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Protocol

qRT-PCR of Microbial Biofilms

Ailyn C. Pérez-Osorio and Michael J. Franklin[1]

Department of Microbiology, Montana State University, Bozeman, MT 59717, USA

[1]Corresponding author (umbfm{at}montana.edu)

INTRODUCTION

Bacteria growing in biofilms often express a different subset of genes compared to the same strains growing planktonically. Quantitative reverse transcriptase real time PCR (qRT-PCR) can be used effectively to quantify the number of RNA transcripts of specific genes from bacteria growing in biofilms. qRT-PCR has a large dynamic range and may be used to verify gene expression data obtained from microarrays. In addition, qRT-PCR is sensitive, and therefore may be used to quantify gene expression from biofilm samples where only a small amount of biological material is available, as in samples obtained by laser capture microdissection microscopy (LCMM). The most commonly used qRT-PCR methods are the SYBR Green and dual-labeled probe (Taqman) approaches. Both approaches use reverse transcription to convert mRNA to cDNA, followed by PCR amplification of the cDNA. This article describes steps involved in aspects of qRT-PCR including (1) primer design, (2) primer and probe performance testing, (3) qRT-PCR using the Corbett Rotor-Gene system, and (4) data export and analysis.

RELATED INFORMATION

The methods described in this article have been optimized for use with RNA that has been isolated from microdissected biofilms (see **Isolation of RNA and DNA from Biofilm Samples Obtained by Laser Capture Microdissection Microscopy** [Pérez-Osorio and Franklin 2008]). For basic details about the agarose gel electrophosis method used to evaluate amplification efficiency, see **Agarose Gel Electrophoresis** (Sambrook and Russell 2006).

The following web resources are particularly useful for designing qRT-PCR primers and probes:

Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) (Rozen and Skaletsky 2000); mfold (http://frontend.bioinfo.rpi.edu/applications/mfold/) (Zuker 2003); IDT OligoAnalyzer (http://www.idtdna.com/scitools/scitools.aspx); and BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

For additional information regarding qRT-PCR using the Corbett Rotor-Gene system and data analysis software, see the Corbett Life Science website (http://www.corbettlifescience.com). A useful source of information from other qRT-PCR users is the discussion group QPCR listserver (http://tech.groups.yahoo.com/group/qpcrlistserver/?v=1&t=search&ch=web&pub=groups&sec=group&slk=1) and the qPCR reference website http://www.gene-quantification.info/).

MATERIALS

Reagents

Primers, forward and reverse, sequence-specific (25X; 7.5 µM)

Primer-probe mix

To make the primer-probe mix, adjust primers and dual-labeled probe to a 75X concentration. For example, if it has been determined by optimization that the primers work best at a final concentration of 300 nM, their concentration in the stock solution should be 22.5 µM. Likewise, for a final 200 nM probe concentration, adjust its stock solution to 15 µM. Mix equal volumes of each of the primers and probe just prior to each assay.

QuantiTect SYBR Green RT-PCR kit (QIAGEN)

- QuantiTect RT Mix (includes Omniscript Reverse Transcriptase and Sensiscript Reverse Transcriptase)
- 2X QuantiTect SYBR Green RT-PCR Master Mix (includes HotStarTaq DNA pol, QuantiTect SYBR Green RT-PCR Buffer, dNTP mix, including dUTP, SYBR Green I, ROX passive reference dye, 5 mM $MgCl_2$)
- RNase-free H_2O

QuantiTect Probe RT-PCR kit (QIAGEN) (optional; for dual-labeled probe-based qRT-PCR)

- 2X QuantiTect Probe RT-PCR Master Mix

QuantiTect RT Mix

RNase-free H_2O

QuantiTect Multiplex RT-PCR Kit (QIAGEN) (optional; for multiplex reactions with three or more primer-probe sets)

RNA (diluted to <5 ng/µL) that has been isolated from microdissected biofilms (see **Isolation of RNA and DNA from Biofilm Samples Obtained by Laser Capture Microdissection Microscopy** [Pérez-Osorio and Franklin 2008])

