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Chapter 3

Real-Time Reverse Transcription PCR as a Tool to Study Virulence Gene Regulation in Bacterial Pathogens

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Abstract

Quantitative real-time PCR (qRT-PCR) is a highly sensitive and reliable method for detection and quantification of DNA. When combined with a prior stage of RNA reverse transcription to generate complementary DNA (cDNA), this is a powerful approach to determine and analyze gene transcriptional expression. Real-time quantitative reverse transcription PCR has become the gold standard method in studying genes expression and virulence regulation under various genetic backgrounds (e.g., in the absence of regulators) or environmental conditions. Here we demonstrate the utilization of this approach to study the transcriptional regulation of the conjugation pilus of the *Salmonella enterica* serovar Infantis virulence plasmid (pESI).

Key words Real-time PCR, Reverse transcription, cDNA, SYBR green dye, ROX, Transcription, Regulation, Gene expression

1 Introduction

Salmonella enterica is a Gram-negative highly diverse bacterial pathogen that is able to infect and cause a disease in a wide range of animal hosts including human. The single biological species *S. enterica* contains more than 2600 distinct biotypes known as serovars that can be associated with diverse animal hosts and may cause different clinical manifestations [1]. Hundreds of genes scattered over the *S. enterica* chromosome and different virulence plasmids are required for its pathogenicity. Such genes may encode secretion pathways, adhesion and invasion factors, host-translocated effectors and toxins, flagella and chemotaxis proteins and more [2]. Such diversity of virulence factors indicates that *Salmonella* pathogenicity is a multifactorial phenotype that is shaped by complex interactions between the pathogen and its host. In order to cause a disease, a pathogen has to tightly regulate multiple virulence pathways and orchestrate the expression of numerous virulence genes in timely and spatial manner in response

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to environmental signals [3]. Therefore, studying virulence gene regulation is essential to understand *Salmonella* biology and its unique pathogenicity.

Real-time quantitative reverse transcription PCR (real-time qRT-PCR) is a well-established technology that has become the method of choice for the detection and quantification of RNA targets [4] that has dramatically changed the way by which gene expression is being measured. Real-time qRT-PCR is based on the inherent quantifiable nature of PCR, making this technique a quantitative as well as a qualitative assay that provides a very wide dynamic range and allows comparison between RNAs with different abundance [5].

In real-time qRT-PCR, RNA isolated from the cells is reverse transcribed into the first strand of cDNA, which now can be used as the template for a subsequently real-time PCR analysis. This technique enables reliable detection and measurement of products generated during each cycle of the PCR process, by combining amplification and detection into a single step. Detection is achieved using different fluorescent chemistries that correlate amplicon (the PCR product) concentration to fluorescence intensity [6]. Thus, generation of the PCR amplicon can be identified at precise points over time and linked with a particular PCR cycle number. When the amplification of the target is first detected, this value is referred to as cycle threshold (C_T), indicating the number of the PCR cycle, at which fluorescence intensity of the generated amplicon is higher than the background. Therefore, the greater the quantity of target DNA in the reaction, the earlier an increase in fluorescent signal will be detected, resulting in a lower C_T.

Currently available fluorescent chemistries to detect the amplification of a target during the real-time PCR reaction can be classified into two main groups. The first group comprises fluorophore-linked oligonucleotides (primer probes) such as the TaqMan chemistry, enabling the detection of specific PCR products only. The second group includes double-stranded DNA (dsDNA) intercalating molecules, such as the SYBR Green 1 or EvaGreen dyes, allowing for nonspecific detection of amplified products due to their ability to bind dsDNA.

To study virulence genes regulation in *Salmonella* we employ the SYBR green 1 dye chemistry and ROX as the passive reference dye. The dsDNA-SYBR Green complex absorbs blue light (λ_{max} = 497 nm) and emits green light (λ_{max} = 520 nm). This way, the fluorescence that is measured after each PCR cycle is proportional to the accumulating dsDNA amplicon [7]. For data analysis we use the comparative C_T method ($\Delta\Delta C_T$) [8], which compares the C_T value of a target RNA of interest with a reference control. This may involve a comparison between two genetic backgrounds (e.g., wild type vs. regulatory mutant) or growth conditions (e.g., growth in rich LB broth vs. minimal media). The C_T

values of both the sample and the reference are normalized to the C_T of an endogenous housekeeping gene that presents a constant expression under the examined experimental conditions (we usually use the *rpoD* or 16S rRNA mRNA).

