Quantitative Real-Time RT-PCR

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Abstract

Quantification of gene expression by real-time RT-PCR has become the gold standard to which all other quantification methods are compared. Two very simple precautions, normalizing the cDNA to contain equal amounts of RNA and validation of the internal control gene, combined with RNA of sufficient quality, will almost certainly guarantee high quality data time after time. Described here is a protocol to normalize the RNA content of multiple cDNA reactions using the Thermo Scientific NanoDrop™ 1000 Spectrophotometer.

Introduction

Quantifying gene expression has become a staple of most biologically-oriented research laboratories. Reverse transcriptase-polymerase chain reaction (RT-PCR) amplifies cDNA following its transcription from RNA. Real-time RT-PCR uses dedicated instrumentation to quantify the amplification process by detecting the fluorescence emitted following each PCR cycle. Proper primer design and good technique coupled with reliable reagents and instrumentation are critical to generating high quality data. As with many multi-step processes, laying down a solid foundation is essential to obtaining consistent and reliable data. Over the years we have found that adherence to two very simple principles will consistently yield high quality data: normalization of each reverse transcription reaction to contain identical amounts of sufficient quality RNA and proper validation of the internal control gene.

The purpose of most quantitative gene expression studies is to compare the gene expression in one sample to another sample. Examples include comparing the gene expression in different cell lines or tissues, studying the time course of drug treatment compared to the untreated control, and measuring the gene expression in diseased versus non-diseased tissues. Real-time PCR data is commonly normalized to an internal control gene. Appropriate genes are typically house-keeping genes whose expression usually does not change under the conditions of the experiment. In order to determine if the internal control gene changes under the conditions of the experiment, the expression of the control gene must be quantified by real-time PCR before quantifying the gene(s) of interest. The most accurate way to validate the internal control gene is to ensure that each reverse transcription reaction in the study contains equivalent amounts of RNA. Otherwise, one cannot conclude if the fluctuation in the internal control gene is due to the experiment or to the differences in RNA that was added to each reaction.

This application note describes how the NanoDrop 1000 is used to normalize the amount of RNA to be added to each reverse transcription reaction. As an example, the expression of U6 RNA will be analyzed by real-time PCR in 33 specimens of human liver tissue. Basic principles of cDNA synthesis, RNA extraction, and PCR will not be described.

RNA Isolation and Quantification

1. The study consisted of 33 human liver specimens; 18 specimens had cirrhosis and 15 specimens were free of cirrhosis. The study was designed to determine whether or not expression of several genes of interest changed with cirrhosis. The first step was to determine the change in expression levels of an internal control gene in the cirrhotic versus noncirrhotic tissues.

2. Frozen liver tissues were pulverized to a powder in a stainless steel mortar and pestle set that was pre-chilled on dry ice. RNA was isolate from each of the liver tissues with Trizol® reagent (Invitrogen) using the manufacturer's protocol. RNA was precipitated as described in the Trizol® protocol and all residual alcohol was evaporated from the RNA pellet. Each pellet was then dissolved in 25 µl of molecular biology grade water, and the sample tubes were placed on wet ice.

3. Using an L-2 pipet (Rainin Instruments), 1 µl of undiluted RNA was placed directly onto the lower measurement pedestal of the NanoDrop 1000. The upper optical pedestal was lowered and the measurement was taken. After each reading, the sample was wiped from both the upper and lower pedestals using a clean Kimwipe. Solvents were not necessary to clean the optical surfaces of the instrument. Each RNA sample was quantified in this manner.

4. Between 50-100 ng of RNA was set aside for analysis using the Agilent 2100 Bioanalyzer. This analysis quantifies the 28S and 18S rRNA species using capillary electrophoresis and applies the RIN (RNA integrity number) algorithm to assess RNA quality. RIN ranges from 1 (poor) to 10 (best). We typically use a RIN of 4-5 as our lower limit of RNA integrity.

5. RNA sample parameters and concentrations from the NanoDrop 1000 were imported into an Excel spreadsheet and expanded to include the last four columns in the table below for RT and real-time PCR reactions.

Sample ID	[RNA] ng/ul	A260	A260/A280	Volume (ul)	Total RNA (ug)	Volume for 1.2ug (ul)	Water (ul)
S1	2551	1.85	1.90	25	63.78	0.470	9.78
S2	2099.13	1.93	2.02	25	52.48	0.572	9.68
S3	2192.62	1.92	2.03	25	54.82	0.547	9.70
S4	2136.48	1.92	2.05	25	53.41	0.562	9.69
S5	1782.58	1.88	1.33	25	44.56	0.673	9.58
S6	1928.28	1.89	1.75	25	48.21	0.622	9.63
S7	1747.47	1.87	1.51	25	43.69	0.687	9.56
S8	1967.65	1.91	1.72	25	49.19	0.610	9.64

Reverse Transcription

6. The proper amount of RNA to be added to each reverse transcription reaction was then determined. We typically add 1 µg of RNA per 50 µl reaction. For most real-time PCR applications, one may easily go as low as 50 ng per reverse transcription reaction.

7. The volume of RNA (in µl) for each reaction was adjusted to contain 1.2 µg of RNA. (Alternatively, one may dilute each of the RNA solutions to the same concentration, e.g. 2 µg/µl, so that all RNA solutions are at the same concentration for future use).

