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Quantitative Real-Time PCR (qPCR) Workflow for Analyzing *Staphylococcus aureus* Gene Expression

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Abstract

Quantitative real-time polymerase chain reaction (qPCR) is a sensitive tool that can be used to quantify and compare the amount of specific RNA transcripts between different biological samples. This chapter describes the use of a "two-step" qPCR method to calculate the relative fold change of expression of genes of interest in *S. aureus*. Using this work-flow, cDNA is synthesized from RNA templates (previously checked for the absence of significant genomic DNA contamination) using a cocktail of random primers and reverse-transcriptase enzyme. The cDNA pools generated can then be assessed for expression of specific genes of interest using SYBR Green-based qPCR and quantification of relative fold-change expression.

Keywords: Real-time PCR, Relative quantification, Livak calculation, RNA, cDNA, SYBR Green

1 Introduction

Quantitative real-time polymerase chain reaction (qPCR) represents an important advancement in molecular biology, whereby the sensitivity of PCR has been combined with the ability to monitor amplification of specific double-stranded DNA products "in real time" at the end of each cycle of the PCR reaction (1, 2). This is achieved by the use of specialized thermocyclers that can measure the fluorescence of specific primer or probe sequences (i.e., "TaqMan" hydrolysis probes, "LightCycler" hybridization probes, molecular beacons) or intercalating dyes (i.e., SYBR Green) to detect the amplified product of interest (3). Regardless of the detection method used, the cycle number at which enough amplified product accumulates to yield a detectable fluorescent signal (termed the "threshold cycle" or "C_T value") is the actual measure used to calculate and quantify the initial amount of template present in a qPCR reaction (4). The amount of starting template in a qPCR reaction and the measured C_T value have an inverse relationship, in that the greater the amount of initial target template, the fewer cycles that are needed to produce detectable fluorescence (2). Because C_T values are measured in the exponential phase of amplification (when reagents are not limiting), they can be used to

reliably and accurately calculate the initial amount of template present in a reaction (4).

In order to utilize qPCR for measuring changes in RNA transcript levels, there are several factors that must be taken into account. For one, it is important to ensure that RNA samples are of high quality and are not contaminated with significant amounts of genomic DNA that may yield false-positive amplification in the downstream qPCR reaction. A second parameter to consider is the choice of a “one-step” (cDNA synthesis and qPCR occur sequentially in the same master mix) verses “two-step” (cDNA is synthesized in a separate reaction before being used as template in qPCR). “Two-step” qPCR typically uses a mixture of oligo(dT) and random primers to amplify the entire cDNA pool, which can be advantageous if subsequent expression studies require the measurement of many different transcripts. In contrast, “one-step” PCR must use a gene-specific oligonucleotide to prime reverse-transcription of the RNA of interest, but this technique cuts out pipetting steps that could potentially introduce experimental error and lesson the accuracy of the overall qPCR (4). Third, a detection method must be chosen, using either fluorescently labeled primers/probes, or intercalating dyes that fluoresce when bound to the double-stranded PCR product. Labeled primers or probes are sequence-specific, but are less cost-effective since a new probe must be synthesized for each gene of interest. Intercalating dyes such as SYBR Green bind indiscriminately to all double stranded DNA products (including potential nonspecific PCR products and/or primer dimers), therefore they lack the target specificity conferred by the labeled primers/probes. However, these dyes are an economical alternative and are preferred for assays requiring testing of many different genes, eliminating the need to design specific probes for each gene of interest. A melt-curve analysis should always be included with qPCR reactions using intercalating dyes, as this will indicate the presence of nonspecific products and/or primer dimers (4, 5). Fourth, a choice must be made between using absolute or relative quantification methods to analyze gene expression data. In absolute quantification the C_T value of each unknown sample is compared to a standard curve of C_T values generated from known amounts of template. By comparison, relative quantification does not calculate the absolute starting quantity of unknown template, but instead reports fold change values compared to a control condition (4). Finally, publication of qPCR results should adhere to the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines, a series of recommendations created to promote publication of sufficient experimental detail that enables the reader to assess the quality of the presented results and/or to be able to reproduce the described experiments (6, 7).

