Analyzing real-time PCR data by the comparative C_T method

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Two different methods of presenting quantitative gene expression exist: absolute and relative quantification. Absolute quantification calculates the copy number of the gene usually by relating the PCR signal to a standard curve. Relative gene expression presents the data of the gene of interest relative to some calibrator or internal control gene. A widely used method to present relative gene expression is the comparative C_T method also referred to as the $2^{-\Delta\Delta C_T}$ method. This protocol provides an overview of the comparative C_T method for quantitative gene expression studies. Also presented here are various examples to present quantitative gene expression data using this method.

INTRODUCTION

Quantifying gene expression levels has become a staple of most molecular biological laboratories. By measuring the amount of cellular RNA, one is able to determine to what extent that particular gene is being expressed. For many genes, the expression levels change dramatically from gene to gene, cell to cell or during various experimental conditions. Some examples of quantitative gene expression studies include: as a validation of protein levels[1]; as a validation of the extent of transcription of a gene[2]; to study the difference in expression of a gene in the diseased state compared to the normal state[3]; change in gene expression during cell differentiation or development[4]; change in expression for cells that are exposed to a chemical substance (e.g., drug, toxin, hormone or cytokine)[5]; quantification of noncoding RNA gene expression[6]; to validate the effectiveness of small interfering RNA[7] or antisense oligonucleotides[8]; and as a diagnostic tool[9].

Real-time RT-PCR is a powerful tool to quantify gene expression. The quantitative endpoint for real-time PCR is the threshold cycle (C_T). The C_T is defined as the PCR cycle at which the fluorescent signal of the reporter dye crosses an arbitrarily placed threshold (**Fig. 1**). By presenting data as the C_T, one ensures that the PCR is in the exponential phase of amplification. The numerical value of the C_T is inversely related to the amount of amplicon in the reaction (i.e., the lower the C_T, the greater the amount of amplicon).

There are several methods of reporting real-time PCR data including presentation of real-time PCR data as absolute or relative expression levels. Absolute expression provides the exact copy number following transformation of the data via a standard curve[10]. The data are typically presented as copy number per cell. In relative quantification, the real-time PCR data is presented relative to another gene often referred to as an internal control. Absolute quantification is required when a precise quantity of amplicon is desired, for example, calculation of viral load[11]. The disadvantage of absolute quantification includes the increased effort to generate standard curves. Furthermore, it is often unnecessary to present data as absolute copy number and relative expression will suffice. For example, if a treatment increases the expression of a particular gene from 10,000 to 50,000 copies per cell, reporting the data as a fivefold increase in gene expression is sufficient.

Several methods have been developed over the years to present the relative gene expression. The efficiency correction method calculates the relative expression ratio from the real-time PCR efficiencies and the C_T (ref. 12). Real-time PCR data has been analyzed using the so-called sigmoidal curve fitting methods that fit the experimental data to an empirical equation and results in the prediction of the PCR efficiency and an estimate of the initial copy number of the amplicon[13,14]. Another method of presenting quantitative real-time PCR data is the comparative C_T method (also known as the $2^{-\Delta\Delta C_T}$ method)[15]. The comparative C_T method makes several assumptions, including that the efficiency of the PCR is close to 1 and the PCR efficiency of the target gene is similar to the internal control gene[15].

There are advantages and disadvantages to each of the methods to analyze relative real-time PCR data. An advantage of the efficiency correction method[12] is that the PCR efficiency of the target and internal control genes are included in the equation and therefore differences in the efficiency between target and internal control will be accounted for in the calculation. The sigmoidal

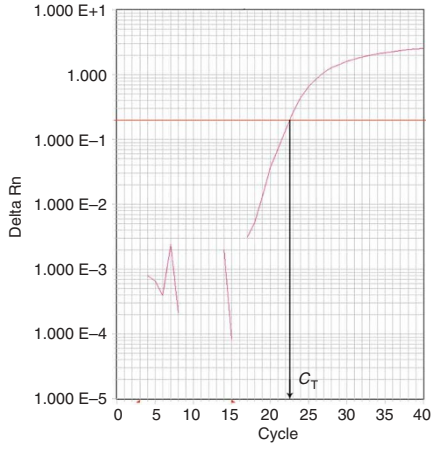

Figure 1 | Real-time PCR output, calculation of C_T. Data is presented from a typical real-time PCR output. The PCR was run for 40 cycles. The point at which the curve intersects the threshold (horizontal red line) is the C_T. The C_T in this example is 22.5.

