

Technical note

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Absolute Quantification of Gene Expression using SYBR Green in the Eco 48 Real-Time PCR System.

Introduction

Gene expression is the process by which genetic information is converted into a functional product. This process uses an intermediate molecule, RNA, which is transcribed from DNA and then used as a template to translate the message into a protein product. Studies of gene expression provide a window into how an organism's genetic makeup enables it to function and respond to its environment.

Real-Time PCR can be used to quantify gene expression by two methods: relative and absolute quantification. The relative quantification method compares the gene expression of one sample to that of another sample: drug-treated samples to an untreated control, for example, using a reference gene for normalization. Absolute quantification is based on a standard curve, which is prepared from samples of known template concentration. The concentration of any unknown sample can then be determined by simple interpolation of its PCR signal (Cq) into this standard curve.

Purpose

This Protocol provides a step-by-step guide for quantifying the level of gene expression of a gene of interest using the absolute quantification method in the Eco 48 Real-Time PCR System. The steps covered in this protocol include:

1. RNA Extraction and Quantification
2. cDNA Synthesis
3. Preparation of Serial Dilutions
4. Real-Time PCR Amplification
5. Data Analysis

Several of these steps are prone to variability that can lead to data inconsistencies. For more detailed discussion of these issues, refer to the Nature Protocol by Nolan, Hands, and Bustin (1).

Step 1: RNA Extraction and Quantification

Optimal quantification of gene expression requires high quality, intact RNA. This implies appropriate sample collection and disruption, as sample dilute it with TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and read it at 260, 280, and 320 nm. Since neither proteins nor nucleic acids absorb at 320 nm, subtract your sample's 320 nm reading from its 260 and 280 readings. This is a good way to eliminate background light scatter caused by dirty cuvettes and dust particles. The purity of your RNA sample

is defined by the A_{260}/A_{280} ratio. Divide the absorbance at 260 nm by the absorbance at 280 nm. A ratio between 1.8 and 2.1 is indicative of highly purified RNA.

Calculate the concentration of your RNA using the following equation:

RNA concentration ($\mu\text{g}/\mu\text{l}$) = $(A_{260} * 40 * D)/1,000$ where **D = dilution factor**

For example, if you dilute 2 μl of your RNA into 498 μl of TE, pH 8.0, and obtain an A_{260} of 1.0, then your RNA concentration is 10 $\mu\text{g}/\mu\text{l}$.

RNA is very unstable. Always keep RNA on ice while working. Unless you are going to use your RNA immediately, store it at -80°C following preparation.

Step 2: cDNA Synthesis

Having total RNA, which is single stranded, is not enough for PCR amplification because Taq polymerase requires a DNA template to work. Therefore, a reverse transcriptase (RT) reaction needs to be performed to synthesize cDNA (complementary DNA) from the RNA template. If you are using a One-Step RT-PCR kit, which already incorporates the RT reaction, go directly to Step 3. Typically 0.1 μg to 1 μg of total RNA is a good starting amount of material (note that some kits allow you to use as high as 5 μg). Always follow your kit manual recommendations.

Below is a standard reverse transcription workflow:

1. Preheat the thermal cycler to 65°C .
2. Combine the following in a 0.2 ml PCR tube:

Component	Amount
Total RNA (up to 5 μg)	n μl
Primers (50 μM oligo dT, or 2 μM gene-specific primer, or 50 ng/ μl random hexamers)	1 μl
Annealing buffer	1 μl
RNase/DNase-free water	to 8 μl

3. Incubate in a thermal cycler at 65°C for 5 minutes, and then immediately place on ice for at least 1 minute. Centrifuge tube briefly.
4. Add the following to the tube on ice:

The slope of the line is a measure of the assay's efficiency. Slopes between -3.1 and -3.6 are considered acceptable (90% and 110% efficient, respectively), while a slope of -3.32 is indicative of 100% efficiency. The R^2 is a measure of the performance of the assay, and is the coefficient of correlation between the data generated and the results expected under ideal conditions. The R^2 should be greater than 0.99. This means that >99% of the total variation in the DNA samples that make up your curve can be explained by the relationship between the Cqs obtained and their respective DNA concentrations. To extract quantification data from any Cq value, the following equation is used:

$$\text{Quantity} = 10(\text{Cq}-b)/m$$

Where b is the y-intercept and m is the slope of the linear regression. The units of quantity are defined by the dilutions used to create the standard curve. If you ran unknown samples in your experiment, the software automatically calculates their quantity/concentration based on the standard curve.

Quantification data is typically depicted as either a weight quantity (e.g., picograms), a concentration (e.g., picograms/microliter), or as "copies" of a given gene. In an absolute quantification experiment like this one, the units used need to be specified by the user because all gene expression data from unknown samples is obtained by interpolating their PCR signals (Cq) into the standard curve. In other words, if the dilutions that make up the standard curve are measured in picograms, the unknown samples will also be measured in picograms. For additional information on how to analyze gene expression data go to: www.PCRmax.com/support. This protocol showed how to perform a gene expression experiment using the absolute quantification method. The same assay used here could be used to perform a relative quantification experiment, where the change in expression of a target gene is determined relative to the expression of a calibrator/normalizer gene.

Troubleshooting

In addition to enabling absolute quantification, running a standard curve is an essential tool for validating a new Real-Time PCR assay and a great starting point to troubleshoot problems you may have with your assay. If the slope, or efficiency, of your standard curve is outside the acceptable range, you can focus on the components that may be causing the problems. For example, if the efficiency is too high (or the slope is higher than -3.1), then there is likely a problem with your template. If the slope is too low

(below -3.6), or the efficiency is too low, then likely you have a problem with your reaction mix.

Template problems are a typical issue when studying gene expression. If RNA degradation is a concern, run a gel to assess the integrity of the RNA. If the RNA shows signs of degradation it is advisable to use another RNA or re-extract. It is also possible to add too much template to the Real-Time PCR assay.

If the concern is with the reaction mix, looking at the melt curve is a good place to start. As a best practice, run a melt curve at the end of every SYBR Green Real-Time PCR experiment. If more than one peak is detectable by melt curve analysis, the assay conditions may need to be optimized.

Under ideal conditions the water control (NTC) should not have any signal as a result of Real-Time PCR amplification. If a signal is detected, one possible cause is that the reagents have been contaminated with template. In this case you would get an amplicon melting at the same temperature as in the wells containing samples. Alternatively, the signal could be a result of primer-dimers or other assay-related artifacts. The detection of more than one peak, or a peak with a different melting temperature than the one in a well with a sample, on a melt curve analysis could explain the detection of a signal in the no template control. To learn more about gene expression study using Real-Time PCR go to www.PCRmax.com to get updates and training materials.

Public Databases with Documented and Validated Real-Time PCR Assays

- RTPrimerDB (<http://medgen.ugent.be/rtprimerdb/>)
- qPrimerDepot (<http://primerdepot.nci.nih.gov/>)
- The Quantitative PCR Primer Database (<http://web.ncicrf.gov/rtp/gel/primerdb/>)

References

1. Nolan T, Hands R and Bustin S (2006) Quantification of mRNA using real-time RT-PCR. *Nature Protocols* 1 (3): 1559-1582
2. Lefever S, Vandesompele J, Speleman F and Pattyn F (2008) RTPrimerDB: the portal for real-time PCR primers and probes. *Nucleic Acids Research* 1-4, October 23 online publication
3. Cui W, Taub D and Gardner K (2006) qPrimerDepot: a primer database for quantitative real time PCR. *Nucleic Acids Research* 35: D805-D809

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