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# Quantification of Densities of Bacterial Endosymbionts of Insects by Real-time PCR

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**[Abstract]** Increased attention has been paid to the endosymbiotic bacteria of insects. Because most insect endosymbionts are uncultivable, quantitative PCR (qPCR) is a practical and convenient method to quantify endosymbiont titers. Here we report a protocol for real-time qPCR based on SYBR Green I fluorescence as well as some tips to prevent possible pitfalls.
**Keywords:** Bacteria, Endosymbionts, Insects, qPCR, Quantification, SYBR Green

**[Background]** Insects often harbor bacterial symbionts of various taxa in their bodies. Such bacterial symbionts (endosymbiotic bacteria) attract great attention because of their profound effects on the host insect. Some bacteria provide essential nutrition to their hosts (Baumann *et al.*, 1995), some confer resistance against parasites (Oliver *et al.*, 2003; Hedges *et al.*, 2008), and some even manipulate reproduction or sex determination of their hosts for their own benefit (Werren *et al.*, 2008; Kageyama *et al.*, 2012). Because most insect endosymbionts are uncultivable, quantitative PCR (qPCR) is a practical and convenient method to quantify endosymbiont titers (Simoncini *et al.*, 2001), possibly complemented by other visualization methods, such as fluorescence *in situ* hybridization (FISH) (Koga *et al.*, 2009) and/or electron microscopy.

## Materials and Reagents

*Note: Reagents differ depending on the qPCR equipment. Here I describe a protocol for absolute quantification using LightCycler® 480 (Roche). For each reaction, two or more technical replicates are strongly recommended.*

1. Pipette tips:
   10-µl tips (*e.g.*, Fukaekasei and Watson, catalog number: 110-201C)
   200-µl tips (Fukaekasei and Watson, catalog number: 1201-705C)
   1,000-µl tips (Fukaekasei and Watson, catalog number: 110-706C)
   To reduce the risk of contamination, use of the filtered tips, *e.g.,*
   10-µl tips (Fukaekasei and Watson, catalog number: 1251-204CS)
   200-µl tips (Fukaekasei and Watson, catalog number: 1252-703CS)
   1,000-µl tips (Fukaekasei and Watson, catalog number: 124-1000S)] is preferable
2. Centrifuge tubes of 1.5 ml (*e.g.*, Fukaekasei and Watson, catalog number: 131-7155C)

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3. Centrifuge tubes of 50 ml [*e.g.*, Falcon 50-ml conical centrifuge tubes (Corning, Falcon®, catalog number: 352070)]
4. LightCycler® 480 Multiwell Plate 96, white (Roche Molecular Systems, catalog number: 04729692001, sealing foils included)
5. Template DNA to be measured (genomic DNA extracted from insects)
6. Template DNA for standard (PCR products or inserted plasmid DNA with known concentration)
7. LightCycler® 480, buffer 1 (2x Master mix) (Roche Molecular Systems, catalog number: 04707516001)
8. LightCycler® 480, buffer 2 ($H_2O$) (Roche Molecular Systems, catalog number: 04707516001)
9. Forward and reverse primers
10. Reaction mix (see Recipes)

## Equipment

1. Spectrophotometer (Bio-Rad Laboratories, model: SmartSpec™ 3000)
2. LightCycler® 480 System (Roche Molecular Systems, model: LightCycler® 480) connected to a computer
3. Centrifuge for 96-well plate (*e.g.*, Kubota, model: PlateSpin II)
4. Pipettes [*e.g.*, PIPETMAN® P10 (Gilson, catalog number: F144802), P20 (Gilson, catalog number: F123600), P100 (Gilson, catalog number: F123615), P200 (Gilson, catalog number: F123601), or P1000 (Gilson, catalog number: F123602)]
5. Laminar flow cabinet

## Software

1. LightCycler® 480 Software, version 1.5.1 (Roche Molecular Systems)
2. Primer3Plus free online software (http://primer3plus.com/)

## Procedure

1. Design primer

   Use a single-copy gene of a target bacterium for primer design (avoid using ribosomal genes because of the possible presence of operonic copies). For example, the bacterial gene *RpoB* (Case *et al*., 2007) is considered suitable. If necessary, design primers for the host (insect) gene to standardize the bacterial titers. Use a single-copy nuclear gene from an autosome (mitochondrial genes are not recommended).