curve fitting models[13,14] have the advantage that PCR efficiency does not need to be calculated by a separate experiment and is estimated during the analysis. Advantages of the comparative C_T method include ease of use and the ability to present data as 'fold change' in expression. Disadvantages of the comparative C_T method include the assumptions of PCR efficiency must hold or the PCR must be further optimized. Disadvantages of the simulated kinetic model include the use of nonlinear regression analysis for the calculations. In addition, it was recently shown that issues relating to fitting the portion of the curve where the fluorescence signal is hidden in the noise band may account for biased estimates of the amplification efficiency[16]. A disadvantage of the efficiency correction method includes that the efficiency of the target and control genes must be determined for each experiment. This is impractical when profiling many hundreds of genes by real-time PCR (see Gene expression profiling).

Eq. 1 shows the final form of the $2^{-\Delta\Delta C_T}$ equation, the derivation of which has been reported previously in Applied Biosystems User Bulletin No. 2 (P/N 4303859) and ref. 15.

$$\text{Fold change} = 2^{-\Delta\Delta C_T} \quad (1)$$

This form of the equation may be used to compare the gene expression in two different samples (sample A and sample B); each sample is related to an internal control gene. Sample A may be the treated sample and sample B, the untreated control; sample A may be the diseased state and sample B, the normal state or sample A is infected with virus and sample B is not. Expanding Eq. 1 to its full form:

$$2^{-\Delta\Delta C_T} = [(C_T \text{ gene of interest} - C_T \text{ internal control})\text{sample A} - (C_T \text{ gene of interest} - C_T \text{ internal control})\text{sample B})] \quad (2)$$

It does not matter which ΔC_T is placed first and which is placed second in Eq. 2, however, the order affects how the results are interpreted. We recommend calling the untreated control or normal (i.e., not diseased), the calibrator or sample B. In this way the data may be interpreted as 'the expression of the gene of interest relative to the internal control in the treated sample compared with the untreated control'.

Most real-time PCR instruments are equipped with either a 96- or 384-well plate configuration. Thus, real-time PCR may be used as a 'low-density array'. Although PCR will never generate the throughput that is achieved by cDNA microarrays, PCR has the advantage of unparalleled sensitivity. Situations exist where one may want to profile several hundreds of genes and for this reason, real-time PCR is ideal. One application is microRNA (miRNA). There presently exists some 530 human and 440 mouse miRNAs[17]. Profiling the mature miRNA using real-time PCR configured in 384-well reaction plates and an assay such as the TaqMan miRNA assays[10] is a convenient method to generate high-quality data on several hundreds of miRNAs. Another example is to make low-density arrays of certain classes of genes (e.g., apoptosis, cell cycle regulation, particular signal transduction pathway). Following the experiment, one may study the expression of various genes within a particular pathway.

Another issue that comes to mind when performing gene expression profiling using real-time PCR is how best to present the data. Most often in gene expression profiling experiments the data are presented as a heatmap[18]. The heatmap allows one to visualize hundreds of data points on a single figure. Data presented as heatmaps are presented as individual data points. When real-time PCR data is to be presented as individual data points it should be presented as $2^{-\Delta C_T}$ or 2^{-C_T} rather than the raw C_T value[15]. When presenting the data from gene expression profiling studies, one would always want to normalize the data to an internal control, so in this case the $2^{-\Delta C_T}$ is appropriate where ΔC_T = (C_T gene of interest − C_T internal control). Heatmaps generally undergo a log transformation. Therefore it is not necessary to use the $2^{-\Delta C_T}$ data and the $-\Delta C_T$ data will suffice.

