

Comparison of qPCR and dPCR methods for the quantification of *Wolbachia* densities and arthropod gene expression

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Introduction

A majority of arthropod species live symbiotically with maternally-transmitted bacteria that can drive evolutionary novelty and ecological diversity (1). However, little is known about the genetics that host and bacterium rely upon to resolve conflict and ensure relative stability between the two domains of life. For instance, inherited and intracellular bacteria are both mutualistic and parasitic, yet high loads of either can lead to reduced host lifespan and fitness (2–4), thereby impeding the transmission of the symbiont and its spread through host populations. Hence, control of symbiont density and transmission can be crucial in resolving conflict in nested ecological relationships.

The bacterial genus *Wolbachia* infects more animal species than any other bacterium on the planet (5). Despite its ability to hijack sexual reproduction and importance in shaping arthropod evolution and vector control strategies, little is known regarding what animal genes regulate such maternally-transmitted, reproductive symbioses and how *Nasonia* parasitoid wasps are an excellent model system for investigating host regulation of *Wolbachia* densities. Two species, *N. vitripennis* and *N. giraulti*, recently shared a common ancestor that diverged ~1 MYA (6). Since that divergence, each species has acquired different *Wolbachia* strains from both the A and B *Wolbachia* phylogenetic supergroups through independent horizontal transfer events (7, 8).

The wVitA *Wolbachia* strain is restricted to the reproductive tissues of *N. vitripennis* wasps and exists at a low density. Intriguingly, upon transfer to the closely related species and naïve host, *N. giraulti*, wVitA increases its densities 100-fold and tissue tropism to all somatic tissues. Importantly, the two species are interfertile, indicating recent evolution of this major phenotypic difference between the species. Recently, a novel, taxon-restricted, unannotated gene was identified in *N. vitripennis* that functions to regulate the densities at which *Wolbachia* are transmitted to the next generation (9). This novel gene, named *Wolbachia* density suppressor (Wds), now provides a unique insight into how animals may evolve mechanisms to manage inherited bacterial infections. Methods using qPCR for assessing gene expression and bacterial counts in *Nasonia* parasitoid wasps have been reported. However, qPCR has limitations including requiring reference materials and the use of dsDNA dye rather than specific fluorescent-probes, which can reduce assay specificity through off-target amplification and limit the ability to multiplex. The goal of this work was to compare performance of quantitative PCR (qPCR) and digital PCR (dPCR) in the quantification of gene expression and *Wolbachia* abundances in *Nasonia* parasitoid wasps.

Materials and Methods

Nasonia parasitoid wasps

Experiments were performed with *Nasonia parasitoid* wasps including *N. vitripennis* strain 12.1(A) and *N. giraulti* strain IntG 12.1(A). *N. vitripennis* 12.1(A) is singly infected with *Wolbachia* bacterial endosymbiont strain wVitA and was derived from the double-infected *N. vitripennis* R511 (wVitA and wVitB) after a prolonged period of diapause (10). *N. giraulti* strain IntG 12.1(A) was generated by backcrossing *N. vitripennis* 12.1(A) females to uninfected *N. giraulti* RV2x(u) males for nine generations (4), producing hybrids with a *N. giraulti* genome and an *N. vitripennis* cytoplasm harboring *Wolbachia* wVitA (now referred to as IntG 12.1). All *Nasonia* were reared at 25°C in constant light on *Sarcophaga bullata* fly hosts reared in house on bovine liver from Walnut Hills Farm (Tennessee, USA).

Experiment 1. RNAi knock-down of *Wolbachia* density suppressor (Wds) gene expression in *N. vitripennis*.

To assess the effectiveness of RNAi to knock-down gene expression, dsRNA was generated and RNAi was performed as described in Funkhouser-Jones & van Opstal (9) targeting the newly identified *Wolbachia* density suppressor gene (Wds) gene that suppresses *Wolbachia* wVitA titers in wasp ovaries. For a negative control injection, dsRNA was generated with *E. coli* targeting the *malE* gene gifted by the Tate lab at Vanderbilt University. *N. vitripennis* female wasps were used for injections. dsRNA was used at a final concentration of 750 ng/μl, and 23 nl was injected into the ventral abdomen of virgin, female *Nasonia* at the red-eyed yellow pupal stage. Once the pupae developed to fully black pupae about three days later, n=10 individual black pupae were collected and stored at -80°C till RNA extraction. RNA was extracted from each sample using Trizol reagent (Invitrogen) with the Direct-zol RNA miniprep kit (Zymo Research) and then treated with the DNA-free DNA removal kit (Ambion) for

45 minutes at 37°C. After ensuring with PCR that all DNA had been removed, RNA was converted to cDNA using the SuperScript VILO cDNA Synthesis kit (Invitrogen).

