Absolute Quantification of Gene Expression using SYBR Green in the Eco™ 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 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 inconsistancies. 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 well as proper isolation and storage of RNA. If you already have purified and quantified RNA, go directly to **Step 2**.

Once total RNA has been purified it needs to be quantified. UV spectroscopy is the traditional method to determine both RNA concentration and purity.

First perform a background correction by reading a blank (TE buffer, pH 8.0) at 260, 280, and 320 nm. To conserve your limiting RNA 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 (µg/µl) = (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 µg/µl.

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 pg 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:**

Component	Amount
2X First Strand Reaction Mix (10 mM $MgCl_2$, 1 mM each dNTP)	10 µl
Reverse Transcriptase	2 µl

5. **Vortex the sample briefly to mix, centrifuge briefly, and incubate as follows:**
   - **If using Oligo dT and/or gene-specific primer: 50 minutes at 50°C**
   - **If using random hexamers: 10 minutes at 25°C, followed by 50 minutes at 50°C**
6. **Terminate the reactions at 85°C for 5 minutes. Chill on ice.**
7. **Proceed directly to Step 3. Otherwise store cDNA at -20°C.**

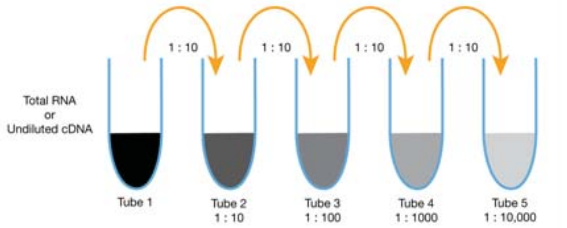
Step 3: Preparation of Serial Dilutions

To ensure a thorough and even coverage of your quantification range, enough dilutions should be prepared to cover the expected range of expression within your samples. Prepare at least a 5-point 10 fold serial standard curve, which can be used by the Eco Real-Time PCR software to determine the concentration of your unknown samples. If using a One-Step RT-PCR kit, prepare your dilutions starting with your RNA sample (from Step 1). If using prepared cDNA (from Step 2), start your dilutions with your cDNA sample:

1. **Pipette 18 µl of Nuclease-free water into 4 microfuge tubes and label them 2 through 5.**
2. **If necessary, thaw your RNA/cDNA sample (tube 1), mix well, and pipette 2 µl into tube 2. Pipette up and down to mix.**
3. **Using a new tip, pipette 2 µl from tube 2 to tube 3. Pipette up and down to mix. Repeat the same process for tubes 4 and 5.**

The serial dilution scheme is depicted in Figure 1, along with the RNA/cDNA dilution in each tube.

Figure 1: Serial Dilution



Step 4: Real-Time PCR Amplification

Standard Real-Time PCR takes between 30 and 120 minutes to run. This protocol is applicable to both standard and fast Real-Time PCR.

1. **Add 2 µl of each dilution point plus 2 µl of water (non-template control, or NTC), in duplicate, to a 48-well plate as shown in Table 1.**
2. **If you are running any unknown samples, add 2 µl of each, preferably in duplicate, to any empty wells (A3 through F8). The software will use the standard curve (A1 through E2) to determine the concentration of any unknown samples.**
3. **Prepare a Real-Time PCR master mix as described below. Make sure to prepare enough, 10-20% more than needed, to account for pipetting inaccuracies.**

Component	For RNA Templates	For cDNA Templates
Nuclease-free water	4 µl	6 µl
2X Real-Time PCR master mix (One-Step kit)	10 µl	-
10X Reverse Transcriptase enzyme (One-Step kit)	2 µl	-
2X Real-Time PCR master mix (Two-Step kit)	-	10 µl
10X Primers (final concentration: 100–900 nM for each primer)	2 µl	2 µl

Table 1: 48-Well Plate for Real-Time PCR Amplification

	1	2	3	4	5	6	7	8
A	Total RNA or cDNA	Total RNA or cDNA	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
B	Tube 2 (1:10)	Tube 2 (1:10)	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
C	Tube 3 (1:100)	Tube 3 (1:100)	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
D	Tube 4 (1:1000)	Tube 4 (1:1000)	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
E	Tube 5 (1:10,000)	Tube 5 (1:10,000)	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
F	Water (NTC)	Water (NTC)	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown

4. **Add 18 µl of master mix to all wells containing dilutions, NTCs, or samples. Pipette up and down to mix, carefully avoiding any bubbles.**
5. **Seal the plate, centrifuge briefly, and place into the Real-Time PCR instrument.**
6. **Choose the appropriate program for your experiment from the proposed protocols. These protocols are just starting point guidance and need to be adjusted based on your reagent provider recommendations.**

RNA Template		cDNA Template	
Fast Real-Time PCR	**Standard Real-Time PCR**	**Fast Real-Time PCR**	**Standard Real-Time PCR**
50ºC for 15 minutes	50ºC for 15 minutes	95ºC for 2 minutes	95ºC for 2 minutes
95ºC for 20 seconds	95ºC for 2 minutes	40 cycles of:	40 cycles of:
40 cycles of:	40 cycles of:	95ºC for 5 seconds	95ºC for 15 seconds
95ºC for 5 seconds	95ºC for 15 seconds	60ºC for 15 seconds	60ºC for 1 minute
60ºC for 15 seconds	60ºC for 1 minute		

7. **On the Plate Setup window specify your (S)tandards as a 5-point, 10-fold serial dilution on wells A1 through E2 as depicted in Table 1. Also specify the (N)o Template Controls (NTC) on wells F1 and F2. Finally if running (U)nknown samples, specify them in the remaining wells (A3 through F8) of the plate.**
8. **Click Start Run. While the instrument is running you can visualize the amplification in real-time in the Monitor Run window. The run will take approximately 40 minutes (Fast) protocol) or up to 90 minutes (Standard protocol).**

Step 5: Data Analysis

Once the run is finished the software automatically opens the Analyze Data window and performs a basic analysis using auto-baseline and threshold settings. Select the Amplification Plot tab. This view of amplification will show spacing of 3.32 cycles between amplification curves for a well-optimized assay. This is because it mathematically takes 3.32 cycles for a 100%-efficient PCR amplification to increase the number of template molecules 10-fold ($2^{3.32} = 10$) in any given sample.

To extract quantification data from Real-Time PCR amplification curves, the results need to be plotted as a linear regression of the Cq values versus the log of the RNA/cDNA quantities. Such a representation is typically called a standard curve. The Eco software automatically generates a standard curve. To view the curve, select the Results tab in the Analyze Data window (Figure 3).

Standard curves provide a wealth of information about a PCR assay. 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.

Figure 2: dRn Amplification Plots of an Example Assay (plus baseline, plus threshold)