A common question that arises regarding C_T data is how to perform calculations using the C_T and more importantly how to obtain the mean ± s.d. in order to perform statistical analysis. Quantitative gene expression using real-time PCR will almost always rely on PCR replicates (typically two to four PCR replicates per cDNA sample). Although there are several different approaches that could be taken, we recommend the following: (i) begin the analysis by taking the mean of these PCR replicates and (ii) then take the mean of the individual samples. It should be emphasized that statistical tests should not be run on the raw C_T data and s.d. should always be calculated after the $2^{-\Delta\Delta C_T}$, $2^{-\Delta C_T}$ or 2^{-C_T} transformation has been performed (see Example 5 in ANTICIPATED RESULTS).

The comparative C_T method has been widely used by scientists[19–21]. The purpose of this protocol is to provide an overview of the comparative C_T method. In addition, we report various tricks and tips that we have learned when using this method to present real-time PCR data. A number of examples of the calculations using the comparative C_T method are provided. Finally, some applications of the method that was not previously reported in our original article[15] including presentation of gene expression profiling data have been added.

MATERIALS

REAGENTS

- Isopropanol
- Ethanol
- Molecular biology grade water
- Chloroform
- Trizol reagent (Invitrogen)
- Glycogen for molecular biology (Roche)
- SYBR green PCR master mix (Applied Biosystems)
- RNase-free DNase I (10 U μl^{-1}; Roche)
- RNA guard (porcine; Amersham Pharmacia Biotech)
- SuperScript II (200 U μl^{-1}; Invitrogen)
- 100 mM dNTP set (Invitrogen)
- BSA (RNase-free; Amersham Pharmacia Biotech)
- Random primers (Invitrogen)
- Optical adhesive cover (Applied Biosystems)
- 96-well real-time PCR plate (Applied Biosystems)
- Phase lock gel (2 ml; Eppendorf)

EQUIPMENT

- DNA Engine Thermal Cycler PTC-0200 (MJ Research)
- 7900 HT real-time PCR instrument (Applied Biosystems)
- NanoDrop spectrophotometer ND-1000 (NanoDrop Technologies)
- Allegra 25R centrifuge with microplate adapters (Beckman Coulter)
- Stainless steel mortar and pestle sets (Fisher)

BOX 1 | ISOLATION OF RNA FROM CULTURED CELLS OR BLOOD

With the exception of Step 5, the protocol for isolating RNA is identical to that described in the Trizol protocol.
1. Remove cells from the −80 °C freezer and place the tubes on dry ice to prevent thawing.
2. Remove one sample from the dry ice and add 1 ml Trizol. It is not necessary to thaw the cells before adding the Trizol.
3. Lyse the cells using a P-1000 pipettor by repeated pipetting until the solution is homogeneous. Incubate at room temperature (20–24 °C) for 5 min.
4. Add 200 µl chloroform, shake by hand 15 times and incubate at room temperature for 3 min.
5. Transfer the mixture to a 2-ml Eppendorf phase lock tube.
6. Centrifuge the phase lock tube in the cold for 15 min at 12,000*g*. Remove the supernatant and place into a 1.5-ml colored, microcentrifuge tube. Colored tubes will enhance visualizing the RNA pellet.
7. Add 500 µl of isopropanol and precipitate the RNA for 10 min at room temperature. This is a good stopping point. If necessary, the samples may be placed in the −20 °C freezer overnight.
8. Place the tubes in a microcentrifuge in the cold. Orient the caps to the outside of the centrifuge's rotor. Centrifuge for 10 min at 12,000*g*. The RNA pellet should be visible at the bottom of the tube on the side that was oriented to the rotor's outside.
9. Decant the supernatant into a 2-ml microcentrifuge tube. It is not necessary to remove all of the supernatant. Add 1,000 µl of 75% ethanol. Centrifuge for 5 min at 7,500*g* in the cold.
10. Decant the supernatant into the same 2-ml microcentrifuge tube used for the isopropanol. Briefly spin the tube containing the RNA for several seconds to bring the residual ethanol to the bottom of the tube.
11. Using a pipette, remove most of the residual ethanol, being careful not to disturb the pellet. To remove the remaining ethanol, place the tubes into a dessicator containing a porcelain platform filled with Drierite. Connect to house vacuum for 5 min. It is not necessary to completely dry the sample because the residual water will not affect the reverse transcription step.
12. Dissolve the RNA pellet in 30–50 µl of molecular biology grade water and place the tubes on ice. RNA may be stored at −80 °C; however, it is recommended that RNA be converted to cDNA on the same day of the isolation.