For RT-qPCR, cDNA was diluted 1:5 in nuclease free water before reactions were setup as described in Table 1 and measured on a CFX96 Real-Time system (BioRad). All reactions were performed in technical duplicates. Two separate qPCR analyses were performed for each sample, one targeting Wds and second targeting the *Nasonia* 60S ribosomal protein L32 (also known as RP49). Wds gene expression was normalized to the *Nasonia* RP49 gene expression in the RNAi knock-down experiment for *N. vitripennis* 12.1 (A *Wolbachia*), and expression values calculated using the $\Delta\Delta C_t$ method of relative quantification with *malE* normalized to 1.0.

of diluted saliva was pipetted onto a glass slide then sampled by swabbing with a clean swab pre-wet with 10 μl of nuclease-free water. Four replicate swabs were made for each dilution; two were randomly assigned to be processed by the EZ2 Connect Fx and two to be processed by the Maxwell FSC.

Table 1. qPCR assay configuration

Component	Volume per 25 μl reaction	Final concentration
Taq Universal SYBR Green SuperMix	12.5 μl	1X
Forward Primer (5 μM)	1 μl	500 nM
Reverse Primer (5 μM)	1 μl	500 nM
Sample	2 μl	-
Nuclease-free water	8.5 μl	-

For digital PCR using the QIAcuity ONE (QIAGEN), 1 μl of cDNA was diluted 1:100 before reactions were setup as described in Table 2. Oligos used to generate the two 10x Primer-Probe solutions are listed in Table 3. All probes in this study were from Integrated DNA Technologies (IDT) and contained a 5' fluorophore and a 3' Eclipse quencher.

Table 2. dPCR assay configuration

Component	Volume per 12 μ l reaction	Final concentration
QIAcuity Probe PCR Kit (4x)	3 μ l	1X
10x Primer-Probe Mix 1 – Wds	1.2 μ l	Fwd Primer = 1.6 μ M Rev Primer = 1.6 μ M Probe = 0.4 μ M
10x Primer-Probe Mix 2 – <i>Nasonia</i>	1.2 μ l	Fwd Primer = 1.6 μ M Reverse Primer = 1.6 μ M Probe = 0.8 μ M
Sample	1 μ l	–
Nuclease-free water	5.6 μ l	–

Table 3. Oligos used in this study

Target	Component	Sequence	Experiment Used
Wds	Forward Primer	5'- ACCTACTGCTGACATCGTTCC-3'	qPCR and dPCR
	Reverse Primer	5'- AGCCCGTCTCTTGTTCACG-3'	qPCR and dPCR
	Probe (yellow)	/5HEX/CG CAT CCG ACA AGT GTG /3MGB-NFQ/	dPCR only
<i>Nasonia</i> – RP49	Forward Primer	5'- CAAGCGTAACTGGAGGAAGC-3'	qPCR and dPCR
	Reverse Primer	5'- CTGCTAACTCCATGGGCAAT-3'	qPCR and dPCR
	Probe (green)	/56-FAM/TT GGA AGT TCT GAT GAT GCA G/3MGB-NFQ/	dPCR only

Samples were loaded into an 8.5K 24-well QIAcuity Nanoplate (QIAGEN) which was then sealed and loaded onto a QIAcuity One, 5plex digital PCR instrument (Cat.No. 911022). The QIAcuity dPCR workflow consists of three steps as follows: a priming/partitioning of the reactions step, PCR cycling step, and imaging step, which are performed automatically by the instrument. PCR amplification was performed directly in the nanoplate by the QIAcuity One instrument and consisted of a heat activation step at 95°C for 2 minutes followed by 40 cycles of denaturation step at 95°C for 5 seconds and a combined annealing and extension step at 55°C for 30 seconds. The qPCR cycling conditions were as follows: 95°C for 3 minutes step followed by 40 cycles of 95°C for 15 seconds and 59°C for 1 minute.

The nanoplate was imaged at default imaging settings consisting of a 500 ms exposure and gain 6 for both the green and yellow detection channels. The dPCR analysis was performed using the QIAcuity Software Suite. A reference dye is included in the master mix, allowing the software to determine which partitions are valid and

analyzable. A volume precision factor (VPF) was applied to the quantitation data to account for variations in partition size for different nanoplate batches.

Resulting Wds expression counts (copies/ μ l) were divided by *Nasonia* counts (RP49), and relative gene expression was normalized to Wds expression in *male* injected females.