Target gene DNA (positive control template for qRT-PCR; see Steps 19, 21, and 23)

Equipment

Computer (Internet-connected)

Ice

Microcentrifuge tubes and caps, 100 µL (Corbett Life Science)

Real-time PCR system

Rotor-Gene 3000 or 6000 (or equivalent) (Corbett Life Science)

PC

Software

METHOD

Primer and Dual-Labeled Probe Design

Primer and dual-labeled probe specificity and performance are crucial to accurate qRT-PCR. Test the properties of primers and probes in silico before purchase. Then evaluate the primer-probe combinations for performance in qRT-PCR.

In Silico Design of PCR Primers and Dual-Labeled Probes

1. Paste the sequence name and gene sequence of interest into the appropriate boxes within the "Main" tab of Primer3Plus.

2. In the "General Settings" tab, modify the following parameters:

   i. Specify product size.
   The recommended product size for SYBR Green analysis is between 190 bp and 250 bp, optimally 225 bp. The recommended product size for dual-labeled probe analysis is between 70 bp and 120 bp, optimally 80 bp.

   ii. Specify primer G+C content of 45%-55%.
   This parameter should be relaxed if a primer sequence cannot be found under this restriction.

   iii. Use the default settings for other parameters in General Settings.

3. In the "Advanced Settings" tab, modify the following parameters:

   i. Set "Max Poly-X" = 3.

   ii. Set "Number to return" = 10.

   iii. Set "Max 3' Stability" = 8.

   iv. (Optional) For additional design stringency, set "Max Self Complementarity" = 3.

   v. (Optional) For additional design stringency, set "Max 3' Self Complementarity" = 0.

   vi. The default settings may be used for other parameters in Advanced Settings.
   The parameters may also be modified or relaxed to obtain primers and probes for difficult gene sequences.

4. Use the "Internal Probe" tab when designing a dual-labeled hybridization probe. Modify internal probe settings as follows:

   i. "Hyb Oligo Size" = minimum 20, optimum 26, maximum 30.

   ii. "Hyb Oligo Tm" = minimum 67, optimum 68, maximum 70.

   iii. "Hyb Oligo Max Poly-X" = 3.

Virtual Evaluation of Primers

5. After the primers have been designed, check them for the following characteristics:
   - Primers should have a G+C content close to 50%.
   - Primers should not contain strings of the same base longer than 3 nucleotides.

- Each primer should not have more than two to three Gs or Cs in the last five bases of its 3' end.
- Primers should not contain a T at the 3' end.
- Primers should not fold into secondary structures, including high-energy hairpins (test with mfold or IDT OligoAnalyzer). The melting temperature (T_m) for secondary structures that do form should be lower than the temperatures used during reverse transcription and cycling.
- Primers should be specific for their target. Run a computer search (e.g., with BLAST) against the genome of the target DNA sequence to verify that each primer and particularly the 8-10 bases of their 3' end are unique.
- Primers should be near the 3' end of the cDNA to avoid RNA degradation problems.

6. Evaluate the target gene sequence.
   - The melting temperature (T_m) for secondary structures that form should be lower than the temperatures used during reverse transcription and cycling.
   - The G+C content should be ~50%.
   - There should be no strings with more than five identical bases in the sequence.

Virtual Evaluation of Dual-Labeled Probes

7. After the probe has been designed, check it for the following characteristics:
   - The probe should have a 3' phosphate group to eliminate the possibility of the probe acting as a primer and extending.
   - The probe-template hybrid should have a T_m that is 8°C -10°C higher than the T_m for the primer-template hybrids.
   - The 5' end of the probe should not have a G residue.
   - The probe sequence should not have more G than C residues. If it does, switch the template strand to which the probe hybridizes.

Primer Performance

8. After evaluating the primers in silico, test their performance in a SYBR Green-based qRT-PCR using each primer at 300 nM (see Steps 19 and 20). The reaction should produce significantly increased fluorescence (as much as 50-fold higher than baseline fluorescence) and a low threshold cycle (C_T) value.