PROCEDURE

1| Isolate RNA as described in **Box 1**.

2| Expose the RNA with DNAse I as described in **Box 2**.

3| Synthesize cDNA as described in **Box 3**.

4| Perform PCR as described in **Box 4**.
▲ **CRITICAL STEP** As stated in the INTRODUCTION, use of the comparative C_T method relies upon the target gene and internal control gene having similar efficiencies.

5| Determine PCR efficiency of gene of interest and internal control gene (**Box 5**).
▲ **CRITICAL STEP** The internal control gene should not change under the experimental conditions. Methodology to validate internal control genes has been described previously in ref. 22.

BOX 2 | DNase TREATMENT

1. Briefly treat the RNA with RNase-free DNase to remove any residual genomic DNA that may be present in the RNA. Prepare the DNase master mix accounting for 1–2 additional reactions.

Ingredient	Per reaction (µl)	For *x* reactions (µl)
RNase-free DNase I (10 U μl^{-1})	1.8	$x \times 1.8$
RNA guard	0.3	$x \times 0.3$
25 mM $MgCl_2$	2.4	$x \times 2.4$
Total	4.5	$x \times 4.5$

2. Add 1.2 µg of RNA, 4.5 µl of master mix and water to 30 µl into 200-µl PCR strip tubes.
3. Mix by gentle flicking, and briefly spin on a minicentrifuge that can handle strip tubes. Using a PCR Thermal Cycler, incubate at 37 °C for 10 min and then 90 °C for 5 min to inactivate the DNase.

BOX 3 | cDNA SYNTHESIS

Ingredient	Per reaction (μl)	For x reactions (μl)
5× SuperScript II buffer	10	$x \times 10$
10 mM dNTPs	5	$x \times 5$
0.1 M DTT	5	$x \times 5$
BSA (RNase-free, optional)	1.25	$x \times 1.25$
Random primers	0.25	$x \times 0.25$
RNA guard	1.25	$x \times 1.25$
Superscript II reverse transcriptase	1.25	$x \times 1.25$
Molecular biology water	1	$x \times 1$
Total	25	$x \times 25$

1. Prepare the master mix shown above, accounting for one to two additional reactions per gene.
2. Add 25 μl of the reverse transcription master mix to 200-μl PCR strip tubes labeled with the date and sample number on the side.
3. Add 25 μl of the DNase-treated RNA to each tube. This will contain 1 μg of RNA.
4. Incubate at the following temperatures using a PCR thermal cycler: 26 °C for 10 min (to allow the random hexamers to anneal), 42 °C for 45 min (reverse transcription) and 75 °C for 10 min (to inactivate the reverse transcriptase).
5. The resulting cDNA may be analyzed immediately by real-time PCR or stored at −20 or −80 °C.

6| Select the appropriate internal control gene for your experiment. Possible examples include 18S rRNA, 7S rRNA, U6 RNA, β actin, GAPDH or small nucleolar RNAs (snoRNAs).

7| Present the real-time PCR data from the replicate cDNAs of the potential internal control genes as 2^{-C_T} (**Box 6**).

8| Perform a Student's *t*-test on the data to determine whether the expression of the internal control gene varies under the experimental conditions.

9| See Example 3 in the ANTICIPATED RESULTS for a hypothetical example to determine whether a gene is suitable for use as an internal control in a drug treatment experiment.

10| Decide which equations to use to analyze your data. If you are comparing the gene expression in one sample to another (e.g., cells treated with ciplatin versus untreated cells[5]), then use the equations for the $2^{-\Delta\Delta C_T}$ method described

BOX 4 | REAL-TIME PCR

1. Perform triplicate PCRs per gene, per cDNA sample.
2. Prepare the following master mix, accounting for one to two additional reactions per gene.

