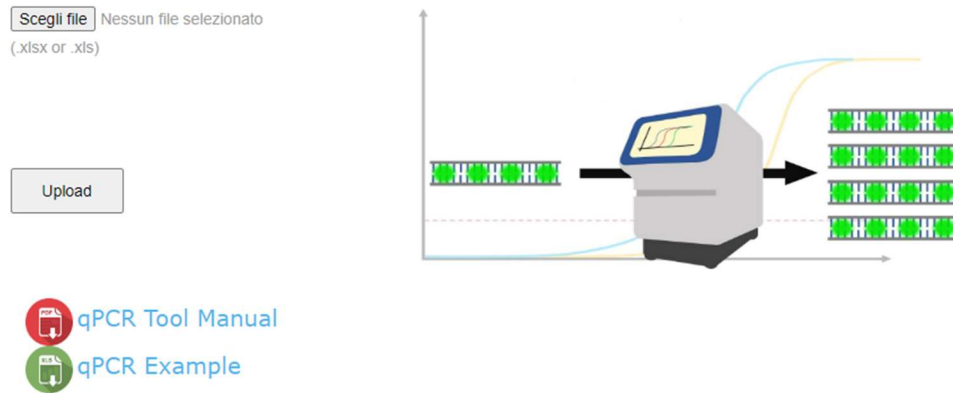
qPCR TOOL

Scegli file | Nessun file selezionato
(.xlsx or .xls)

Upload

qPCR Tool Manual
qPCR Example

Figure 1. Upload screen. Click on 'Upload' button after the selection of the dataset file ('Scegli file' button),

Reference Condition	⌄
Reference Gene	⌄
Analysis Method	⌄
Statistical Method	⌄

Submit

Figure 2. Selection screen. All the selections in the drop down menus are compulsory. Click on 'Submit' button after the selections.

4. Results

The results of the analysis are reported in three different files from the **Download screen**, as shown in Fig. 3.

Relative Expression

Barplot

Statistical Significance

Figure 3. Download screen. Results are directly downloadable from Download screen.

Relative Expression is reported in the first XLSX file, with the format as in Tab. 2 for $\Delta\Delta C_T$ method; **relative fold change** is reported in the first XLSX file, with the format as in Tab. 3 for ΔC_T method.

group	gene	normalized	calibrated	relative_expression	error	lower	upper
WT	gbx2	17.57833333	0	1	0.867811423	0.547977505	1.824892
KO	gbx2	18.39555556	0.817222222	0.567533626	0.710782042	0.346756717	0.928877
WT	th	4.52	0	1	0.777971722	0.583186116	1.714718
KO	th	4.721111111	0.201111111	0.869880355	0.647692485	0.555245001	1.362807

Table 2. Relative expression XLSX file from $\Delta\Delta C_T$ method. The reference gene is dropped.

For the $\Delta\Delta C_T$ method, the columns represent:

- *group*: the condition
- *gene*: the gene of interest (the reference gene is dropped);
- *normalized*: C_T (or the average C_T value) of genes of interest after subtracting the C_T value of the reference gene;
- *calibrated:* the normalized average C_T value of genes of interest after subtracting the C_T value of the reference group;
- *relative_expression:* the genes of interest expression, normalized by a reference gene and calibrated by a reference group;
- *error:* the standard deviation of the relative expression;
- *lower:* the lower interval of the relative expression;
- *upper:* the upper interval of the relative expression.

group	gene	calibrated	fold_change	error	lower	upper
WT	actin	0	1	0.523297	0.69578	1.437236
KO	actin	-0.08889	1.063551	0.48354	0.760673	1.487025
WT	gbx2	0	1	0.692284	0.618873	1.615839
KO	gbx2	0.728333	0.603601	0.520961	0.420654	0.866113
WT	th	0	1	0.575674	0.670973	1.490373
KO	th	0.112222	0.925162	0.430923	0.686273	1.247207

Table 3. Relative fold change XLSX file from ΔC_T method. The reference gene is not dropped.

For the ΔC_T method, the columns represent:

- *group*: the condition
- *gene*: the reference gene and the genes of interest
- *calibrated:* the normalized average C_T value of genes after subtracting the C_T value of the reference group;
- *fold_change:* the relative fold change, normalized to the reference group;
- *error:* the standard deviation of the relative fold change;
- *lower:* the lower interval of the relative fold change;
- *upper:* the upper interval of the relative fold change.

A **qualitative barplot** for the $\Delta\Delta C_T$ method is in Fig. 4, downloadable in PDF format, with conditions paired per genes of interest (see relative expression values in Tab. 2). The only difference with the ΔC_T method is the absence of the plots for the reference gene (not shown).
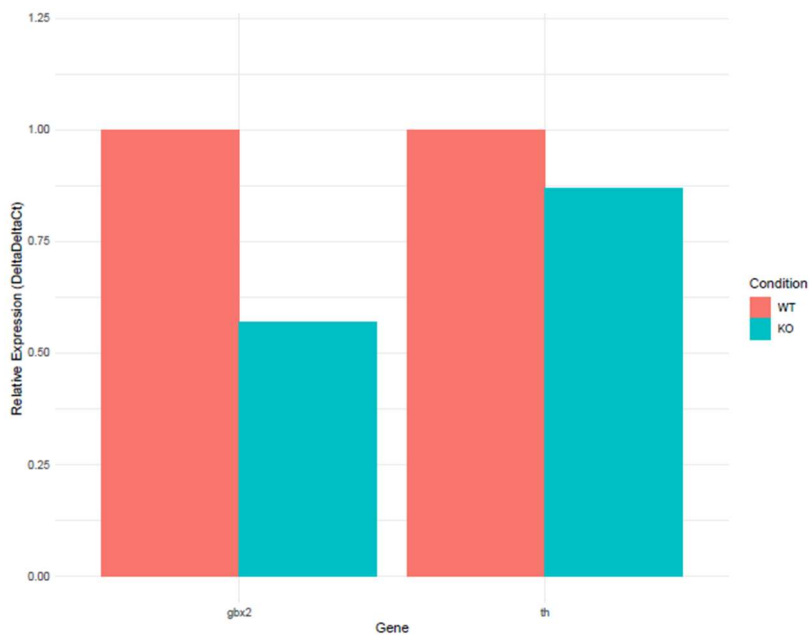

Figure 4. Barplot for $\Delta\Delta C_T$ method. Conditions are paired per gene of interest.