See Troubleshooting.

9. Evaluate amplicon characteristics by melt curve analysis (see Step 20ii). It is important for the melt curve profile to have only one peak.
The melt curve is a measure of the change in fluorescence of the PCR products as temperature increases; a melt curve of a reaction that produces a single amplicon will have only one peak, at the T_m *of the specific PCR product.*

10. Check the specificity of RT-PCR product(s) by agarose gel electrophoresis (see **Agarose Gel Electrophoresis** [Sambrook and Russell 2006]). A single band, representing the specifically amplified product, should be visible. Use a gel that can resolve this band from nonspecifically amplified bands.

11. Check the reaction efficiency. Create a standard curve by performing qRT-PCR with template that has been serially diluted over five to seven orders of magnitude. Use the slope of the standard curve to calculate efficiency:
Efficiency = $10^{(-1/\text{slope})}-1$

The resulting efficiency should fall between 90% and 110%.

12. Ensure good assay linearity and sensitivity. Linearity is measured by the r^2 value derived from the standard curve regression analysis. Sensitivity refers to the last standard that retains linear characteristics, giving a measure of the detection and quantification limits of the assay.

13. Optimize and/or redesign primer pairs that fail to perform as desired.

Dual-Labeled Probe Performance

14. After evaluating the primers and probe in silico, test primer performance in the absence of the probe by using the SYBR Green-based assay (Steps 19 and 20).

15. Select a primer pair with good performance characteristics, and use them to test the probe in dual-labeled probe-based qRT-PCR using the probe at 200 nM (Steps 21 and 22). The reaction should produce a two- to fivefold increase in fluorescence and a low C_T value.

See Troubleshooting.

16. Optimize and/or redesign primer-probe sets that fail to perform well.

Multiplex Assay Performance

17. To determine if primer-probe sets can be used together in multiplex reactions, select two or more primer-probe sets with excellent performance characteristics in singleplex reactions (see Steps 14-16). Test their performance in a multiplex reaction using dual-labeled probe-based qRT-PCR as described in Steps 21 and 22.
Ensure that all primer-probe sets have similar reaction efficiencies in singleplex reactions before using them together in a multiplex reaction (see Step 11).

18. Check the performance of the primer-probe sets in the multiplex reaction; their reaction characteristics should be similar to those obtained in singleplex reactions. They should each produce a two- to fivefold increase in fluorescence and a low C_T value. Each primer-probe set should show good reaction efficiency (see Step 11).
See Troubleshooting.

qRT-PCR

Three methods for qRT-PCR are described here: the SYBR Green method (Steps 19 and 20), the dual-labeled probe approach (Steps 21 and 22), and the multiplex dual-labeled probe approach (Steps 23 and 24). Use the primers and probes designed in Steps 1-7, and follow the steps below in combination with the recommendations from the thermal cycler's manufacturer.

SYBR-Green-Based qRT-PCR

19. Assemble the following 25-µL reaction in a 100-µL microcentrifuge tube on ice:
If the RNA volume is changed, ensure a final reaction volume of 25 µL by adjusting the amount of RNAse-free H_2O.

In addition to the samples to be analyzed, include a negative control reaction without RNA, a DNA contamination control reaction for each sample without RT mix, and a positive control reaction in each assay.

RNAse-free H_2O	5.25 µL
2X QuantiTect SYBR Green RT-PCR Master Mix	12.5 µL
25X Forward primer	1 µL
25X Reverse primer	1 µL
QuantiTect RT mix	0.25 µL
RNA	5 µL

20. Perform the RT-PCR.

i. Program the thermal cycler to perform the following reverse transcription and PCR cycles. Acquire fluorescence data during the 72ºC extension.

No. of cycles	Reverse Transcription	Denaturation	Annealing	Extension
1	30 min at 50ºC			
1		15 min at 95ºC[a]		
45		15 sec at 94ºC	30 sec at annealing temperature (ideally 60ºC)[b]	30 sec at 72ºC

[a]The initial 15 min at 95ºC is to activate the HotStarTaq DNA polymerase.