Ingredient	Per reaction (μl)	For *x* reactions (μl)
2× SYBR green reagent	12.5	$x \times 12.5$
Forward/reverse primer mix (50 μM each)	0.125	$x \times 0.125$
Molecular biology grade water	7.375	$x \times 7.375$

3. Dilute the cDNA 1:50 or 1:100 by first placing molecular biology grade water into a disposable sterile basin. Add 99 μl of the water into the PCR strip tube using a multichannel pipette.
4. Add 1 μl of cDNA to the labeled strip tubes using a multichannel pipette (e.g., Rainin L8-10). Use a multichannel pipette (e.g., Rainin L8-200) to mix the solution 20 times. For efficient mixing, set the pipette at 75% of the solution's volume (75 μl in this example).
5. Recap the undiluted cDNA with new strip caps to prevent cross-contamination.
6. Use a fine tip marker to mark the plate to the location of the different master mixes.
7. Use the repeating pipette (Rainin, E12-20) to add 20 μl of master mix to each sample. Add one row at a time.
8. Add 5 μl of dilute cDNA to each of the wells using a multichannel pipette.
9. Add the optical adhesive cover and seal using the sealing tool. Perform a brief spin (up to 1,500 r.p.m.) on a centrifuge equipped with a 96-well plate adapter.
10. Perform PCR using the real-time instrument per the manufacturers' protocol. Typically, 40 cycles of 15 s at 95 °C and 60 s at 60 °C followed by the thermal dissociation protocol for SYBR green detection.

BOX 5 | DETERMINE THE PCR EFFICIENCY OF GENE OF INTEREST AND INTERNAL CONTROL GENE

1. Make tenfold serial dilutions of cDNA and then amplify the cDNA using primers to both the gene of interest and internal control.
2. Plot the C_T (y-axis) versus log cDNA dilution (x-axis). Determine the slope of the line.
3. PCR efficiency is then calculated by the equation $m = -(1/\log E)$, where m is the slope of the line and E is the efficiency[23].
4. Although no hard and fast rules apply as to what makes the efficiency of the genes similar enough to use the comparative C_T method, a rough guide is that they should be within 10% of each other (1.8× to 2.2×).
5. If one gene is outside of the 1.8× to 2.2× range, then the PCR conditions should be optimized until they are within 10% of 2. This may be achieved by adjusting the primer and/or the $MgCl_2$ concentrations in the reaction.
6. If the PCR efficiency is not optimized following adjustment of the PCR conditions, then it is recommended to design new primers.

in the INTRODUCTION. Situations where it would be advisable to modify this approach are (i) for the presentation of real-time PCR data as individual data points (**Box 6**) or (ii) for the presentation of real-time PCR data generated from gene expression profiling (continue to Step 11).

Gene expression profiling

11| Select the primers to amplify the genes of interest and internal control genes for the profiling experiment. Profiling experiments may be performed in 96- or 384-well plates.

12| Perform Steps 1–8 in the general protocol.

13| Approximate the efficiencies of the different amplifications.

14| One of the issues that arises as the number of genes are increased is how would one calculate the PCR efficiency on so many different genes? It is not practical to perform analysis like that presented in **Box 5** on several hundreds of different genes.

15| In these situations, the PCR efficiency may be approximated by the shape of the PCR amplification plot. This type of analysis should only be performed on the logarithmic PCR amplification plots and not the linear plots. Primers that produce a nearly identically shaped reaction plot will be expected to have similar efficiency. On the contrary, those that do not produce a similar shape may need to be redesigned or the PCR further optimized. See **Figure 2** for an example.

16| Present the data as $-\Delta C_T$ and plot the heatmap as described[18]. An example is shown in **Figure 3**.