Experiment 2. Phenotyping the variation in *Wolbachia* densities in *N. vitripennis* and *N. giraulti* containing the same *Wolbachia* infection originating from *N. vitripennis* (wVitA).

To assess the effect of a maternal genotype on offspring *Wolbachia* densities, *N. vitripennis* 12.1 (A) and *N. giraulti* IntG12.1A were hosted with 3 females to 1 male for three days and allowed to lay eggs. After 10-12 days, n=10 pools of 5 red-eyed yellow female pupae per independent mating setup were collected on the same day into sterile eppendorf tubes. Samples were stored at –20°C until DNA extraction. DNA was extracted ►

using a modified Gentra Puregene Tissue Kit (QIAGEN) in which 0.5 µl of Proteinase K (QIAGEN) was added to sample in the cell lysis buffer and incubated at 55°C for 3 hours in a shaking water bath. DNA was eluted in 100 µl of DNA hydration buffer. Quantitative PCR (qPCR) was performed as previously described (9) on the CFX96 Real-Time system (Bio-Rad) machine (annealing temp: 59°C). *Wolbachia* DNA copy number and density in two *Nasonia* species were determined using *Wolbachia groEL* and *Nasonia NvS6K* primers and calculated based on the following standard curve equations: *groEL*: $y = 3.367x + 35.803$ and *NvS6K*: $y = 3.455x + 35.908$, where y = averaged Ct value between technical duplicates and x = log starting quantity of template DNA. *Wolbachia* density was calculated by dividing *groEL* copy number by *NvS6K* copy number for each sample.

For dPCR using the QIAcuity ONE (QIAGEN), 1 µl of each sample was used to setup dPCR as demonstrated in Table 4 before being loaded into an 8.5K 24-well QIAcuity Nanoplate (QIAGEN) which was then sealed and loaded onto a QIAcuity One (Cat.No. 911022). Oligos used to generate the two 10x Primer-Probe solutions are listed in Table 5.

Digital PCR setup was performed as described in Experiment 1. *Wolbachia (groEL)* densities were measured as copies/µl after the run and densities were calculated by dividing *Wolbachia* counts by *Nasonia* counts (*NvS6K*).

Table 4. dPCR assay configuration

Component	Volume per 12 µl reaction	Final concentration
QIAcuity Probe PCR Kit (4x)	3 µl	1X
10x Primer-Probe Mix 1 – <i>Wolbachia groEL</i>	1.2 µl	Fwd Primer = 1.6 µM Rev Primer = 1.6 µM Probe = 0.4 µM
10x Primer-Probe Mix 2 – <i>Nasonia NvS6K</i>	1.2 µl	Fwd Primer = 1.6 µM Reverse Primer = 1.6 µM Probe = 0.4 µM
Sample	1 µl	–
Nuclease-free water	5.6 µl	–

Table 5. Oligos used in this study

Target	Component	Sequence	Experiment Used
<i>Wolbachia groEL</i>	Forward Primer (QTF1)	5'-CAACCTTACTTCCTATTCTTG-3'	qPCR and dPCR
	Reverse Primer (QTR1)	5'-CTAAAGTGCTTAATGCTTCACCTTC-3'	qPCR and dPCR
	Probe (yellow)	/5HEX/TG GTA AAC CTT TGG TTA TTA TTG C/3MGB-NFQ/	dPCR only
<i>Nasonia – NvS6K</i>	Forward Primer (QTF4)	5'-GGCATTATCTACAGAGATTTGAAACCAG-3'	qPCR and dPCR
	Reverse Primer (QTR4)	5'-CAAAGCTATATGACCTTCTGTATCAAG-5'	qPCR and dPCR
	Probe (green)	/56-FAM/GT TAC TGC TAA TTA TCT TCT TTT TTC ATT A/3MGB	dPCR only

Results and Discussion

Digital PCR (dPCR) offers a simple and accurate method for quantifying absolute template counts with more sensitivity compared to standard quantitative PCR (qPCR) methods. dPCR also permits the measurement of the absolute number of molecules generated at the endpoint of PCR for sample target quantification without the need for a standard curve calculated from cycle threshold (Ct) values and therefore improves the accuracy and confidence of results. Furthermore, the use of specific fluorescent probes for each sample target increases the specificity of target measurements with no off-target binding effects as one might see using non-specific DNA binding dyes. As a result, multiple samples can also be multiplexed using different fluorescently labeled probes and quantified in a single reaction without the need to perform independent measurements of each target as is required by qPCR.

