How to Analyze Real Time qPCR Data?

Mohammad Najafi*
Biochemistry Department, Razi Drug Research Center, Cellular and Molecular Research Center, Tehran University of Medical Sciences, Tehran, Iran

Introduction

The comparative transcriptome and genome studies are the fields of molecular biology that describe the biological responses and the organism load. These studies are often based on the high throughput techniques and also the copy-number limited (qPCR) and semi-quantitative (RT-PCR) PCR methods. In the PCR-based methods, the analytical sensitivity is dependent on the end-point and kinetic analyses so that Real-Time qPCR is known as a high accuracy technique. The Real-Time qPCR is a time-dependent method to determine the primary RNA or DNA copies, based on the signal detection limitations in the exponential phase [1].

Data Analysis

The data analyses are commonly performed in “fold change” and “relative expression” procedures [2,3].

The fold change procedure

It shows absolute CT (or Copy) number ratio of target (T) and reference (R) genes in the experiment (Exp) and control (Con) studies without the consideration of statistical tests. Similar to northern blotting technique, the target gene changes ($\Delta$CT$^T$) between the groups (Exp-Con) are normalized to that for the reference gene ($\Delta$CT$^R$) [4]. The procedure needs the standard curves (serial log10-dilution) for the calculations of application efficiency (aE) and copy numbers as presented in the following part:

$$CT\ Ratio = \left( \frac{E_1}{E_2} \right)^{\Delta CT},$$

where, $E = aE + 1$ & $aE = 0.95 - 1.05$

$\Delta CT^T = CT^T_{(Con)} - CT^T_{(Exp)}$

$\Delta CT^R = CT^R_{(Con)} - CT^R_{(Exp)}$

If $E_1 \approx E_2$, then:

$$CT\ Ratio = \left( \frac{E_1^{\Delta CT^T}}{E_2^{\Delta CT^R}} \right)$$

$\Delta CT = \Delta CT^T - \Delta CT^R$

The relative expression procedure

In this procedure, the CT (or Copy) numbers of samples are adjusted in the relative mode. Similar to the above procedure, the application efficiencies (aE) and copy numbers are calculated using the standard curves and the normalized sample ratios are adjusted to the mean of con-defined group so that they can be compared statistically.

The steps are indicated in the following subsections:

Determination of CT (Cycle Threshold) number: It is the intersection between threshold line and logarithmic amplification plot and, is used in both procedures. The CT numbers reported at the range of 17-27 are suitable to analyze.

CT ratio of target and reference genes: The CT numbers of target genes (CT$^T$) are normalized by these prepared for reference genes (CT$^R$) in all samples (S).

$$CT\ Ratio = \left( \frac{E_1}{E_2} \right)^{\Delta CT^R}$$

where, $E = aE + 1$ & $aE = 0.95 - 1.05$

$\Delta CT^T = CT^T_{(Con)} - CT^T_{(Exp)}$

$\Delta CT^R = CT^R_{(Con)} - CT^R_{(Exp)}$

If $E_1 \approx E_2$, then:

$$CT\ Ratio = \left( \frac{E_1^{\Delta CT^T}}{E_2^{\Delta CT^R}} \right)$$

$\Delta CT = \Delta CT^T - \Delta CT^R$

Adjusting CT: The mean of CT$^T$ (mCT$^T$) is calculated in the Con-defined group (or Zero group) and considered to be equal to 1 (mCT$^T_{(Con)}$=1). Other CT$^T$ values are adjusted on the basis of mCT$^T_{(Con)}$. This approach is able to adjust two variables, $\Delta$CT and E.

$$Adjusted\ CT^T = \frac{CT^T_{(Con)}}{mCT^T_{(Con)}}$$

The relative expression plots (obtained for several groups) are important to show statistical differences. Based on the non parametric distribution of the data, the CT$^T$ values may be adjusted with the median of CT$^T_{(Con)}$ (medCT$^T_{(Con)}$). Although, it usually has not effect on comparative statistical results and the adjusted data distribution but, it can affect the fold reports on the plots. Moreover, the data adjustment with medCT$^T_{(Con)}$ may introduce the large deviations around the measures of central tendency (Mean or Median) dependent on the distance between the median and the mean in each group.

Statistical tests: The non parametric distribution of the adjusted CT$^T$ values is usually evaluated with One-Sample Kolmogorov-Smirnov Test. Furthermore, the data distribution between subgroups can be evaluated using Median Test. In most studies, they are significantly lower than 0.05 so that the non parametric analyses must be performed with Mann Whitney U, Kruskal-Wallis (Independent samples), Wilcoxon and Friedman (Related samples) tests. On the other hand, the data must be evaluated with student t, ANOVA (Independent samples) Pairs, ANCOVA (Related samples) tests [5,6].

*Corresponding author: Dr. Mohammad Najafi, Biochemistry Department, Razi Drug Research Center, Cellular and Molecular Research Center, Tehran University of Medical Sciences, Tehran, Iran, Tel/Fax: 982188622742; E-mail: nbssmsbn@tums.ac.ir

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In conclusion, the fold changes show absolutely the between-group alterations while the relative changes present statistically between-group differences after adjusting the data in the real time qPCR data analyzing procedures. The statistical tests are dependent on the study method and data distribution.

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