ANTICIPATED RESULTS

Example 1

The mean C_T of the *HOXD10* gene in treated and untreated samples was 24.6 and 27.5, respectively. The mean C_T of the 18S rRNA in the treated and untreated samples was 9.9 and 9.8, respectively. What is the fold change in expression of the *HOXD10* gene due to treatment?

$$\text{Fold change due to treatment} = 2^{-\Delta\Delta C_T}$$
$$= 2^{-[(24.6-9.9)-(27.5-9.8)]}$$
$$= 8$$

BOX 6 | PRESENTATION OF REAL-TIME PCR DATA AS INDIVIDUAL DATA POINTS

The $2^{-\Delta\Delta C_T}$ method described in the INTRODUCTION is not necessary in situations where the data from a real-time PCR experiment are to be presented as individual data points or normalized individual data points. This is common during gene expression profiling, when one wants to determine the variation in the measurements from replicate PCR runs or when determining whether the internal control gene varies under the conditions of the experiment. In these situations, data may be presented either as $2^{-\Delta C_T}$ or 2^{-C_T}. The normalized, individual data point (e.g., $2^{-\Delta C_T}$) is identical to the $2^{-\Delta\Delta C_T}$ (Eq. 1); only the sample is not related to a calibrator (hence, only one ΔC_T is present in the equation). The 2^{-C_T} form of the equation may be considered the non-normalized, individual data point. The only difference between $2^{-\Delta C_T}$ and 2^{-C_T} is whether one wants to include an internal control or not. Almost always the C_T from a PCR should be compared to an internal control. Exceptions include when one wants to determine whether the internal control gene changes during the experiment (Example 3 in ANTICIPATED RESULTS) or to determine the variation in individual PCRs (see ref. 15 for an example).

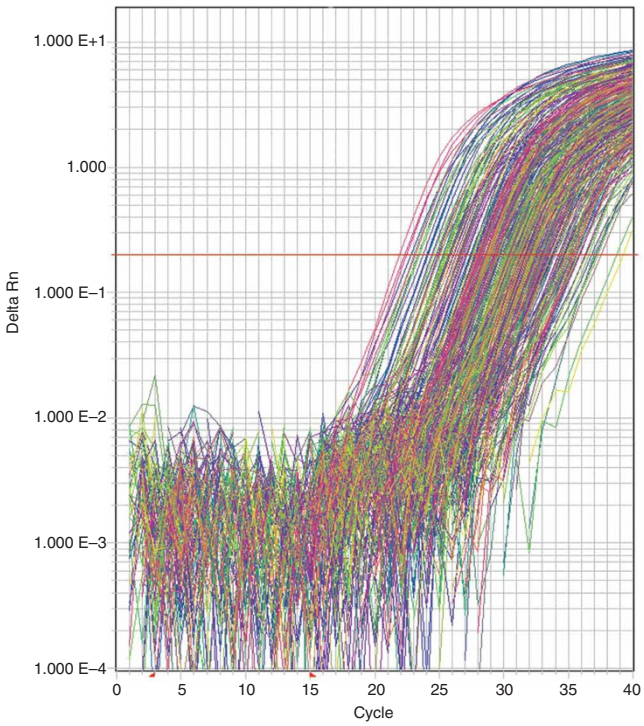

Figure 2 | Real-time PCR plots from gene expression profiling. The expression of 366 different genes was profiled in the identical sample of cDNA. The shapes of the amplification plots are similar, demonstrating similar PCR efficiency.

This is interpreted as the expression of *HOXD10* mRNA was increased by eightfold due to treatment. If the first ΔC_T is greater than the second ΔC_T, then the value of $2^{-\Delta\Delta C_T}$ will be <1. This implies that there was a reduction in the expression due to treatment. Taking the negative inverse of $2^{-\Delta\Delta C_T}$ will provide us with the fold change reduction in expression.

Example 2

The mean C_T of the *HOXD10* gene in treated and untreated samples was 26.5 and 24.9, respectively. The mean C_T of the 18S rRNA internal control in the treated and untreated samples was 9.7 and 9.9, respectively. What is the fold change in expression of the *HOXD10* gene due to treatment?

$$\text{Fold change due to treatment} = 2^{-\Delta\Delta C_T}$$
$$= 2^{-[(26.5-9.7)\ -\ (24.9-9.9)]}$$
$$= 0.287$$
$$\text{Fold change due to treatment} = \frac{-1}{0.287} = -3.5$$

This is interpreted as the expression of *HOXD10* mRNA was reduced by 3.5-fold due to treatment.