8. The appropriate volume of RNA solution was then added to ensure the presence of 1.2 µg of RNA and additional water was added to make a final volume of 10 µl. Residual genomic DNA was removed by incubating the RNA solution with 15 units of RNase-free DNase I in 2 mM $MgCl_2$ (30 µl total volume) for 10 min at 37° C followed by 5 min at 90° C to inactivate the DNase.

9. 25 µl of the reaction was removed (to contain 1 µg of RNA) and the reverse transcription reaction was then assembled (50 µl final volume) as suggested in the SuperScript II protocol (Invitrogen). We typically use random primers to prime the reverse transcription. The cDNA products were isolated, purified, and then stored at -20° or -80° C.

Real-Time PCR

10. An important thing to remember about real-time PCR is that 'a little goes a long way.' cDNA is very stable when properly stored and can potentially be used for essentially an unlimited number of PCR reactions.

11. The cDNA was diluted (1:50 or 1:100) in molecular biology grade water. 5 ul of the diluted cDNA was added along with the reagents listed in the real-time PCR reagent kit (we routinely use the SYBR Green core reagent kit from Applied Biosystems) and gene specific primers (25 µl final volume).

12. Real-time PCR was performed using 40 cycles on a real-time PCR instrument. If using SYBR green, always perform the thermal denaturation protocol following the 40 cycles of PCR. This will allows one to determine if there is more than one product in the reaction. This step is not necessary if using TaqMan detection. Duplicate reactions per cDNA sample were performed, however, we routinely perform triplicates and occasionally quadruplicate reactions. Always include a 'no template' control reaction by substituting water for cDNA. If one is developing a new assay it is wise to include a minus rRT control reaction (containing all of the components of the reverse transcription reaction except enzyme) to ensure that genomic DNA has not contaminated the reaction.

Data Analysis

13. The threshold cycle number (C_T) is the quantitative endpoint for real-time PCR. The mean C_T was calculated from the duplicate, triplicate, etc. reactions. We present the mean C_T data as 2^{-CT}.

14. Data was plotted on a graph to provide a visual reference (Fig. 1). For statistical analysis, a t-test was performed to determine if there was a difference in the expression of the two groups, i.e. cirrhotic versus noncirrhotic livers. The mean expression from one group is divided by the mean expression of the other group to report the fold change in gene expression. The fold change in expression between both groups should ideally equal one for a properly validated internal control gene.

15. If one is unsure of which internal control gene to use, several different internal control genes should be tested and compared to determine which gene is suitable. We routinely use 18S rRNA or U6 RNA. The primers for these genes are as follows:

    18S rRNA
    5' GTAACCCGTTGAACCCCATT 3' (forward)
    5' CCATCCAATCGGTAGTAGCG 3' (reverse)

    U6 RNA
    5' CTCGCTTCGGCAGCACA 3' (forward)
    5' AACGCTTCACGAATTTGCGT 3' (reverse)

16. If the internal control gene does not change, then one is free to quantify the gene of interest by repeating steps 10 – 12 above. If the internal control gene changes then select another internal control gene and repeat steps 10 – 14.

Results

The mean U6 RNA expression in the cirrhotic and noncirrhotic livers was 2.94×10^{-5} and 2.78×10^{-5}, respectively (fold change = 1.058, $P = 0.571$). Since there was no change in the expression of the U6 RNA between the groups of liver tissues (Fig. 1), U6 RNA may be used as an internal control gene in these studies.

U6 RNA Expression in Cirrhotic and Non-Cirrhotic Livers

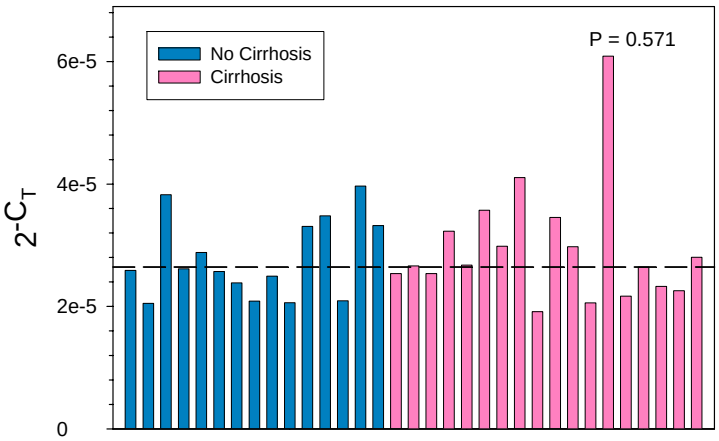

Figure 1. Total RNA was isolated from 33 specimens of human liver; 18 patients had cirrhosis and 15 patients were free of cirrhosis. One µl of RNA from each specimen was quantified on the NanoDrop 1000. A cDNA library was made from each of the RNA samples; all 33 cDNA reactions were normalized to contain 1 µg of total RNA. The U6 RNA expression was determined using real-time PCR and gene specific primers to U6 RNA. There was no significant difference in the U6 RNA expression between the cirrhotic and noncirrhotic groups ($P = 0.571$, student's t-test). Dashed line is the median value.

Conclusion

Quantitative real-time RT-PCR requires normalization of cDNA to reflect the same amount of initial RNA in each reaction. This is to ensure that any differences in RT-PCR output are that of actual RNA expression levels and not simply differences in the amounts of input cDNA template. By requiring only 1 ul of sample, the NanoDrop 1000 proved ideal for Q-RT-PCR normalization. Using the described method of template normalization enabled us to identify an appropriate internal control. By normalizing RNA samples in this manner and using an appropriately identified control gene, differences in RNA expression levels from two different sets of specimens can be determined with confidence.