qPCR has become popular in the field of *S. aureus* biology as a means of measuring changes in gene expression, as well as a validation tool for RNA microarray (8–11) and RNA-seq studies (12–14). The two-step qPCR workflow presented in this chapter has been optimized for analyzing *S. aureus* gene expression in our lab by relative quantification using the Livak method (15). The typical experimental workflow is (1) "quality-control" check of RNA samples for genomic DNA contamination, (2) cDNA synthesis from RNA templates using reverse-transcriptase, (3) detection of transcripts of interest using qPCR, and (4) quantification of relative fold-change expression.

2 Materials

1. Nuclease-free PCR water, non-DEPC treated (*see* **Note 1**).
2. Forward and Reverse Primer pairs for reference gene and target gene (*see* **Note 2**).
3. Bio-Rad iQ™ SYBR® Green Supermix (2× concentrate).
4. Ambion RNase away (*see* **Note 3**).
5. DNAase/RNase-free water (*see* **Note 3**).
6. 70 % ethanol (*see* **Note 3**).
7. PCR cabinet with filtered airflow and UV light (*see* **Note 3**).
8. Pipettes (*see* **Note 3**).
9. Vortex Genie.
10. DNase/RNase-free aerosol barrier pipette tips (*see* **Note 3**).
11. 1.7 ml microcentrifuge tubes, sterile, DNase/RNase free.
12. RNA Template (*see* **Note 4**).
13. Ambion Turbo-DNase kit (*see* **Note 4**).
14. Bio-Rad iScript™ cDNA Synthesis Kit.
15. Thermocycler.
16. Thin-walled PCR tubes, sterile, DNase/RNase free.
17. qPCR machine (*see* **Note 5**).
18. qPCR well plate (*see* **Note 5**).
19. Film for qPCR plate (*see* **Note 5**).

3 Methods

For all steps below, all samples and reaction components should be thawed and stored on ice while in use. Perform all work in a PCR cabinet using DNase/RNAse-free pipettes and filter tips (*see* **Note 3**).

3.1 Check of RNA Samples for Genomic DNA Contamination

1. Dilute each RNA sample in sterile nuclease-free water (non-DEPC treated) to a final concentration of 37.5 ng/μl in a volume of 20 μl. Place diluted samples on ice until step 5 below.
2. Set up a master mix containing all of the components (but do not add template) listed in Table 1, column 1, using the reference gene primer set. This master mix should contain enough volume to analyze each RNA template, a no template control (NTC), and one extra sample to account for pipetting error. In this example experimental setup, the master mix recipe in column 2 of Table 1 is designed for triplicate 15 μl qPCR reactions per template, and is enough to analyze two RNA templates, one positive control (genomic DNA), one no template control (NTC; nuclease free water), and one extra sample to account for pipetting error.
3. Vortex master mix from step 2 above, and aliquot into mini master mixes for each RNA sample, DNA template, and NTC. Each mini master mix should have enough volume for setting up triplicate qPCR reactions per template, plus one extra volume to account for pipetting error. In this experimental example, 52 μl of the master mix is aliquoted into 4 × 1.7 ml microcentrifuge tubes.
4. Add the appropriate template (RNA sample, DNA sample, or NTC) to each mini master mix. In this experimental example, 8 μl of template is added to 52 μl of mini master mix.
5. Vortex each mini master mix and aliquot in triplicate into wells of the qPCR plate. In this experimental example, 15 μl of each

Table 1
Sample qPCR reaction setup for analyzing four templates (two RNA samples, one DNA sample, one NTC)

Component (initial concentration)	Amount to add to master mix (260 μl total volume) (μl)	Volume per mini master mix (60 μl total volume) (μl)	Final volume per 15 μl reaction (μl)	Final concentration or amount per 15 μl reaction
Sterile, nuclease free water (non-DEPC treated)	50	10	2.5	N/A
Forward primer (2.5 μM)	30	6	1.5	0.25 μM
Reverse primer (2.5 μM)	30	6	1.5	0.25 μM
iQ™ SYBR® Green Supermix (2×)	150	30	7.5	1×
Template (37.5 ng/μl)	Not added at this step	8	2	75 ng

mini master mix is aliquoted in triplicate to the wells of the qPCR plate (*see* **Note 6**).