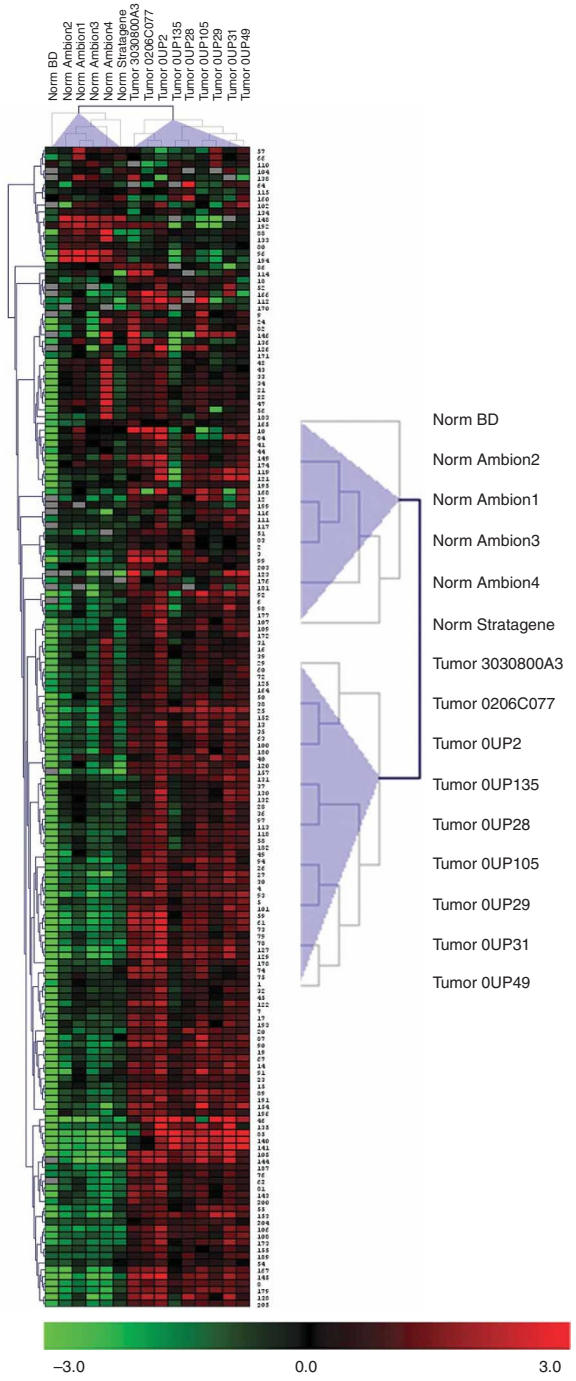

Figure 3 | Real-time PCR expression profiling of microRNA in pancreas tissue. The expression of over 225 mature microRNAs was profiled in six normal pancreases (norm) and nine pancreatic tumors using a real-time PCR assay. The heatmap was generated by a log transformation of the real-time PCR data presented as $\Delta C_T(C_{T\ \text{miRNA}} - C_{T\ 18S\ \text{rRNA}})$.

Example 3

The gene expression is to be compared in cell cultures that are treated with a hypothetical drug to those that are untreated. The mean C_T from replicate runs of an internal control gene are 27.2, 27.0 and 27.4 (treated samples) and 26.2, 26.3 and 26.0 (untreated samples). What is the fold change in expression of the internal control in the treated versus the untreated samples? Does this gene serve as a useful internal control in this experiment? First calculate the mean C_T values as 2^{-C_T}.

Treated	Untreated
$2^{(-27.2)} = 6.49E{-}09$	$2^{(-26.2)} = 1.30E{-}08$
$2^{(-27.0)} = 7.45E{-}09$	$2^{(-26.3)} = 1.21E{-}08$
$2^{(-27.4)} = 5.65E{-}09$	$2^{(-26.0)} = 1.49E{-}08$
Mean = 6.53E−09	Mean = 1.33E−08

The fold change in the internal control in the treated samples compared to the untreated sample is: 6.53E−09/1.33E−08 = 0.490. Drug treatment reduced the expression of the internal control by 2.04-fold so it would not make a good internal control gene.

