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Title:

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Date:

2021-09-01

Citation:

Lau, M. J., Hoffmann, A. A. & Endersby-Harshman, N. M. (2021). A diagnostic primer pair to distinguish between wMel and wAlbB Wolbachia infections. Plos One, 16 (9 September), <https://doi.org/10.1371/journal.pone.0257781>.

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RESEARCH ARTICLE

A diagnostic primer pair to distinguish between *wMel* and *wAlbB* *Wolbachia* infections

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OPEN ACCESS

Citation: Lau M-J, Hoffmann AA, Endersby-Harshman NM (2021) A diagnostic primer pair to distinguish between *wMel* and *wAlbB* *Wolbachia* infections. PLoS ONE 16(9): e0257781. <https://doi.org/10.1371/journal.pone.0257781>

Editor: Luciano Andrade Moreira, Fundacao Oswaldo Cruz Instituto Rene Rachou, BRAZIL

Received: June 21, 2021

Accepted: September 9, 2021

Published: September 23, 2021

Peer Review History: PLOS recognizes the benefits of transparency in the peer review process; therefore, we enable the publication of all of the content of peer review and author responses alongside final, published articles. The editorial history of this article is available here: <https://doi.org/10.1371/journal.pone.0257781>

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Data Availability Statement: All relevant data are within the manuscript and its [Supporting Information](#) files.

Funding: Ary A. Hoffmann is funded by the National Health and Medical Research Council

Abstract

Detection of the *Wolbachia* endosymbiont in *Aedes aegypti* mosquitoes through real-time polymerase chain reaction assays is widely used during and after *Wolbachia* releases in dengue reduction trials involving the *wMel* and *wAlbB* strains. Although several different primer pairs have been applied in current successful *Wolbachia* releases, they cannot be used in a single assay to distinguish between these strains. Here, we developed a new diagnostic primer pair, *wMwA*, which can detect the *wMel* or *wAlbB* infection in the same assay. We also tested current *Wolbachia* primers and show that there is variation in their performance when they are used to assess the relative density of *Wolbachia*. The new *wMwA* primers provide an accurate and efficient estimate of the presence and density of both *Wolbachia* infections, with practical implications for *Wolbachia* estimates in field collected *Ae. aegypti* where *Wolbachia* releases have taken place.

Introduction

The bacterium, *Wolbachia*, is providing an increasingly popular method to inhibit dengue virus transmission in the mosquito, *Aedes aegypti*. *Wolbachia*-infected populations involving the *wMel* strain have now been successfully established in *Ae. aegypti* in regions including northern Australia, Brazil and Indonesia [1–3], while *wAlbB*-infected *Ae. aegypti* have been established in Malaysia [4]. Detection of the *Wolbachia* endosymbiont in *Ae. aegypti* mosquitoes is a standard requirement for good laboratory practice during *Wolbachia* mosquito releases in dengue reduction programs and for tracking *Wolbachia* invasions in the field [4, 5]. Real-time polymerase chain reaction (real-time PCR) and High Resolution Melt (HRM) assays (SYBR® equivalent/non-probe) have been developed that enable detection and *Wolbachia* density estimation for the strain of interest [6–8]. However, difficulties can arise in using these assays when there is a need to detect *Wolbachia* and distinguish between multiple *Wolbachia* strains. In experiments where superinfected lines are used [9], or where mosquitoes carrying different single infections need to be distinguished for experiments or in field collected samples [10], several real-time PCR assays using different primer pairs are currently required. Given that both *wMel* and

(1132412, 1118640, www.nhmrc.gov.au). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

wAlbB strains are now actively being used in field releases and that each strain may have advantages in particular situations, the requirement for multiple strain identification is likely to increase in the foreseeable future.

In previous work, we have used a *Wolbachia*-specific primer pair, *wI* [7], which targets a conserved locus VNTR-141 containing tandem repeats [11]. This pair of primers works efficiently in amplifying *wMel* and *wMelPop* infections in a real-time PCR and HRM assay, but achieves poor amplification of *wAlbB* [10]. As well as being used for *Wolbachia* detection, primers are needed for quantification of *Wolbachia* density in mosquitoes. There are various *Wolbachia* specific primers for *wMel*, *wAlbB* or *wMelPop* [9, 12–14], but currently there is no standardized assay for *Wolbachia* screening that is comparable between strains and that can be used to compare results between laboratories. Although cross-laboratory comparability may not be a realistic aim when using a SYBR® equivalent/non-probe-based assay, the use of extra internal controls can make these assays robust for relative density estimates, improving consistency within laboratory experiments [7, 10].

In this study, we developed a diagnostic primer pair that can detect and distinguish between the *wMel* and *wAlbB* infections and also provides an estimate of *Wolbachia* density. In addition, we assessed primer efficiency of some other published primers for *Wolbachia* in mosquitoes. We also tested quantification cycle (C_q) [15] value differences between primers for different *Wolbachia* strains to assess primer suitability for relative *Wolbachia* density estimation.

Materials and methods

Diagnostic primer design

To develop the new primers, we screened for sequence differences between the *wMel* and *wAlbB* strains and then focused on the sequences of a DNA-directed RNA polymerase subunit beta/betagen with locus tag WD_RS06155 in *wMel* and its analogue in *wAlbB*. We then developed a new pair of primers designated *wMwA* (Table 1) to distinguish *Wolbachia wMel* and *wAlbB* in a single run of a real-time PCR assay, based on two base-pair mismatches at the 3'-end of each primer, which resulted in the T_m peak for *wAlbB* being separated from that of *wMel*. We checked the specificity of this primer pair by an initial test of six males and six females for each strain with different *Wolbachia* infection type (*wMel*- or *wAlbB*-infected or uninfected). Subsequent testing was done with female mosquitoes only.