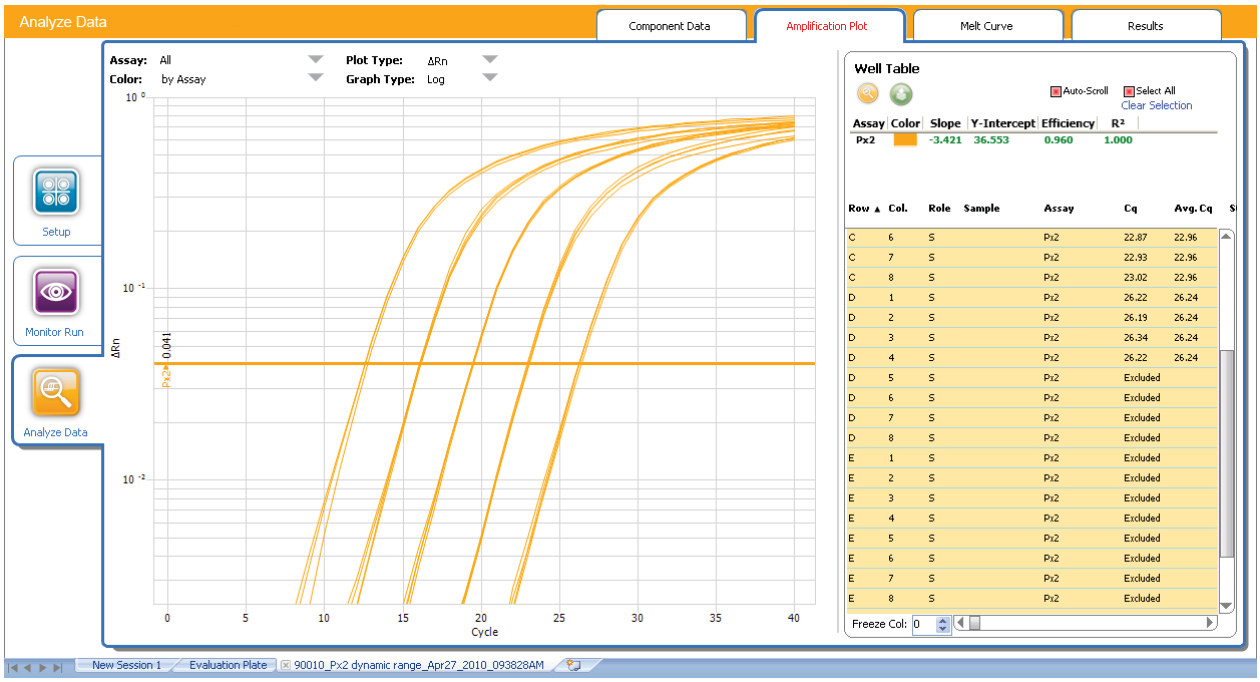
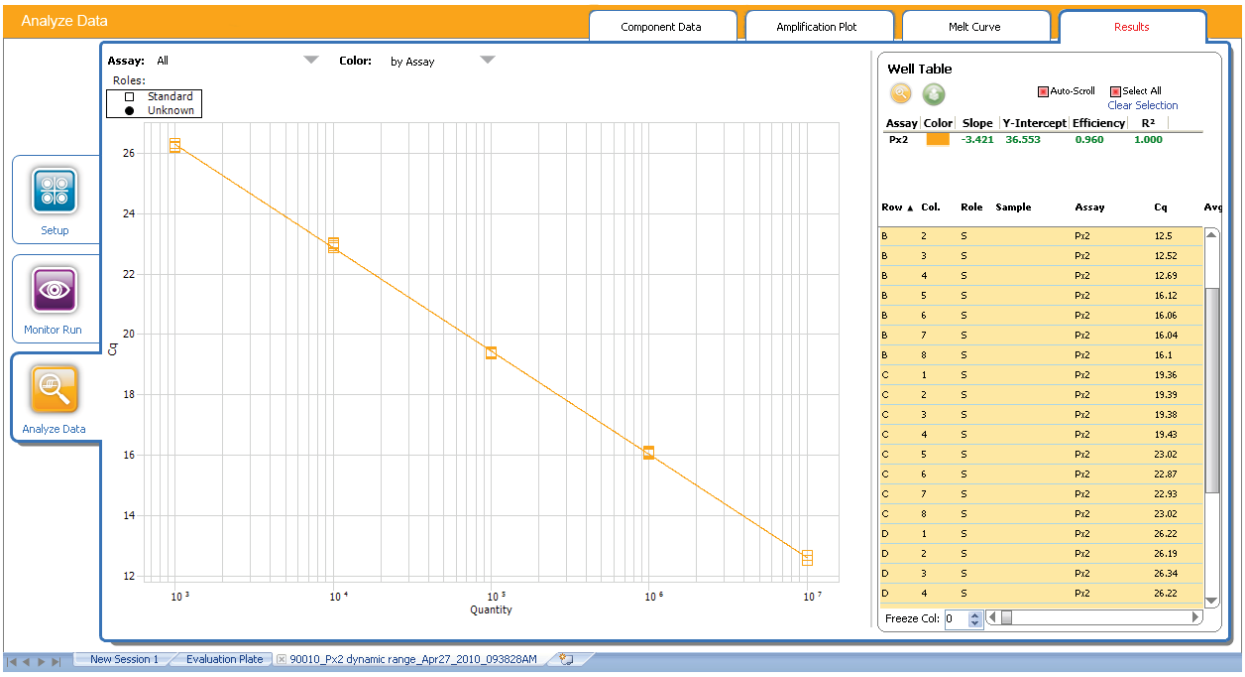


Figure 3: An Example Standard Curve



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:

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

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: http://www.illumina.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.illumina.com/ecoqpcr 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.ncifcrf.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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Pub. No. 570-2010-003 Current as of 26 July 2010

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