Example 4

The expression of the BCL-2 and 18S rRNA genes were quantified in a group of 20 HER2 positive breast cancer patients and in 20 HER2 negative breast cancer patients. What is the mean ± s.d. for the HER2 expression in each group? What is the difference in the Bcl-2 expression in the HER2 positive compared with the HER2 negative patients?

Because these are different patient samples, there is no means to justify which HER2 positive sample is compared with which HER2 negative sample and therefore the $2^{-\Delta\Delta C_T}$ method cannot be used. It is best to calculate the mean ± s.d. for each group as individual data points using

$$2^{-\Delta C_T}\left[2^{-(C_{T\ \mathrm{Bcl-2}} - C_{T\ 18S\ \mathrm{rRNA}})}\right].$$

The s.d. may be calculated from these data. If the mean ± s.d. $2^{-\Delta C_T}$ (HER2 positive group) is $1.03 \times 10^{-6} \pm 9.87 \times 10^{-8}$ and the mean ± s.d. $2^{-\Delta C_T}$ (HER2 negative group) is $2.06 \times 10^{-8} \pm 2.43 \times 10^{-9}$, then the difference in the Bcl-2 expression in the HER2 positive compared with the HER2 negative patients is $1.03 \times 10^{-6}/2.06 \times 10^{-8}$ or 50-fold. A Student's *t*-test may also be performed on the data to determine whether the difference is statistically significant.

Example 5

As another example, let us assume that three mice were treated with an experimental drug and three mice were untreated. cDNA was synthesized from the livers of each mouse and triplicate PCRs were performed on each sample of cDNA. The mean C_T (treated mice) from the triplicate PCRs for the target and internal control gene are 27.2, 28.0, 27.8 (target gene) and 24.2, 24.7 and 24.9 (internal control gene). The mean C_T (untreated mice) from the triplicate PCRs for the target and internal control gene are 22.8, 23.0, 22.4 (target gene) and 24.8, 25.0 and 24.7 (internal control gene). What are the mean ± s.d. and the coefficient of variation (CV) for the relative amount of target gene in both groups of mice? What is the difference in expression of the target gene in the liver of the treated mice compared to the untreated mice?
Using the $2^{-\Delta C_T}$ method gives:

Treated mice	Untreated mice
$2^{-(27.2-24.2)} = 0.125$	$2^{-(22.8-24.8)} = 4.00$
$2^{-(28.0-24.7)} = 0.102$	$2^{-(23.0-25.0)} = 4.00$
$2^{-(27.8-24.9)} = 0.134$	$2^{-(22.4-24.7)} = 4.92$

The mean ± s.d. (CV) is: 0.120 ± 0.0168 (13.9%) (treated mice). The mean ± s.d. (CV) is: 4.31 ± 0.534 (12.4%) (untreated mice). The fold change in expression between the treated and untreated mice is: 0.120/4.31 = 0.0278; fold change due to treatment = −1/0.0278 = −35.9.

An example to approximate the PCR efficiencies from reactions using multiple genes is shown in **Figure 2**. In this example, the expression of 366 different genes was profiled in the identical sample of cDNA. As one can see from the figure, the shapes of the amplification plots are similar to one another, in particular the slope of the geometric phase of amplification, suggesting that the PCR efficiencies are approximately the same. An example of gene expression profiling data that was generated using real-time PCR is shown in **Figure 3**. This particular data set is the expression of over 225 mature miRNAs that were profiling using the TaqMan miRNA assays (Applied Biosystems)[10]. The $-\Delta C_T$ data was used in this example to generate the heatmap.

Conclusion

Real-time quantitative PCR has become a staple of most biologically oriented laboratories. Some investigators are often mystified as to how one deals with the data generated from real-time PCR experiments. The comparative C_T method is a widely used method to present real-time PCR data. Using some of the tricks, tips and examples presented here will hopefully demystify the method and allow more investigators to successfully use it for analyzing real-time PCR data.

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