To demonstrate this approach we will show the transcription regulation of *pilV*, encoding the minor pilin subunit of the conjugative pilus of the virulence-resistance plasmid, pESI in *Salmonella enterica* serovar Infantis under different environmental conditions and genetic backgrounds (Figs. 1 and 2) [9, 10].

2 Materials

1. Luria–Bertani (LB) broth (Lennox) For 1 L: 10 g tryptone, 5 g yeast extract, 10 g NaCl (pH 7.0).
2. Minimal media [e.g., N-minimal media containing 80 mM MES (pH 5.8) or 100 mM Tris–HCl (pH 7.0), 5 mM KCl, 7.5 mM $(NH_4)SO_4$, 0.5 mM K_2SO_4, 337 μM K_2HPO_4/KH_2PO_4, 20 mM $MgCl_2$, 38 mM glycerol, and 0.1% Casamino acids].
3. RNA stabilization reagent (e.g., the QIAGEN RNAprotect Bacteria Reagent).
4. RNA purification kit (e.g., the QIAGEN RNeasy Mini Kit).
5. TE buffer (Tris–EDTA, 30 mM Tris, 1 mM EDTA, pH 8.0) containing 15 mg/mL lysozyme from chicken egg white and 20 mg/mL Proteinase K.
6. RLT buffer (from the RNeasy Mini Kit) containing β-mercaptoethanol (10 μL of β-ME per 1 mL of RLT buffer).
7. Analytical grade ethanol absolute.
8. 70% ethanol.
9. RNase-free DNase I.
10. Sodium acetate (3 M, pH 5.2).
11. DEPC-treated water.
12. Spectrophotometer (e.g., NanoDrop).
13. Reverse transcription kit for qRT-PCR.
14. FastStart Universal SYBR Green Master (ROX).
15. Real-time PCR system.

3 Methods

3.1 RNA Purification

RNA isolation is based on the QIAGEN RNeasy Mini Kit protocol [11] with various adjustments. Other established protocols (e.g., phenol–chloroform extraction or TRIzol reagent) or kits from other manufacturers can be used as well for this purpose.