6. Apply plastic film to seal the top of the qPCR plate (*see* **Note 7**).
7. Transfer sealed qPCR plate to qPCR machine, and run qPCR reaction, using denaturation, annealing/extension times and temperatures that have been optimized for the primers and templates to be analyzed (*see* **Note 8**).
8. Examine the average C_T values generated from each qPCR reaction. The NTC reaction should not yield a C_T value (or a very high C_T value close to 40). In order to proceed with cDNA synthesis, each RNA template should yield a C_T value of 30 or larger. If RNA C_T values are less than 30, an Ambion Turbo-DNase treatment may be performed to eliminate residual DNA contamination.

3.2 cDNA Synthesis from RNA Templates

1. Based on the RNA concentration of each sample (*see* **Note 4**), calculate the volume for each that contains 0.75 μg RNA.
2. Setup a 20 μl cDNA synthesis reaction in a thin-walled PCR tube for each RNA sample using the Bio-Rad iScript™ cDNA Synthesis Kit and recipe listed in Table 2 (*see* **Note 9**).
3. Transfer cDNA reactions to a thermocycler programmed as follows: 25 °C for 5 min, 42 °C for 30 min, 85 °C for 5 min, 4 °C hold.
4. When cDNA synthesis reactions are complete, use cDNA immediately for qPCR or store at −20 °C until you are ready to proceed with qPCR (*see* **Note 9**).

3.3 qPCR Detection of Target and Reference Gene Expression

1. Set up a master mix for each of the reference primers and the target primers, as outlined in Fig. 1. Each master mix will contain all of the components listed in Table 1, column 1, with the exception of template, which will be added in step 4 below.

Table 2
Recipe for cDNA synthesis

Reaction component	Volume added per 20 μl reaction	Final concentration or amount per 20 μl reaction
5× iScript reaction mix (contains polyA and random hexamer primers)	4 μl	1×
iScript reverse transcriptase enzyme	1 μl	
RNA template	Volume necessary to add 0.75 μg RNA to reaction	37.5 ng/μl
Sterile, nuclease-free water (non-DEPC treated)	Volume necessary to bring reaction volume to 20 μl	