   Generally, primers which are 18-23 mer in length are used. The product size should be 80-300 bp (optimum: 80-150 bp). The desired melting temperature ($T_m$) is 55-65 °C, with a maximum

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difference of 3 °C in the $T_m$ of the two primers. It is strongly recommended using a software, such as Primer3Plus free online software (http://primer3plus.com/), to design primers.

Some of the primers that have been successfully used to detect insect endosymbionts are given in Table 1.

**Table 1. Examples of primers for endosymbiotic bacteria that have been successfully used for qPCR**

| Target bacterium (host insects) | Target gene | Primer name | Nucleotide (from 5' to 3') | Amplicon length (bp) | Reference |
|---|---|---|---|---|---|
| *Spiroplasma* (*Drosophila hydei*; *Orius sauteri*) | *dnaA* | dnaA109F | TTAAGAGCAGTTTCAAAATCGGG | 137 | Kageyama *et al.* (2006); Watanabe *et al.* (2014) |
| | | dnaA246R | TGAAAAAAACAAACAAATTGTTATTACTTC | | |
| *Spiroplasma* (*Mallada desjardinsi*) | *spoT* | spoT-MDF | TGCCAATTCCATATGAAGAC | 222 | Hayashi *et al.* (2016) |
| | | spoT-r | CACTGAAGCGTTTAAATGAC | | |
| *Rickettsia* (*Bemisia tabaci*; *M. desjardinsi*) | *gltA* | glt375-F | TGGTATTGCATCGCTTTGGG | 200 | Caspi-Fluger *et al.* (2011); Hayashi *et al.* (2016) |
| | | glt574-R | TTTCTTTAAGCACTGCAGCACG | | |
| *Wolbachia* strain *w*Cl (*Eurema mandarina*) | *wsp* | wsp81F | TGGTCCAATAAGTGATGAAGAAAC | 232 | Narita *et al.* (2007) |
| | | wHecFem1 | ACTAACGTCGTTTTTGTTTAG | | |
| *Wolbachia* strain *w*Cl (*Eurema mandarina*) | *wsp* | wHecFem2 | TTACTCACAATTGGCTAAAGAT | 398 | Narita *et al.* (2007) |
| | | Wsp691R | AAAAATTAAACGCTACTCCA | | |

2. Prepare DNA template

   Extract DNA from the whole body or a particular tissue/organ of the insect. Usually, 50-100 ng of DNA per reaction is needed. The use of purified DNA, such as that extracted using the phenol chloroform method (Green and Sambrook, 2012) or a commercial kit, such as DNeasy® Blood & Tissue Kit (QIAGEN), is recommended. The concentration of the extracted DNA can be measured using a spectrophotometer. Confirm that $A_{260}/A_{280}$ is approximately 1.8 and $A_{260}/A_{230}$ is > 1. If possible, DNA extracted from insects treated with antibiotics should also be used as a control, because this will indicate whether the amplified DNA is derived from the bacterial or the host genome (see Note 1 for details).

3. Prepare standard DNA

   For absolute quantification, DNA samples with known copies of target genes are required. The DNA samples could be PCR products or inserted plasmids. The number of copies can be estimated by mass concentration measured using a spectrophotometer with Avogadro constant $N_A = 6.022 \times 10^{23}$ copies/mol, assuming that the molecular weight of 1 bp dsDNA is approximately 660 g/mol. Use a serial dilution of standard DNA, such as $10^8$, $10^7$, $10^6$, $10^5$, $10^4$, and $10^3$ copies.

4. Prepare the reaction mix (see Recipes), vortex for 5 sec, and dispense 20 µl into each well. Apply 5 µl of DNA template (sample or standard DNA) or buffer 2 (negative control) (Figure 1).