The third file in XLSX format contains the **statistical significance** between the different conditions per gene, as shown in Tab. 4.

gene	estimate	p_value	lower	upper
gbx2	0.817222	0.04449	0.023383	1.611062
th	0.201111	0.599315	-0.60567	1.007897

Table 4. Statistical Significance XLSX file. P-values are related to reference condition.

For $\Delta\Delta C_T$ and ΔC_T methods, the columns represent:

- *gene*: the gene of interest (the reference gene is dropped);
- *estimate:* the estimated statistic for each term;
- *p_value:* the p-value for each term;
- *lower:* the low value of 95% confidence interval;
- *upper:* the high value of 95% confidence interval.

5. Hint with Prism

Unfortunately, it is not possible to add the calculated p-values directly to the barplot for now (because of some issues with the R package used for this aim). However, we provide a way to redo the barplot with statistical significance and error bars on **GraphPad Prism** (tested ver. 9.5.1) [3]. Taking into account Tab.2 and Tab. 4:

a) Open Prism;
b) *CREATE -> Grouped ->*
    a. *Data table*: Enter or import data into new table
    b. *Options*: Enter and plot error values already calculated elsewhere, and select - Mean, SD, N -
c) Put genes as row names, conditions as column names, respective relative expression values as mean, error as SD, and number of samples per condition as N, such as in Fig. 5;

Table format: **Grouped**		Group A			Group B		
		WT			KO		
⬛	✕	Mean	SD	N	Mean	SD	N
1	gbx2	1	0.87	6	0.57	0.71	9
2	th	1	0.78	6	0.87	0.65	9

Figure 5. Insert value on Prism table. The table format is 'Grouped', with genes as row names and conditions as column names. The error in Tab.2 is the standard deviation (SD).

d) From the left field *Graphs*, click on the name of the dataset, from *Grouped* Graph family, select the type *Summary data*, with *Interleaved bars Plot* Mean with SEM;
e) From the upper field *Draw*, select *Manually add lines with text* and the preferred way to show the statistical significance (i.e. stars or p-values)
f) Move manually the brackets for the p-values, selected from Tab. 4;
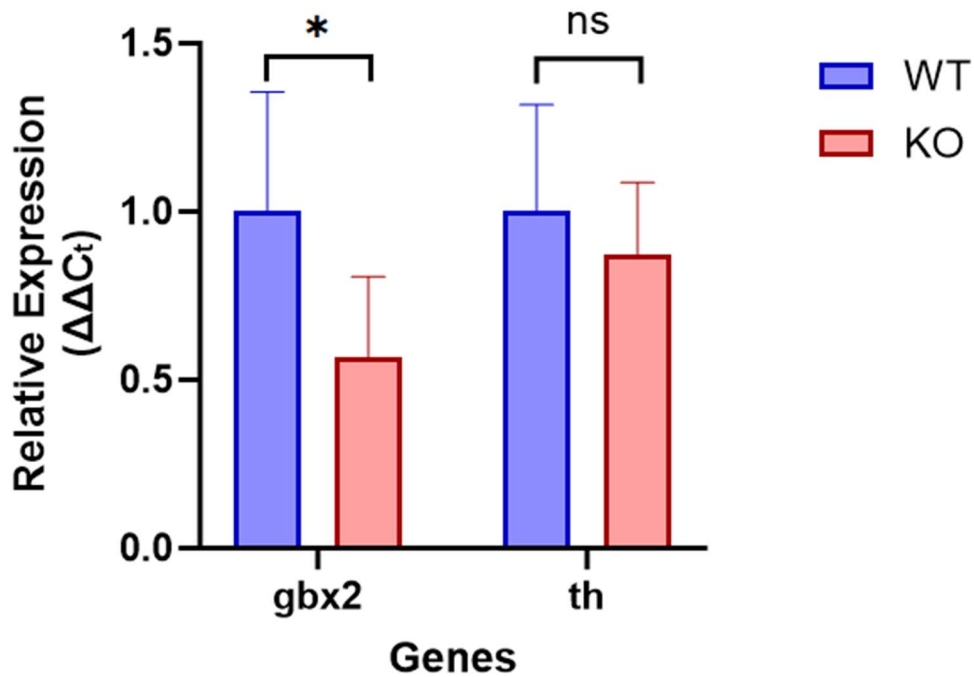g) Customize your graph with labels and colors, as the example shown in Fig. 6.

Figure 6. Barplot from Fig. 4 replotted with Prism. The graph is plotted automatically from Prism table in Fig. 5. X label, Y label, brackets with statistical significance, and colors should be modified manually.

6. References

[1] Ahmed M, Kim DR. **pcr: an R package for quality assessment, analysis and testing of qPCR data**. *PeerJ*. 2018 Mar 16;6:e4473. doi: 10.7717/peerj.4473. PMID: 29576953; PMCID: PMC5858653

[2] *https://cran.r-project.org/web/packages/pcr/vignettes/qpcr_analysis.html*

[3] *https://www.graphpad.com*