[b]Annealing temperature depends on primer melting temperatures.

ii. Verify the specificity and identity of the RT-PCR products by performing a melt curve analysis: Add a melting step that ramps from 72ºC to 99ºC, rises by 1ºC in each step, and waits for 30 sec on the first step and 5 sec each step afterward.

iii. Set the gain of the SYBR Green channel at 10 and do not perform a gain optimization.

Dual-Labeled Probe-Based qRT-PCR

21. Assemble the following 25-µL reaction in a 100-µL microcentrifuge tube on ice:
If the RNA volume is changed, ensure a final reaction volume of 25 µL by adjusting the amount of

RNAse-free H_2O.

In addition to the samples to be analyzed, include a negative control reaction (one without RNA), a DNA contamination control reaction (one without RT mix), and a positive control reaction in each assay.

RNAse-free H_2O	6.25 µL
2X QuantiTect Probe RT-PCR Master Mix	12.5 µL
Primer-probe mix	1 µL
QuantiTect RT mix	0.25 µL
RNA	5 µL

22. Perform the RT-PCR.

i. Program the thermal cycler to perform the following reverse transcription and PCR cycles. Acquire fluorescence data during the annealing step.

No. of cycles	Reverse Transcription	Denaturation	Annealing/Extension
1	30 min at 50ºC		
1		15 min at 95ºC[a]	
45		15 sec at 94ºC	60 sec at annealing temperature (ideally 60ºC)[b]

[a]The initial 15 min at 95ºC is to activate the HotStarTaq DNA polymerase.

[b]Annealing temperature depends on primer melting temperatures.

ii. Perform a gain optimization on the tube position with highest template concentration before the first acquisition.

Multiplex Dual-Labeled Probe-Based qRT-PCR

23. Assemble the following 25-µL reaction in a 100-µL microcentrifuge tube on ice:
If the RNA volume is changed or more primer-probe mixes are used, ensure a final reaction volume of 25 µL by adjusting the amount of RNAse-free H_2O.

In addition to the samples to be analyzed, include a negative control reaction without RNA, a DNA contamination control reaction for each sample without RT mix, and a positive control reaction in

each assay.
For multiplex reactions that use three or more primer-probe sets, the QuantiTect Multiplex RT-PCR kit is available.

RNAse-free H_2O	5.25 µL
2X QuantiTect Probe Master Mix	12.5 µL
Primer-probe mix A	1 µL
Primer-probe mix B	1 µL
QuantiTect RT mix	0.25 µL
RNA	5 µL

24. Perform the RT-PCR.

i. Program the thermal cycler to perform the following reverse transcription and PCR cycles. Acquire fluorescence data during the annealing step.

No. of cycles	Reverse Transcription	Denaturation	Annealing/Extension
1	30 min at 50ºC		
1		15 min at 95ºC[a]	
45		15 sec at 94ºC	30 sec at annealing temperature (ideally 60ºC)[b]

[a]The initial 15 min at 95ºC is to activate the HotStarTaq DNA polymerase.

[b]Annealing temperature depends on primer melting temperatures.

ii. Perform a gain optimization on the tube position with highest template concentration before the first acquisition.

Data Analysis

Two methods for analyzing the qRT-PCR data with Corbett's Rotor-Gene software are presented here. For the Standard Curve Analysis (Steps 25-32), a standard calibration curve must be included in the assay. In Comparative Quantitation Data Analysis (Steps 33-38), the relative concentrations of samples are determined without a calibration curve and without threshold lines. Consult the help menu of the Rotor-Gene software for more information about these analysis methods.

Standard Curve Analysis

25. Determine the starting amounts for each dilution of the standard curve. Assign a concentration to each standard within the "Edit Sample" menu of the Rotor-Gene software.
These assigned concentrations can be arbitrary, following the dilution pattern of the sample (i.e., 100, 10, and 1 ng/µL, for a 10-fold dilution series). They can also be relative concentrations derived from transcript-calculated copies when using transcript-specific standards.