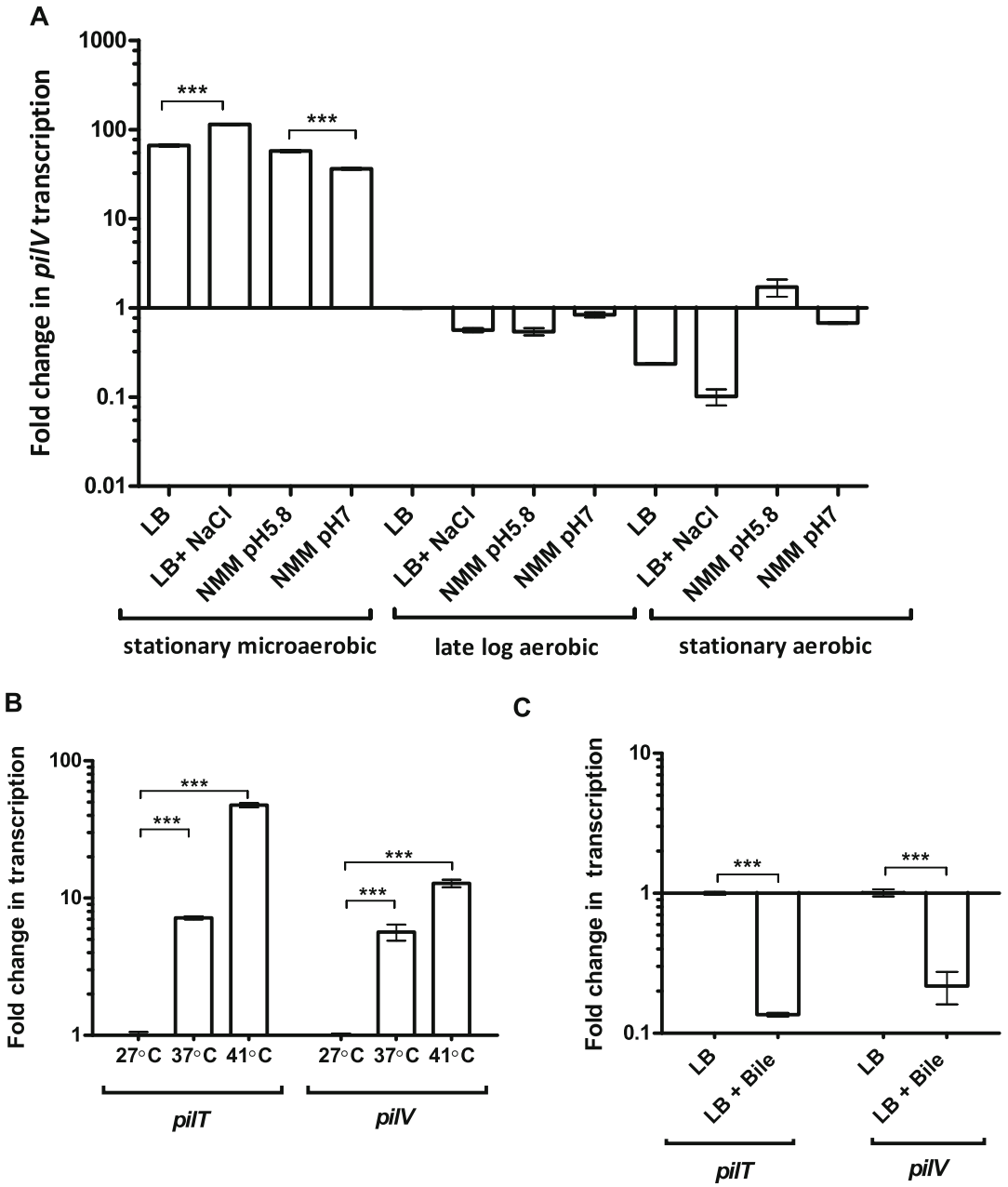

Fig. 1 The transcription of pESI pilus is induced in response to microaerobic, physiological temperature and repressed by bile. Real-time qRT-PCR was used to study the expression of *pilV*, encoding the minor pilin subunit of the conjugative pilus of the virulence-resistance plasmid, pESI in *Salmonella enterica* sv Infantis. (**a**) qRT-PCR shows the fold change in *pilV* transcription under different growth conditions. RNA was extracted from *S.* Infantis st. 119944 cultures grown in LB, LB supplemented with 0.3 M NaCl, N-minimal medium pH 5.8 and N-minimal medium pH 7.0. Differences in *pilV* expression are shown relative to the transcription of *pilV* in LB late-logarithmic culture grown under aerobic conditions. Induction of *pilV* under microaerobic growth conditions can be appreciated. (**b**) Fold change in the transcription of *pilV* and *pilT* grown in LB under microaerobic conditions at 37 °C and 41 °C relative to 27 °C. Induction of both genes at 41 °C can be seen. (**c**) Fold change in the transcription of *pilV* and *pilT* grown under microaerobic conditions at 37 °C in LB supplemented with 4% bile salts (sodium cholate) is shown. Repression of gene expression can be seen in the presence of bile. All RT-PCR results show the mean and standard deviation of three to six biological repeats. *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$. This figure is reproduced from ref. [9] with permission from the publisher