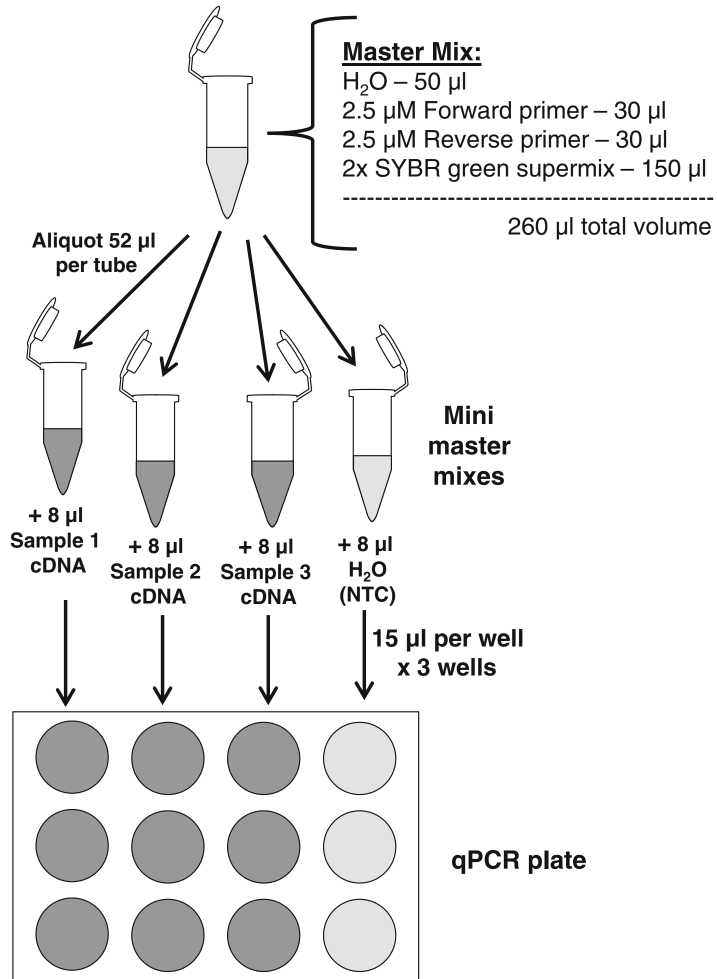

Fig. 1 Schematic of qPCR setup

The master mix should contain enough volume to analyze each cDNA template, a no template (negative) control, and one extra sample to account for pipetting error. In this example experimental setup, the master mix recipe in column 2 of Table 1 is designed for triplicate 15 µl qPCR reactions per template, and is enough to analyze three cDNA templates, one NTC control, and one extra sample to account for pipetting error.

2. Vortex each master mix from step 1 above, and aliquot each into mini master mixes for each cDNA sample and NTC. Each mini master mix should have enough volume for setting up triplicate qPCR reactions per template, plus one extra volume to account for pipetting error. In this experimental example, 52 µl of each master mix is aliquoted into 4 × 1.7 ml microcentrifuge tubes.

3. Add the appropriate template (cDNA, or sterile nuclease-free water for NTC) to each mini master mix. In this experimental example, 8 μl of template is added to 52 μl of each mini master mix.
4. Vortex each mini master mix, and aliquot in triplicate into wells of the qPCR plate. In this experimental example, 15 μl of each mini master mix is aliquoted in triplicate to the wells of the qPCR plate (*see* **Note 6**).
5. Apply plastic film to seal the top of the qPCR plate (*see* **Note 7**).
6. Transfer sealed qPCR plate to qPCR machine, and run qPCR reaction, using denaturation, annealing/extension times and temperatures that have been optimized for the primers and templates to be analyzed (*see* **Note 8**).
7. Examine the average C_T values generated from each qPCR reaction (cDNA templates and NTC). The NTC reaction should not yield a C_T value (or a very high C_T value close to 40). Also examine the melt curve to ensure the presence of one distinct peak per primer set (*see* **Note 10**).

3.4 Quantification of Relative Fold-Change Expression Using the Livak Method (15) (See Note 11)

1. For the experimental setup being analyzed, one of the cDNA samples must be assigned as the calibrator, and the rest of the cDNA samples as the "test" samples. For the example data graphed in Fig. 2, the aerobic 2 h growth cDNA sample was assigned as the calibrator, whereas the 6 and 12 h aerobic samples and all low-oxygen samples were considered the "test" samples.
2. Normalize the C_T value of each target gene to that of its corresponding reference (ref) gene C_T value for each test sample and the calibrator sample using the following formulas:

$$\Delta C_T(\text{test}) = C_T(\text{target, test}) - C_T(\text{ref, test})$$
$$\Delta C_T(\text{calibrator}) = C_T(\text{target, calibrator}) - C_T(\text{ref, calibrator})$$

3. Normalize the ΔC_T of each test sample to the ΔC_T of the calibrator using the following formula:

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

4. Calculate the expression ratio for each test sample $\Delta\Delta C_T$ using the Livak equation:

$$2^{-\Delta\Delta C_T} = \text{Normalized expression ratio}$$

5. The normalized expression ratio for each test sample can be graphed as the fold-change relative to the calibrator sample (*see* Fig. 2), or alternatively can be presented in a table. The example data presented in Fig. 2 were obtained by following the qPCR