26. Identify and label unknown samples within the "Edit Sample" menu.

27. When acquiring data from multiple channels (i.e., in multiplex assays), assign a page to each channel within the "Edit Sample" menu; this will allow a separate analysis per channel.

28. Under the "Analysis" window, select the "Quantitation" tab. Select the appropriate channel and choose a page to analyze.

29. Ensure that all standards have been selected for analysis, and then use the "Autofind" feature located on the right side of the screen to calculate the position of the threshold line.

30. When running a series of assays over a period of time, select the threshold from an assay with good linearity and low variability (<12%) among standards.

31. Select all samples and export the data from the "Quantitative Result" window into a spreadsheet program.

32. Combine technical replicates by calculating the geometric mean or by using the "replicate calculated value" given by the Rotor-Gene software.

Comparative Quantitation Data Analysis

33. Under the "Analysis" window of the Rotor-Gene software, and under the "Other" tab, select the "Comparative Quantitation" tool.

34. Select the appropriate channel and choose a page to analyze.

35. Select all samples and export the data from the "Comparative Quantitation" window into a spreadsheet program.

36. Combine technical replicates by calculating the geometric mean or by using the "replicate calculated value" given by the Rotor-Gene software.

37. Consider using samples with a reaction amplification above 1.6, and screen for unusually low

takeoff numbers.
A 100% efficient reaction has a reaction amplification value of 2.

38. Log-transform (LT) the C_T values using the following equation:

$LT = (amplification^{-takeoff})*10^{10}$

TROUBLESHOOTING

Problem: Primers do not perform well at 300 nM.

[Step 8]

Solution: Optimize the working concentrations of the primers.

1. Develop a matrix in which one primer is tested at various concentrations while the other primer is kept at one concentration. Do this for both primers.

2. Final primer concentrations to test are: 50 nM, 100 nM, 300 nM, 600 nM, and 900 nM.

3. Choose primer concentrations that result in the highest increase in fluorescence and the lowest C_T value.

Problem: Primer-probe set does not perform well.

[Step 15]

Solution: Optimize the working concentration of the probe.

1. Develop a matrix in which both primers are kept at a constant concentration while the concentration of the probe is modified.

2. Final probe concentrations to test are: 50 nM, 100 nM, 200 nM, and 300 nM.

3. Select the primer-probe combination that results in the highest increase in fluorescence and the lowest C_T value.

Problem: Primer-probe sets perform worse in multiplex reactions than in singleplex reactions.

[Step 18]

Solution: Optimize the multiplex reaction by varying the ratios between primer sets. To do this, keep primer and probe concentrations constant but alter the ratio between sets until all sets have reaction characteristics similar to those obtained in singleplex reactions.

DISCUSSION

Because qRT-PCR is an important approach for studying gene expression of bacteria in their native environments, it has been applied to many studies in environmental microbiology (Fey et al. 2004; Roussel et al. 2006; Matsuda et al. 2007). qRT-PCR is very sensitive (it can detect as few as 100 RNA transcripts), so it is particularly useful for biofilm research, where gene expression can be quantified directly from very little starting material, as in subsections of biofilms obtained by LCMM (Lenz et al. 2008). For such quantitation to be accurate, it is important that appropriate protocols be used (Livak and Schmittgen 2001; Bustin 2004; Nolan et al. 2006).

This article describes two qRT-PCR methods: the SYBR Green and dual-labeled probe approaches. In SYBR Green-based qRT-PCR, a fluorescent dye binds to double-stranded DNA in the PCR, and a C_T value is obtained during exponential increase in the number of amplicons. In the dual-labeled probe approach, a probe specific for the PCR product, containing a fluorescent label and a fluorescence-quenching molecule, is degraded by *Taq* polymerase as the amount of PCR product increases. This degradation results in an increase in fluorescence intensity from which a C_T value may be obtained.

Although the SYBR Green approach is cost-effective, the dual-labeled probe approach allows analysis of multiple genes simultaneously (multiplexing), because different fluorescently labeled probes can be used for different genes.

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