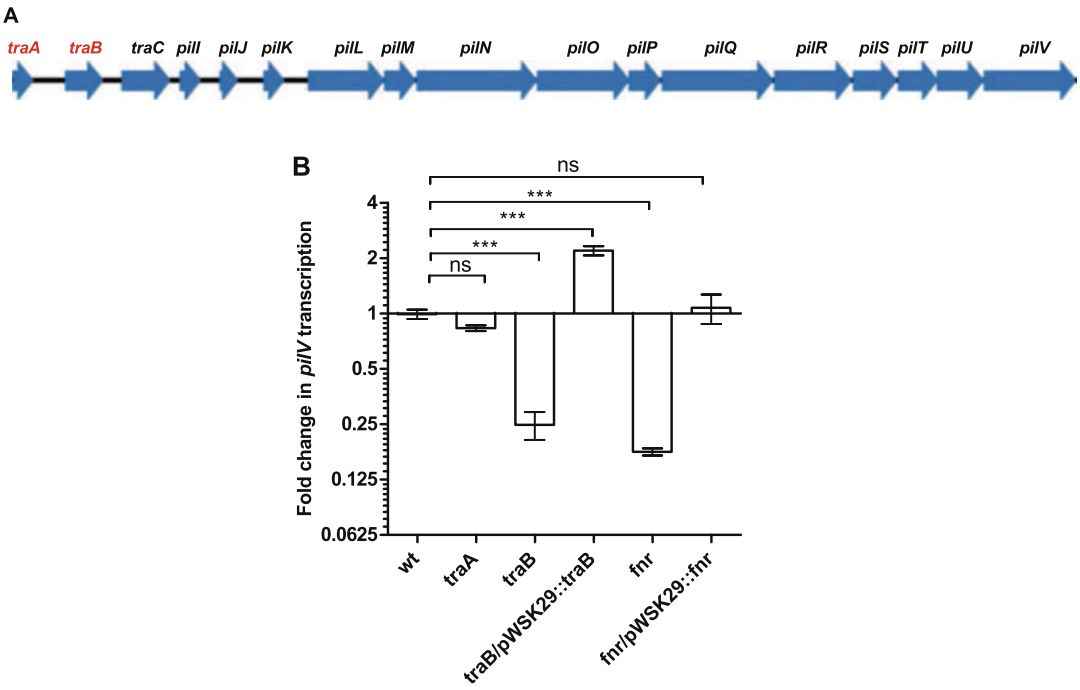

Fig. 2 pESI pilus transcription is regulated by TraB and FNR. (**a**) Gene organization of the *pil* cluster encoded on pESI. Arrowheads show location and orientation of the different ORFs. Putative regulatory genes are shown in red. (**b**) RNA was extracted from *S.* Infantis st. 119944 (wt), its derivative mutants (*traA*, *traB*, and *fnr*) and complemented strains *traB*/pWSK29::*traB* and *fnr*/pWSK29::*fnr* cultures grown in LB under microaerobic conditions at 37 °C. qRT-PCR analyses were conducted to determine the fold change in the transcription of *pilV* in the indicated backgrounds relative to the wild-type strain. One-way ANOVA with Dunnett's Multiple Comparison Test was implemented to determine statistical significance. *, $P < 0.005$; **, $P < 0.001$; ***, $P < 0.0001$; ns, not significant. A positive regulatory role in controlling *pilV* expression was found for the regulators Fnr and TraB. This figure is reproduced from Aviv et al. 2016 [9] with permission from the publisher (ASM Journals)

1. Grow appropriate *Salmonella* cultures at the desired experimental growth conditions.
2. Measure the optical density (OD_{600}) of the cultures using a spectrophotometer and normalize (using the growth media) all cultures to $OD_{600} \sim 1$.
3. Transfer 0.5 mL (~4×10^8 CFU) from the normalized cultures into 1.5 mL microcentrifuge test tubes (*see* **Note 1**).
4. Immediately add 1 mL of RNAprotect Bacteria Reagent and mix well by vortex for 5 s (*see* **Note 2**).
5. Incubate for 5 min at room temperature (*see* **Note 3**).
6. Centrifuge for 10 min, 11,000 × *g* at room temperature using a microcentrifuge.