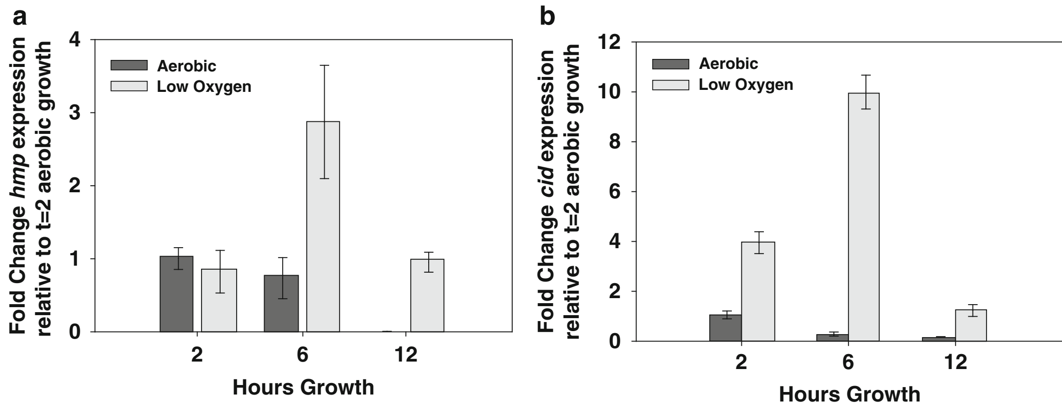

Fig. 2 Sample qPCR data analyzing *hmp* (**a**) and *cidA* (**b**) gene expression in *S. aureus*. Total RNA was isolated at the indicated time points from $n = 3$ biological replicates of UAMS-1 grown under highly aerated (*dark grey bars*; 250 rpm, 1:10 volume-to-flask ratio) or low-oxygen (*light grey bars*; 0 rpm, 7:10 volume-to-flask ratio) conditions. Quantitative real-time PCR was performed on reverse-transcribed cDNA from each sample using *hmp* (**a**) and *cidA* (**b**) specific primers. The Livak method was used to determine relative fold-change expression of each gene, using *sigA* expression as the reference gene and the 2 h aerobic sample as the calibrator. Error bars = Standard Error of the Mean

workflow presented in this chapter, and the resulting data indicated that both the *cidA* and *hmp* genes are more highly expressed during low-oxygen growth relative to aerobic growth. These results correlated very well to previous qPCR expression data for these two genes published by other groups (16, 17).

4 Notes

1. It is recommended that non-DEPC-treated nuclease-free sterile water be utilized for qPCR-based applications, as trace amounts of DEPC that have not been inactivated are thought to have the potential to inactivate the PCR polymerase and inhibit the qPCR reaction.
2. Primer design for qPCR: It is recommended that primer design for genes of interest be performed with software such as Primer3Plus (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi), which has a preprogrammed qPCR option. In general, qPCR primers should amplify a target product of 75–200 bp, as smaller products tend to have higher efficiencies but less specificity. At the same time, qPCR products need to be large enough to make them distinguishable from primer dimers. Primers must also be designed for a reference gene, which by definition should be expressed at a similar level over all of the different experimental conditions you plan to test (18). Our lab has found that primers specific for the *S. aureus* sigma-A (*sigA*)

gene satisfy this criteria under most of the experimental conditions we have tested. Other researchers have also utilized *sigA* as a reference gene (17). Alternatively, it has recently been suggested that the geometric mean of several reference genes be used for normalization of *S. aureus* gene expression studies using qPCR (19). For both target and reference primers, it is recommended that these be tested in a traditional PCR reaction using genomic DNA as template, and analyzed by gel electrophoresis to verify amplification of the correctly sized product and the absence of nonspecific products or primer dimers. In addition to the primer considerations stated above, each target and reference primer pair must be used to generate a standard curve using serial dilutions of cDNA template, in order to calculate the efficiency of each primer set (4). This analysis will also give you an idea of how much you need to dilute your sample cDNA template (if at all), to ensure that the C_T values fall into the linear range of the curve. Hallmarks of a good standard curve include linearity ($R^2 > 0.98$), efficiency (E) between 90 and 105 %, and an ideal slope of −3.3 (4).

