Quantitative Real Time PCR Protocol

Stack Lab

Overview

Real-time quantitative polymerase chain reaction (qPCR) differs from regular PCR by including in the reaction fluorescent reporter molecules that increase proportionally with the increase of DNA amplification in thermocycler. There are two types of fluorescent chemistries for this purpose: double strand DNA-binding dyes and fluorescently labeled sequence specific probe/primer. SYBR Green I dye and TaqMan® hydrolysis probe are the common examples for these two, respectively. The SYBR Green method doesn't need fluorescently labeled probe/primer and costs much less than the TaqMan method. The protocol that follows is intended for use with the SYBR Green I method unless otherwise specified. The key equipment for qPCR is a specialized thermocycler with fluorescence detection modules which is used to monitor and record the fluorescence in real-time as amplification occurs.

A typical workflow of qPCR for gene expression measurement involves RNA isolation, reverse transcription, qPCR assay development, qPCR experiment and data analysis. Special attention is needed for preventing RNA degradation. For reliable quantification, always include samples for standard curves and measure proper reference genes, of which the expression is constant in your experimental conditions, as endogenous control.

Materials and equipment

Reagents for RNA extraction and cDNA synthesis: Trizol from Invitrogen or RNeasy from Qiagen; Chloroform; isopropyl alcohol; 5% Ethanol; RNAsefree (or DEPC-treated) water; SuperScript II reverse transcriptase and buffer; oligo$(dT)_{12\text{-}18}$ or random hexamer primer; dNTP; RNase inhibitor.

Reagents for qPCR: SYBR green I qPCR mixture from ABI (this mixture includes dNTP, Taq DNA polymerase, reaction buffer and the fluorescent dyes, use TaqMan qPCR mixture instead depending on your chemistry of choice); custom-made gene specific primers from Integrated DNA Technology (and custom-made gene specific fluorescent labeled probe from ABI for TaqMan chemistry). Plastic: white strip qPCR tube (200µl volume) with optical clear strip caps or white 96-well qPCR plates with optical clear seal sheets and press applicator. Two pieces of similar qPCR equipment, Bio-Rad iCycler iQ and iQ5,

are available for use.

Primers and probes

The size of amplicon in qPCR reaction is between 75-200bp to ensure higher amplification efficiency. For primer and probe design in this purpose, the software *Primer Express v2.0* from ABI is highly recommended, especially for TaqMan chemistry, which needs both sequence specific primers and fluorescence labeled probe. Vector NTI, ApE, and free web-based software like *PrimerQuest* on IDTdna.com and *Primer3* (http://fokker.wi.mit.edu/primer3/input.htm) or any other software may work fine too. You may use published primers in the literature or deposited in online qPCR primer banks. However, always double check the validity of sequences with NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) or similar tools. Primers spanning exon-exon boundary have the advantage of avoiding amplification from genomic DNA source. Other general principles in primer selection include: design primers with a GC content of 50–60%; to match up the two steps method in cycling, choose annealing temperature close to 60^{o}C; avoid repeats of Gs or Cs longer than three bases and avoid secondary structure; have Gs and Cs on 3' ends of primers.

Experimentation

1. RNA extraction: Refer to TRIZOL or RNeasy kit manual for detail. To avoid RNA degradation in storage, go immediately to reverse transcription for cDNA synthesis. RNA/cDNA hybrid is stable. A typical procedure for RNA extraction is as following,

1) Homogenization: Use 1 ml of Trizol reagent per 0.5-1x10^{7} cells (or 50-100mg tissue, or 10cm^{2} surface). Apply directly after PBS washing adherent cells.
2) Phase separation: Incubate at RT for 5 min. Add 0.2 ml of chloroform per 1 ml of TRizol. Shake tubes vigorously for 15 sec, incubate for 3 min. Spin down at 4 ^{o}C for 15 min 12,000 x g in a desktop centrifuge. Remove and keep the colorless upper aqueous phase, about 600 µl.
3) Precipitation: Add 0.5 ml of isopropyl alcohol, incubate 10 min at RT, centrifuge 12000xg for 10 min at 4 ^{o}C. Remove the supernatant, keep the RNA pellet.
4) Wash: Add 1ml of 75% ethanol, vortex the sample, then 7,500xg for 5 min
5) Re-dissolving the RNA: Air-dry the RNA pellet for 10 min, add 30 ul RNase free water to dissolve it.