7. Remove the supernatant and add 115 μL of TE buffer containing 15 mg/mL lysozyme and 20 mg/mL Proteinase K and carefully resuspend the pellet by pipetting.
8. Incubate at room temperature for 10 min and gently mix by inverting the tube every 2 min (*see* **Note 4**).
9. Add 350 μL of RLT buffer containing β-mercaptoethanol and mix.
10. Add 250 μL of analytical grade absolute ethanol and mix (*see* **Note 5**).
11. Transfer the samples into the RNeasy Mini Spin column placed in a 2 mL collection tube and centrifuge for 1–2 min at 11,000 × *g* in a table top centrifuge. Make sure that all liquid has passed through the column before discarding the flow-through.
12. Add 350 μL of buffer RW1 to the column. Centrifuge for 1 min at 11,000 × *g* in a microcentrifuge.
13. Prepare DNase I RNAse-free Stock Solution by dissolving lyophilized DNase I in 550 μL of RNase-free water. Place on ice the required amount for the subsequent steps and store the rest in aliquots at −20 °C (*see* **Note 6**).
14. Prepare the DNase I Incubation Mix by adding 10 μL of DNase I Stock Solution to 70 μL RDD buffer (supplied with the RNeasy Mini Kit) and mix gently (*see* **Note 7**).
15. For on-column DNase I treatment, add 80 μL DNase I Incubation Mix directly to the RNeasy column membrane and incubate for 15 min at room temperature inside biosafety cabinet, to protect from potential contaminations (*see* **Note 8**).
16. Add 500 μL buffer RPE (supplied in the RNeasy Mini Kit) to the RNeasy spin column and centrifuge for 1 min at 11,000 × *g* in a microcentrifuge. Repeat this stage once more.
17. Place the RNeasy spin column in a new 1.5 mL collection tube and add 40 μL of RNase-free water directly to the spin column membrane.
18. Incubate for 10 min at room temperature and centrifuge for 1 min at 11,000 × *g* in a microcentrifuge to elute the RNA. Place the purified RNA on ice.
19. Measure the RNA concentration using spectrophotometer and take about 2 μg of RNA for a secondary DNase I treatment in solution (*see* **Note 9**).
20. Mix the following ingredients in a 1.5 mL test tube for a secondary DNAse I digestion: ~2 μg of the purified RNA, 10 μL buffer RDD, and 2.5 μL DNase I Stock Solution and top up to a final volume to 100 μL with RNase-free water.

21. Incubate at room temperature for 10 min. Avoid long incubation times, which may compromise RNA integrity.
22. Perform ethanol precipitation of the DNAse I-treated RNA by adding 10 μL of 3 M sodium acetate (pH 5.2) and 250 μL of ice-cold ethanol absolute. Mix well and incubate for ≥3 h at −20 °C.
23. Centrifuge at 11,000 × *g* for 20 min at 4 °C in a refrigerated microcentrifuge. Carefully remove the supernatant without disturbing the pellet (which may be invisible).
24. Add 750 μL of ethanol 70%, invert the tube a few times and centrifuge at maximal speed for 2 min at 4 °C.
25. Remove supernatant and leave the tube open inside a biosafety cabinet (not on ice) for ~15 min, until the entire ethanol has evaporated.
26. Add 40 μL of RNase-free water and resuspend the RNA by pipetting. Place the RNA samples on ice.
27. Measure RNA concentration using NanoDrop spectrophotometer or equivalent and store unused RNA at −80 °C. Avoid repetitive freezing and thawing of the RNA samples.

3.2 Reverse Transcription of the RNA (cDNA Synthesis)

1. Into a clean RNAse-free 0.2 mL PCR tube add 150–200 ng purified DNAse I-treated RNA, 4 μL of iScript Reverse Transcription Supermix (Bio-Rad Laboratories) or any other cDNA synthesis mix of choice (*see* **Note 10**), and RNase-free water to a final volume of 20 μL.
2. Insert the tubes in a Thermal Cycler and run the following program: priming for 5 min at 25 °C, reverse transcription for 30 min at 42 °C, reverse transcriptase inactivation for 5 min at 85 °C.
3. Store the cDNA at −20 °C for short periods or at −80 °C for long-term storage.

3.3 Preparation of the Samples for RT-PCR

1. Dilute each one of the PCR primers (forward and reverse) to a final concentration of 30 pmol/μL in a RT-PCR primers mix (*see* **Note 11**).
2. Place a 96-Well optical real time PCR plate or proper PCR tubes on ice. For each reaction add 10 μL of SYBR Green reagent, 0.8 μL of the primers mix, and 7.2 μL of RNase-free water.
3. To each reaction add 2 μL of the cDNA (made in 3.2) to make a total volume of 20 μL per reaction. We recommend including at least three replicates for each target.
4. In addition, set up a control reaction to test for DNA contamination. To prepare this control, dilute 150–200 ng purified RNA (before the reverse transcription stage) in 20 μL RNase-

free water that will be used as a control in the absence of a reverse transcription step. Take 2 μL from this RNA suspension into 18 μL of the real-time PCR mix (as in **step 2**).