3. Nuclease-free work environment considerations: To minimize degradation of RNA and cDNA samples, it is imperative to maintain a clean workspace that is free from contamination by DNase and RNase enzymes. The first safe-guard to protecting these samples is wearing gloves and changing them often throughout the experiment. If RNA or cDNA degradation appears to be problematic in the qPCR workflow, the use of additional precautions such as wearing disposable sleeve protectors over your lab coat, and performing all steps of the experiment under a PCR cabinet equipped with UV light and filtered airflow is highly recommended. Prior to working with RNA or cDNA, all pipettes and work surfaces should be wiped down thoroughly with Ambion RNase Away. As this solution is irritating to skin and can rust metal surfaces, it is important to wash the pipettes and work surfaces a second time with 70 % (vol/vol) ethanol (made with nuclease-free water) or 100 % nuclease-free water to remove the residual RNase Away. All disposable plastics to be used in the qPCR workflow (tubes, aerosol barrier pipette tips) should also be certified DNase/RNase free and autoclaved before use (if not already presterilized).
4. RNA samples can be isolated from *S. aureus* by any optimized method of preference, as long as the concentration of RNA is ≥70 ng/μl and the A_{260}/A_{280} ratio is ≥2.0. Our lab utilizes the FASTPREP lysing matrix B and Qiagen RNeasy kit according to a previously published protocol, and elute the RNA using non-DEPC-treated nuclease-free water (20). For RNA to be analyzed by qPCR we routinely incorporate a second

DNase treatment following RNA isolation, using the Ambion Turbo DNase kit. RNA samples should be stored at −80 °C prior to cDNA synthesis and qPCR analysis. Although we have not encountered any issues with degradation of RNA upon repeated freeze-thawing of samples, RNA can be stored as working aliquots to minimize this concern.

5. Use the plates and sealing film that are compatible with the qPCR platform to be used in the experiment. Our lab routinely uses the Illumina Eco real-time PCR instrument, which has a small 48-well plate format.
6. It is important to minimize the introduction of air bubbles into the wells of the qPCR plate, as these can sometimes interfere with fluorescent detection by the qPCR instrument. Air bubbles can be removed by gently tapping the plate, or, if there is access to a centrifuge and rotor compatible with the qPCR plate, a short, low-speed spin should greatly reduce or eliminate air bubbles from the wells.
7. When applying the sealing film to the plate, it is helpful to use the edge of a pipette box lid or some other solid edged surface to smooth out the film evenly and securely over the plate. Wrinkles in the applied sealing film can sometimes interfere with fluorescent detection by the qPCR instrument, and if the sealing film is not secured properly, evaporation can occur in the wells during the qPCR reaction.
8. In our lab we use the Illumina Eco qPCR machine, and with most *S. aureus* primers and templates, the program (including melt curve analysis) outlined in Table 3 works well.
9. cDNA synthesis reactions may be scaled up to 40 μl if a large amount of cDNA template is required. If this option is pursued, 1.5 μg RNA template should be added to each 40 μl cDNA synthesis reaction. For best results, repeated freeze-thawing of

Table 3
Sample qPCR cycling protocol

Step	# Cycles	Temperature	Time
Polymerase activation	1	95 °C	3 min
Data collection	40	95 °C (denaturation)	15 s
		58 °C (annealing/extension)	30 s
Melt curve	1	95 °C	15 s
		58 °C	15 s
		58 °C + 0.5 °C/s	
		95 °C	15 s

cDNA samples should be avoided. Therefore it is recommended that cDNA be stored at −20 °C in working aliquots.

10. When performing qPCR using non-sequence specific detection methods such as SYBR Green, it is essential that a melt curve is performed at the end of the qPCR reaction. This step detects the melting temperatures (based on the size and nucleotide composition) of each PCR product formed in each well of the qPCR plate. One distinct peak on the melting curve generally indicates that only one PCR product is being synthesized and detected. However, multiple peaks indicate the presence of nonspecific products and/or formation of primer dimers, which can artificially lower the C_T of the reaction. In this latter scenario, the qPCR primers should be redesigned.
11. To use the Livak calculation for relative quantification in qPCR, the efficiencies of the reference and target primers must be within 5 % of each other, and both efficiencies should ideally be close to 100 %. The efficiencies of each primer set can be calculated by performing a standard curve with serial dilutions of cDNA template as described in **Note 2**. If the efficiency of each primer set is high (greater than 85 %) but not within 5 % of each other, an alternative relative fold-change calculation can be performed, such as the Pffafl method (21).

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