The goal of this work was to determine the useability of qPCR versus dPCR for quantification of gene expression and bacterial counts in *Nasonia parasitoid* wasps, an important animal model for studying the evolutionary genetics of host-microbe interactions. To accomplish this, we compared measurements obtained from qPCR and dPCR of wasp gene expression after RNA interference (RNAi) against the recently discovered *Wolbachia* density suppressor gene (Wds) in *N. vitripennis* as described previously (9) and to compare *Wolbachia* counts and densities in two independent wasp species, *N. vitripennis* and *N. giraulti*, harboring the same *Wolbachia* infection (wVitA) (4).

Previously, RNAi has successfully been implemented in *Nasonia* to investigate the role of specific genes on *Wolbachia* density (9). We repeated RNAi by injecting dsRNA targeted at the *Wolbachia* density suppressor (Wds) gene and a control gene, *malE*, originating from *E. coli* that should have no effect on Wds expression. We measured gene expression first by qPCR and noted a significant 40% knockdown of Wds gene expression compared to the *malE* injection control (Figure 1).

This result is consistent with previous findings (9). We then quantified the same samples via dPCR and measured a significant knockdown effect resulting in a 27% reduction in gene expression of Wds compared to the *malE* injection control.

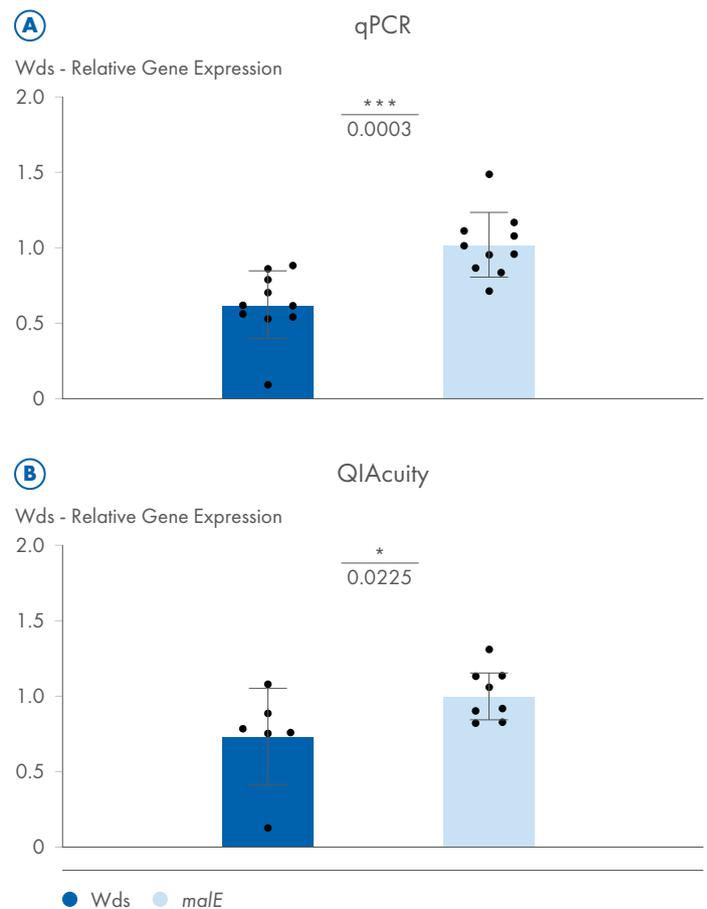


Figure 1. dPCR and qPCR quantify reduced levels of Wds in RNAi knockdown.

Comparison of qPCR data using a CFX96 Real-Time (Bio-Rad) system to dPCR using the QIAcuity ONE (QIAGEN) system of RNAi knockdown efficiency of the x density suppressor gene (Wds). **A** qPCR data of the relative gene expression of Wds in black pupae of *N. vitripennis* injected with either dsRNA targeting the Wds gene or dsRNA targeting a foreign *E. coli* gene, normalized to Wds expression in *malE* injected females. *** $p=0.0003$, Mann-Whitney U test.

B dPCR data of the relative gene expression of the same samples with expression normalized to Wds expression in *malE* injected females. * $p=0.0225$, Mann-Whitney U test.

Next, we quantified the counts and densities of *Wolbachia* bacteria in the two divergent wasp species *N. vitripennis* and *N. giraulti*, that markedly vary in *Wolbachia* abundance. It has been reported previously

that introgression of wVitA into the new *N. giraulti* (IntG) genomic background results in a two-order-of-magnitude increase in the number of *Wolbachia* present and spread of the infection outside of the reproductive organs to other somatic tissues (4). To quantify this, we extracted DNA from pupal offspring of each wasp species and first used qPCR to calculate *Wolbachia* copy number and density. We noted a significant increase in *Wolbachia* copy

number and density in *N. giraulti* (IntG) harboring wVitA compared to *N. vitripennis* 12.1(A) (Figure 2A). Average *Wolbachia* densities increased 94% from 0.065 in *N. vitripennis* to 6.150 in *N. giraulti*. We then quantified the same samples via dPCR and measured the same significant trend where *Wolbachia* densities significantly increased 62% from 0.044 to 2.753.