5. Additional control should include PCR reaction with no template. Instead of cDNA add 2 μL of RNAse-free ddH_2O. Make sure to include this type of control for every amplified target. This reaction controls for the lack of DNA contamination in any of the used reagents.
6. If using 96-well plate, cover the top with an adhesive PCR plate foil and centrifuge briefly (~30 s) at 500 × *g* to collect residual liquid from the sides of the wells.

3.4 Real-Time PCR Reaction

1. Place the plate in the RT-PCR instrument.
2. Set the correct parameters according to the type of experiment performed, in this case Quantitation-Comparative C_T ($\Delta\Delta C_T$).
3. Real-time cycling conditions are as follow: 95 °C for 10 min; and 40 cycles of 95 °C for 15 s, 60 °C for 1 min.
4. Make sure to include a melt (dissociation) curve analysis of the amplified targets.
5. Examine the melting temperatures from the dissociation curve in order to assess the homogeneity of the PCR products and to determine the specificity of the PCR reaction (*see* **Note 12**).

3.5 Data Analysis

In order to analyze the results we use the comparative threshold method ($2^{-\Delta\Delta C_T}$). This involves comparing the C_T values of the sample of interest (a mutant or a certain growth condition, particular treatment, etc.) with a reference (e.g., the wild-type strain or standard growth conditions). The C_T values of both the sample and the reference are normalized to the C_T values of an appropriate endogenous housekeeping gene determined for each condition or background (*see* **Note 13**).

Steps for calculating the fold change of expression levels:

1. Calculate the average C_T of the endogenous housekeeping gene, target and the reference.
2. Calculate the delta C_T (ΔC_T) of the target gene by subtracting the average C_T of the endogenous housekeeping gene from the C_T value of the target.
3. Calculate the delta C_T (ΔC_T) of the reference by subtracting the delta C_T of the endogenous housekeeping gene from the C_T value of the reference.
4. Calculate the delta delta C_T ($\Delta\Delta C_T$) value by subtracting the delta C_T (ΔC_T) of the reference from the delta C_T (ΔC_T) of the target gene using the following formula: $\Delta\Delta C_T = [\text{delta}]\ C_{T\ \text{target}} - [\text{delta}]\ C_{T\ \text{reference}}$.
5. Calculate the fold change of expression as $2^{-\Delta\Delta C_T}$.

4 Notes

1. Applying higher amount of cells may block the column and result in a lower RNA yield.
2. RNAprotect Bacteria Reagent should be added to the bacteria culture in a 2:1 ratio (V/V).
3. At this stage, one can store the RNAprotect-treated culture at −80 °C or continue to **step 6**.
4. Mix gently by inverting up and down the tube. Do not use vortex at this stage.
5. From hereafter perform all the following steps in a biosafety cabinet (to prevent contamination of the samples) and use only RNase-free reagents. Use only filtered pipette tips and make sure to wear gloves and replace them frequently.
6. When preparing the DNase I stock solution, inject the RNase-free water into the vial using a needle and syringe. Mix gently by inverting the vial. Aliquot the DNase I solution and store at −20 °C.
7. This amount is sufficient for one sample. For more than one sample, multiply the amounts by the number of samples you need to treat.
8. Make sure to add the DNase I Incubation Mix directly to the RNeasy spin column membrane. DNase digestion will be incomplete if part of the mix stays on the walls or the O-ring of the spin column.
9. In our hands, a second treatment of DNAse I in solution is required to eliminate all traces of DNA from *Salmonella* cultures grown in rich media.
10. If using other cDNA synthesis kit, the volume of the reagents may need to be adjusted according to the manufacture protocol.
11. Design the RT-PCR primers with an annealing temperature of 55–60 °C to amplify a150–200 bp fragment.
12. This step is very important especially when using the SYBR green 1 dye because it lacks sequence specificity. If a PCR product results in the no-template and no-reverse transcription controls, make sure that these amplicons have a different melting curve than the experimental samples. If they share the same Tm, it may indicate a genomic DNA contamination in the RNA samples. Primer-dimers may also give a detectable signal, but these will have a different melting curve than the target amplicon.
13. Select an endogenous housekeeping gene, which have similar C_T values under the studied growth conditions or genetic backgrounds (e.g., 16 s rRNA, *rpoD*, or *dnaK*).

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