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| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | ST10$^{8}$ rep.1 | ST10$^{8}$ rep.2 | Sample2 rep.1 | Sample2 rep.2 | Sample10 rep.1 | Sample10 rep.2 | Sample18 rep.1 | Sample18 rep.2 | Sample26 rep.1 | Sample26 rep.2 | Sample34 rep.1 | Sample34 rep.2 |
| B | ST10$^{7}$ rep.1 | ST10$^{7}$ rep.2 | Sample3 rep.1 | Sample3 rep.2 | Sample11 rep.1 | Sample11 rep.2 | Sample19 rep.1 | Sample19 rep.2 | Sample27 rep.1 | Sample27 rep.2 | Sample35 rep.1 | Sample35 rep.2 |
| C | ST10$^{6}$ rep.1 | ST10$^{6}$ rep.2 | Sample4 rep.1 | Sample4 rep.2 | Sample12 rep.1 | Sample12 rep.2 | Sample20 rep.1 | Sample20 rep.2 | Sample28 rep.1 | Sample28 rep.2 | Sample36 rep.1 | Sample36 rep.2 |
| D | ST10$^{5}$ rep.1 | ST10$^{5}$ rep.2 | Sample5 rep.1 | Sample5 rep.2 | Sample13 rep.1 | Sample13 rep.2 | Sample21 rep.1 | Sample21 rep.2 | Sample29 rep.1 | Sample29 rep.2 | Sample37 rep.1 | Sample37 rep.2 |
| E | ST10$^{4}$ rep.1 | ST10$^{4}$ rep.2 | Sample6 rep.1 | Sample6 rep.2 | Sample14 rep.1 | Sample14 rep.2 | Sample22 rep.1 | Sample22 rep.2 | Sample30 rep.1 | Sample30 rep.2 | Sample38 rep.1 | Sample38 rep.2 |
| F | ST10$^{3}$ rep.1 | ST10$^{3}$ rep.2 | Sample7 rep.1 | Sample7 rep.2 | Sample15 rep.1 | Sample15 rep.2 | Sample23 rep.1 | Sample23 rep.2 | Sample31 rep.1 | Sample31 rep.2 | Sample39 rep.1 | Sample39 rep.2 |
| G | NTC rep.1 | NTC rep.2 | Sample8 rep.1 | Sample8 rep.2 | Sample16 rep.1 | Sample16 rep.2 | Sample24 rep.1 | Sample24 rep.2 | Sample32 rep.1 | Sample32 rep.2 | Sample40 rep.1 | Sample40 rep.2 |
| H | Sample1 rep.1 | Sample1 rep.2 | Sample9 rep.1 | Sample9 rep.2 | Sample17 rep.1 | Sample17 rep.2 | Sample25 rep.1 | Sample25 rep.2 | Sample33 rep.1 | Sample33 rep.2 | Sample41 rep.1 | Sample41 rep.2 |

**Figure 1. Example of sample sheet.** ST: Standard DNA with known concentration, NTC: Non-template control ($H_2O$). When working with two replicates, 41 samples can be measured.

5. Seal the multiwell plate with sealing foil and centrifuge at 900 *x g* for 30 sec.
6. A typical PCR program may be as follows:
   a. Initial denaturation for 30 sec at 95 °C.
   b. Denaturation for 5 sec at 95 °C.
   c. Annealing for 10-30 sec at X °C (X depends on $T_m$).
   d. Extension for 10-20 sec (depends on product length, 1 min $kb^{-1}$) at 72 °C.
   e. Return to step (b) for 40 cycles.
   f. Melting at 60 °C-95 °C.

   If the $T_m$ of both the primers is considerably high (> 65 °C), a two-step PCR can be used as follows:
   a. Initial denaturation for 30 sec at 95 °C.
   b. Denaturation for 5 sec at 95 °C.
   c. Annealing and extension for 10-30 s at X °C (X depends on $T_m$)
   d. Return to step (b) for 40 cycles.
   e. Melting at 60 °C-95 °C.