6) (Optional)Use RNAse-free DNAse treatment (Kit available from Qiagen) depending on downstream applications.

2. cDNA synthesis: A typical reaction for reverse transcription is listed below,

Oligo dT 0.5µg/µl 1µl
dNTP mix 10mM each 1µl
Total RNA 5µg and DEPC-water 10µl
Mix the above and incubate at 65 ^{o}C for 5 min

Then add,
5x First strand buffer 4µl
0.1 M DTT 2µl
RNAsin 40U/µl 1µl
SuperScriptII (200U/µl) 1µl

Incubate at 42 ^{o}C for 50 min
Denature the enzyme at 70 ^{o}C for 15 min
Store the cDNA product at -20 ^{o}C for later use. Usually, you can dilute 10-50 fold and then use 5 µl each as template for one qPCR reaction.

3. Preparing qPCR components: Take all due caution to prevent contamination from plasmid DNA and previous PCR product by using micropipette tips with barrier. Use triplicates or quadruplicates in qPCR reaction for every sample you have and these are technically replicates required to monitor machine error. To minimize variation in pipetting, always prepare master mixtures containing everything except for template first and then dispense to individual wells or tubes. Add templates at the final step. Seal covers firmly. Collect all liquid at the bottom by brief centrifugation.

A typical qPCR reaction tube using SYBR Green I chemistry has components in 30 µl of volume as in the following table. Note that SYBR green mixture has optimized amount of DNA polymerase, dNTP, reaction buffer and dyes.

Components	*Concentration*	*Use*	*Final concentration.*
SYBR Green I mixture	2X	15 µl	1X
Primer Forward	10 µM	0.50	167 nM

		µl	
Primer Revers	10 µM	0.50 µl	167 nM
ddH2O		9 µl	
cDNA Template		5 µl	

4. Set up your thermal cycler protocol and plate layout.

Bio-Rad iCycler iQ and iQ5 need 2 separate files before running, one for thermal cycling and the other for plate layout. Since real time qPCR uses short amplicons, it is recommended to use two steps method (95^{o}C and 60^{o}C)for thermocycling instead of the three steps (95^{o}C, 55^{o}C, 72^{o}C) in regular PCR. Careful primers and amplicon choice could avoid the need to optimize annealing temperature. A melting curve step should be added to end of cycles on Bio-Rad iCycler or iQ5.

A typical qPCR experiment thermocycle protocol is as following,

	Cycle1 X 1 repeat	*Cycle2 X40 repeats*	
	Step1	*Step 1*	*Step2*
Temperature	95^{o}C	95^{o}C	60^{o}C
Dwell time	5min	0.5min	1min

A typical qPCR experiment layout is as in the table below. There are 5 samples of serially diluted standard (S1, S2, S3, S4, and S5) for creating a standard curve, 2 unknown experimental samples (U1 and U2) and 1 ‘no template’ control (NTC) for monitoring contamination in reagent preparation, all in triplicates. Also on plate layout settings, you need mark these wells (from A1 to H3) as SYBR Green I or FAM (similar excitation and emission with the former) as their fluophore and specify signal collection filter accordingly. You may accommodate samples for reference gene in the same plate as your samples for gene of interest as long as they are running on the same thermal cycling protocol.

