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How to Analyze Real Time qPCR Data?

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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-limited quantitative (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 (ΔCT_T) between the groups (Exp-Con) are normalized to that for the reference gene (ΔCT_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:

$$CT\ Ratio = \frac{(E_T)^{\Delta CT_T}}{(E_R)^{\Delta CT_R}}$$

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

$$\Delta CT_T = CT_{T(Con)} - CT_{T(Exp)}$$

$$\Delta CT_R = CT_{R(Con)} - CT_{R(Exp)}$$

$$If\ E_T \cong E_R, \qquad thus:$$

$$CT\ Ratio = \frac{(E)^{\Delta CT_T}}{(E)^{\Delta CT_R}}$$

$$CT\ Ratio = (E)^{\Delta CT_T} \times (E)^{\Delta CT_R}$$

$$CT\ Ratio = (E)^{-(\Delta CT_R - \Delta CT_T)}$$

$$\Delta\Delta CT = \Delta CT_R - \Delta CT_T$$

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).

$$CT\ Ratio = \frac{(E_T)^{\Delta CT_T}}{(E_R)^{\Delta CT_R}}$$

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

$$\Delta CT_T = CT_{T(Con)} - CT_{T(Exp)}$$

$$\Delta CT_R = CT_{R(Con)} - CT_{R(Exp)}$$

$$If\ E_T \cong E_R, \qquad thus:$$

$$CT\ Ratio = \frac{(E)^{\Delta CT_T}}{(E)^{\Delta CT_R}}$$

$$CT\ Ratio = (E)^{\Delta CT_T} \times (E)^{\Delta CT_R}$$

$$CT\ Ratio = (E)^{-(\Delta CT_R - \Delta CT_T)}$$

$$\Delta\Delta CT = \Delta CT_R - \Delta CT_T$$

Adjusting CT_s: The mean of CT_s (mCT_s) is calculated in the Con-defined group (or Zero group) and considered to be equal to 1 ($mCT_{s(Con)}$=1). Other CTs values are adjusted on the basis of $mCT_{s(Con)}$. This approach is able to adjust two variables, ΔCT and E.

$$Adjusted\ CT_S = \frac{CT_S}{mCT_S(Con)}$$

The relative expression plots (obtained for several groups) are important to show statistical differences. Based on the non parametric distribution of data, the CTs values may be adjusted with the median of $CT_{s(Con)}$ ($medCT_{s(Con)}$). 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 $medCT_{s(Con)}$ 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 CTs 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].

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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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