   *Notes:*
   *i. Data acquisition should be performed at the extension step in each cycle (necessary for generating an amplification curve) and continuously performed at the final melting (necessary for generating a melting curve; Figure 2).*
   *ii. When preparing mixtures, special care should be taken to avoid contamination. It is recommended to use particular pipettes for this purpose in a clean environment, such as in a laminar flow cabinet.*

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*iii. A conserved bacterial gene can be chosen if it is not necessary to classify the bacteria. If the focus is on a specific family, genus, or species, the target gene should be accordingly chosen. Genes that have multiple copies in the genome, such as many outer membrane protein genes, should be avoided. Notably, strain-specific and SNP-containing genes should be avoided because it may compromise the experiment.*

*iv. When designing primers, the specificity of the primer to the target gene is important. A BLAST search for possible existence of similar nontarget sequences would be helpful for selecting specific primers. It is also desirable that the products be approximately of the same size.*

## Data analysis

After each reaction, check the melting curve to confirm that the correct products have been amplified. Samples with cycle threshold (Ct) value of > 35 should be considered uninfected or infected at an undetectable level.

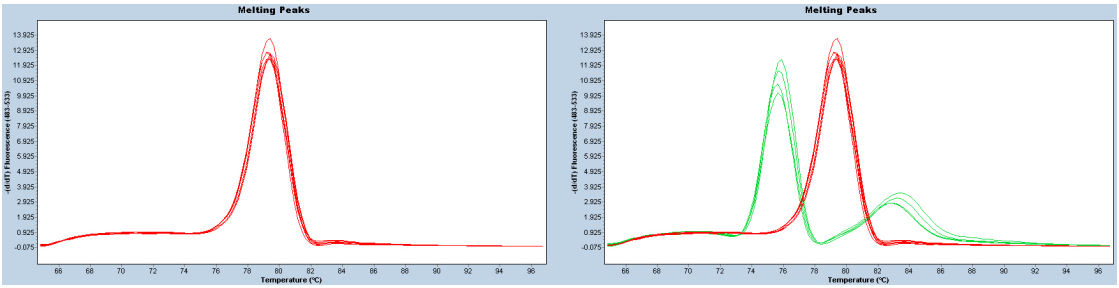


**Figure 2. Melting curves.** Melting curves should be uniform (red lines on the left panel). Two or more patterns (*e.g.*, red and green lines on the right panel) indicate that PCR products are different.

## Notes

1. For detection of any bacterial endosymbiont, it is risky to rely only on PCR and qPCR because of possible pseudo-positives (although most can be identified using melting curves) or the amplification of fragments arising due to horizontal gene transfer (HGT). It is known that many insect species harbor bacterial genome fragments of various sizes in their chromosomes (Dunning Hotopp *et al.*, 2007). Therefore, it is desirable to use multiple target genes to reduce the probability of targeting an HGT gene from the insect genome. If possible, visualize the targeted bacterium using FISH before or after examining the bacterial titers by qPCR. It is helpful to also test insects treated with antibiotics (tetracycline hydrochloride is effective for almost all bacterial species) to demonstrate whether this eliminates/decreases the endosymbiont population.

2. To standardize the bacterial titers, it is desirable to use the mass concentration of the total genomic DNA as a denominator. However, this may lead to an imprecise estimate of bacterial density because the contribution of bacterial DNA to the total genomic DNA may be significant. For example, *Wolbachia pipientis* (genome size: *ca.* 1.2 Mb) whose density is relatively low compared with other obligate endosymbionts, usually yields approximately 10-50 times the number of copies as that of the insect nuclear DNA (Goto *et al*., 2006). Because the insect genome size is generally 100-300 Mb, bacterial DNA may account for as much as 3.3%-50% of the total genomic DNA, which is too large a proportion to be ignored.
3. For standardization of bacterial titers, mitochondrial sequence copies are not recommended, because the density of mitochondria can change according to environment, host physiology, and use of antibiotics. Notably, tetracycline treatment influences mitochondrial metabolism and mtDNA density two generations after treatment in *Drosophila simulans* (Ballard and Melvin, 2007).

## Recipes

1. Reaction mix
   In each well (25-µl reaction), add the following reagents:
   Buffer 1 (2x buffer), 12.5 µl
   Buffer 2 ($H_2O$), 2.5 µl
   Primer A (10 µM), 2.5 µl
   Primer B (10 µM), 2.5 µl
   Usually, for one plate (96 wells), a premix can be prepared in a 50-ml tube by adding reagents in the following volumes:
   Buffer 1 (2x buffer), 1,250 µl
   Buffer 2 ($H_2O$), 250 µl
   Primer A (10 µM), 250 µl
   Primer B (10 µM), 250 µl

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