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	S1									
B	S2	S2	S2									
C	S3	S3	S3									
D	S4	S4	S4									
E	S5	S5	S5									
F	U1	U1	U1									
G	U2	U2	U2									
H	NTC	NTC	NTC									

Data Analysis

At the end of a qPCR running with SYBR green chemistry, check the appearance of single peak melt curve first. Melt curve is generated by increasing the temperature from 55^{o}C to 95^{o}C in tiny increments and monitoring the intensity of fluorescence at each step. As the dsDNA denatures (melts), the fluorescence decreases. A plot is therefore created using the negative first derivative of fluorescence intensity as a function of temperature. You should see a characteristic single sharp peak at melting temperature (Tm) of your specific amplicon.

The amount of PCR product in ideal conditions will double at the end of each cycle during exponential amplification stage, that is 2^{n}, where n= number of cycles. Quantification of initial inputs in qPCR assay is based on the number of cycles when fluorescence intensity reaches a threshold set automatically or manually in logarithmic amplification stage. This is called threshold cycles (C_T) and it's required for all quantification methods. The final amount of qPCR product in the tube at the end of last cycle doesn't matter and is not used for any calculation. Usually the software running qPCR equipment is able to perform some automatic analysis and provide C_T value at least.

When expression level was denoted in certain units, like, ng, copies or arbitrary units, it is called absolute quantification, but more commonly gene expression level is denoted in number of folds as compared to your biological control sample (calibrator, and expression level is taken as "1"). The latter method is called relative quantification. To accurately compare gene expression level in different samples, separate standard curves for gene of interest (GOI) and reference gene (ref) are usually required in either absolute or relative quantification methods.

Endogenous reference genes (usually house-keeping gene such as GAPDH,

β-actin, PGK, HPRT etc.) are commonly included in qPCR assay to be detected with an aliquot of the same samples as your GOI uses and is quantified to normalize any possible variation from starting materials, although in some situation equal amounts of starting materials in mass unit may justify the void of this step. The efficiency of reverse transcription between GOI and ref genes may vary and therefore pose a concern about accuracy in quantification, which is a question not well addressed yet. But the amplification efficiency (E) variance in different genes is well considered in qPCR quantification methods. E has to be calculated from the slope of standard curves. Estimation of amplification efficiency without the need of standard curves (such as sigmoidal curve-fitting on amplification plot) is not readily available yet.

$$E=10^{-1/slope}$$

Optimal slope of qPCR standard curve is -3.32, which translates into E=2 and refers to the doubling of DNA amount. However amplification efficiency is frequently presented as a percentage

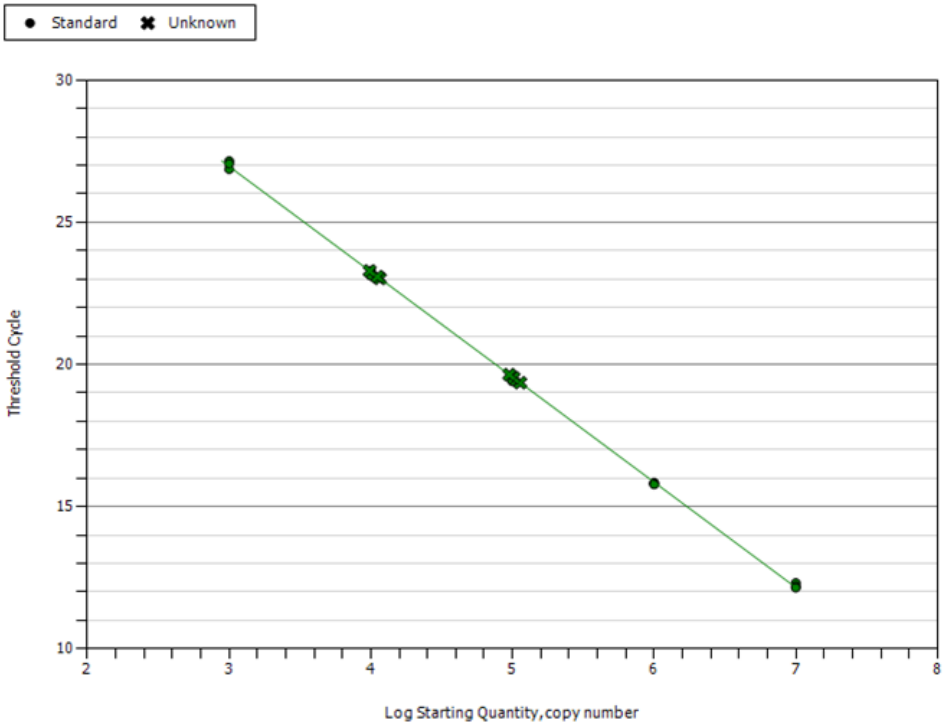
$$\% \text{ Efficiency} = (E - 1) \times 100\%$$