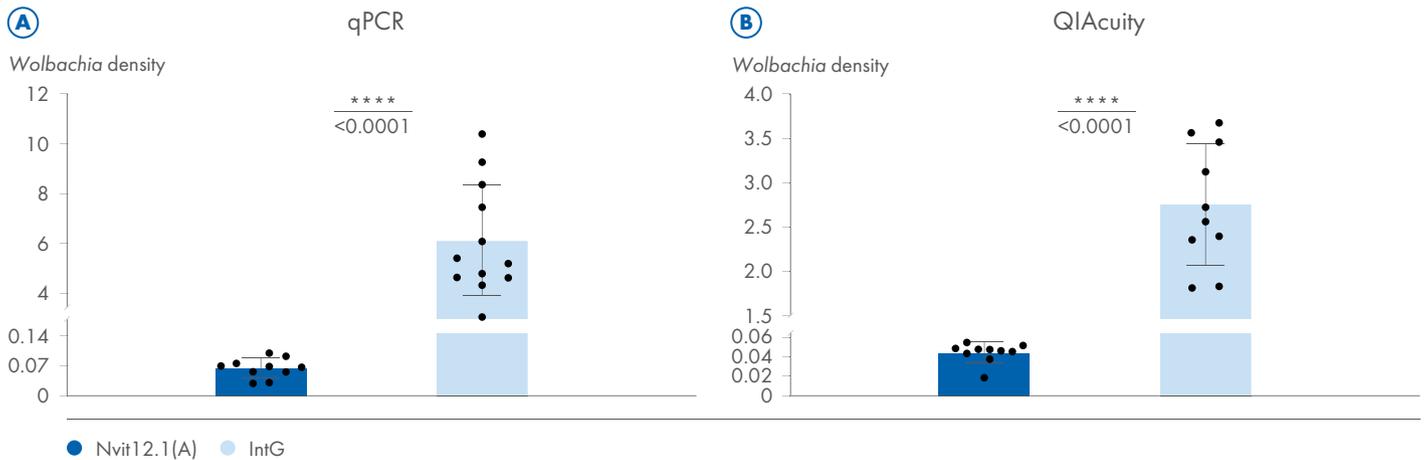


Figure 2. dPCR and qPCR measured increased *Wolbachia* densities compared to *Nasonia*.

Comparison of qPCR data using a CFX96 Real-Time (Bio-Rad) system to dPCR using the QIAcuity ONE (QIAGEN) system to calculate the phenotypic variation of *Wolbachia* densities in two *Nasonia* species, *N. vitripennis* and *N. giraulti*, harboring the same *Wolbachia* infection wVitA. **A** qPCR data of *Wolbachia* density relative to *Nasonia* copy number and **B** dPCR data of *Wolbachia* density relative to *Nasonia* copy number in the two wasp lines. *** $p < 0.0001$, Mann-Whitney U Test.

Conclusion

We sought to perform a comprehensive study, comparing dPCR and qPCR, that looked at both gene expression from different host genetic backgrounds (RNA as starting material) and bacterial densities residing in these host systems (DNA as starting material). The data presented herein demonstrates that the QIAcuity digital PCR platform is capable of confirming significant trends identified in

RNAi gene expression analyses and *Wolbachia* abundance analysis in *Nasonia* parasitoid wasps by qPCR while providing several technical benefits. Lower trends in data values may represent increased specificity of target sample quantification that is diminished in qPCR data that relies on non-specific DNA binding dyes.

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Product	Contents	Cat. no.
QIAcuity Probe PCR Kit	1 ml 4x concentrated QIAcuity Probe Mastermix, 2x 1.9 ml Water	9003220
QIAcuity Nanoplate 8.5k 24-well (10)	10 QIAcuity Nanoplate 8.5k 24-well, 11 Nanoplate Seals	19598
QIAcuity Four Platform System	Four-plate digital PCR instrument for detecting up to 5 fluorescent dyes, notebook computer, barcode scanner, roller, USB flash memory and QIAcuity Software Suite: includes installation, training, and 1 preventive maintenance visit, 1 year warranty on labor, travel, and parts	952034

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