The hallmarks for an optimized qPCR assay include linear standard curve (R^2 > 0.980 or r > |–0.990|), high efficiency (90-105%) and consistency across replicate reactions.

Absolute quantification:

Following is an example of using standard curve to determine the copy number of a particular gene as shown in the figure. In this example, we were determining the copy number of the gene in two samples U1 and U2. A 10-fold dilution series was made from a sample of plasmid solution with known copies of the gene, resulting in a set of 5 standards containing 10^2–10^7 copies of the gene. The standards and the samples were run in the same plate and in same condition.

A standard curve was then constructed with logarithm of the initial copy number of the standards plotted along the x-axis and their respective C_T values plotted along the y-axis. The equation for the linear regression line (y = mx + b) is shown at the bottom the graph. Based on the equation for the linear regression, we can determine the quantity of an unknown sample whenever its Ct value is available.



$y = –3.50x + 37.20$; $r^2 = 0.999$

Furthermore, you may measure a housekeeping gene in the same way and use that result to normalize the value from your gene of interest.

Relative quantification:

This frequently-used method presents expression levels in number of folds as compared to expression level of calibrator which is usually the biological control sample (taken as expression of “1”). The results from above absolute quantification method could be transformed into relative quantification simply by dividing all the results with the quantity of calibrator. More commonly relative quantification is calculated by Livak method or Pfaffl method. The actual operation of these quantification methods could be performed by qPCR software or by some setup in *Microsoft Excel*.

Livak Method

This is also called $2^{-\Delta\Delta C_T}$ method and is the seemingly easiest way to do relative quantification as it uses only C_T values as the following:

1) Normalize the C_T of GOI to that of ref, for both the test sample and the calibrator sample. This is ΔC_T:

$$\Delta C_{T(test)} = C_{T(GOI, test)} - C_{T(ref, test)}$$
$$\Delta C_{T(calibrator)} = C_{T(GOI, calibrator)} - C_{T(ref, calibrator)}$$

2) Calculate the difference between the ΔC_T of the test sample and the ΔC_T of the calibrator, so called $\Delta\Delta C_T$:

$$\Delta\Delta C_T = \Delta C_{T(test)} - \Delta C_{T(calibrator)}$$

3) Calculate the expression ratio:

$$\text{Expression ratio (folds)} = 2^{-\Delta\Delta C_T}$$

However the Livak method has an often untrue assumption that both GOI and reference gene have the same amplification efficiency (E) of 2, a condition that is rarely met in practice. Livak Method is actually a special case of the following method, when $E_{GOI}=E_{ref}=2$.

Pfaffl Method

In most situations, the amplification efficiency of GOI and reference gene is different, for relative quantification, the proper calculation is Pfaffl method as in following formula,

$$\text{Expression ratio (folds)} = [(E_{GOI})^{(C_T(GOI, calibrator) - C_T(GOI, test))}]/[(E_{ref})^{(C_T(ref, calibrator) - C_T(ref, test))}]$$

Ending Remark

Double stranded DNA dye (SYBR green I) based qPCR is a powerful tool for gene expression assay and it is relatively easy to develop. Careful experimental design and correct data analysis will provide reliable results. However SYBR green chemistry is not suitable for multiplex qPCR assays where amplification of more than one target is performed simultaneously in a single reaction tube. TaqMan and other fluorescent probe/primer based chemistry should be considered when such needs arise.