Sample preparation

The *wMel* and *wAlbB*-infected *Ae. aegypti* were tested for strains transinfected previously [16, 17]. The *wMel* strain was collected from Cairns, Australia in 2019 from regions that had been invaded several years earlier [2, 12], while the *wAlbB* strain was derived from a *wAlbB* infected strain crossed to an Australian background and maintained in the laboratory [13]. An uninfected strain was developed from *Ae. aegypti* eggs collected in Cairns, Queensland, Australia prior to *Wolbachia* releases [10, 18].

Female mosquitoes of *wMel*-infected [17], *wAlbB*-infected [16] and uninfected were reared with TetraMin® fish food tablets in reverse osmosis (RO) water until the adult stage [19], and then were killed in absolute ethanol before Chelex® DNA extraction. In the standard procedure, DNA of an individual female was extracted in 250 μ L 5% Chelex® 100 Resin (Bio-Rad Laboratories, Hercules, CA) and 3 μ L of Proteinase K (20 mg/ mL, Bioline Australia Pty Ltd, Alexandria NSW, Australia). The Chelex® 100 Resin solution containing DNA was centrifuged at 12500 rpm for 5 min and DNA solution was pipetted from the supernatant.

Table 1. Primers for detection of *Wolbachia* strains and estimation of density.

Primer name	<i>Wolbachia</i> status target	Targeted locus	Forward	Reverse	Amplicon size (bp)	Source
<i>mos</i>	uninfected	AF154067	AGTTGAACGTATCGTTTCCCGCTAC	GAAGTGACGCAGCTTGTGGTCGTCC	77	[7]
<i>aeg</i>	uninfected	AF154067	ATCAAGAAGCGCCGTGTCCG	CAGGTGCAGGATCTTCATGTATTCCG	66	[7]
<i>w1</i>	<i>wMel</i>	VNTR 141 region	AAAATCTTTGTGAAGAGGTGATCTGC	GCACTGGGATGACAGGAAAAGG	16	[7]
<i>wsp</i>	<i>wMel</i> , <i>wAlbB</i> -infected	WD_RS04815	GCATTTGGTTAYAAAATGGACGA	GGAGTGATAGGCATATCTTCAAT	139 (<i>wMel</i>), 136 (<i>wAlbB</i>)	[9]
<i>wMel</i>	<i>wMel</i> -infected	WD_RS02275	CAAATTGCTCTTGTCTGTGG	GGGTGTTAAGCAGAGTTACGG	68	[9]
<i>wAlbB</i>	<i>wAlbB</i> -infected	DEJ70_RS01110	CCTTACCTCCTGCACAACAA	GGATTGTCCAGTGGCCTTA	109	[9]
<i>wMwA</i>	<i>wMel</i> , <i>wAlbB</i> -infected	WD_RS06155	GAAGTTGAAGCACAGTGTACCTT	GCTTGATATTCCTGTAGATTTCATC	155 (both)	Newly designed

<https://doi.org/10.1371/journal.pone.0257781.t001>

LightCycler® efficiency test

After extraction, DNA concentration was measured using a Qubit™ 1X dsDNA HS Assay Kit and Qubit™ 2.0 fluorometer (ThermoFisher Scientific, Waltham, MA USA), and then diluted ten times before making a three-fold dilution series to test the efficiency of currently-used *Wolbachia* primers in a real-time PCR assay (Table 1). We also diluted the solution six times before making a three-fold dilution series to investigate the influence of Chelex®-extracted DNA concentration.

For the real-time PCR and HRM, we used a LightCycler® 480 High Resolution Melting Master (HRMM) kit (Roche; Cat. No. 04909631001, Roche Diagnostics Australia Pty. Ltd., Castle Hill New South Wales, Australia) and IMMOLASE™ DNA polymerase (5 U/μl) (Bio-line; Cat. No. BIO-21047) as described by Lee et al. (2012) (S1 Table). We used 384-well plates with white wells (SSI Bio, Lodi CA USA, Cat. No. 3430–40), and the PCR conditions for DNA amplification beginning with a 10-minute pre-incubation at 95°C (Ramp Rate = 4.8°C/s), followed by 40 cycles of 95°C for 5 seconds (Ramp Rate = 4.8°C/s), 53°C for 15 seconds (Ramp Rate = 2.5°C/s), and 72°C for 30 seconds (Ramp Rate = 4.8°C/s).

Three technical replicates were run for each sample of each dilution and a graph was produced showing the log₃ [dilution factor] (x-axis) against mean C_q (y-axis) and a linear trend line (y = mx + c) was fitted. Slope (m) and R² values were recorded so that PCR amplification efficiency (E) could be evaluated with the equation:

$$E = (3^{\frac{1}{\text{slope}}} - 1) \times 100\%$$

Compare with Chelex® extraction, we also purified DNA from the above Chelex® 100 Resin solution using the PureLink™ Quick PCR purification Kit (Invitrogen Cat. No. K3100-01), in which the binding buffer B2 was used. In addition, a different DNA extraction method was used: female mosquitoes were homogenized individually in 100 μL STE buffer (10 mM Tris-HCl pH8, 100 mM NaCl, 1mM EDTA), and then incubated at 95°C for 10 minutes. After these extractions, 10 μL supernatant was pipetted into 90 μL ddH₂O and made a three-fold dilution series.

Primer quantification cycle comparison and density estimation

Following the efficiency study, we used a mixture of young (4±1days since eclosion) and old (38±1days since eclosion) female mosquitoes and tested for C_q value differences between primers for different *Wolbachia* strains to assess suitability for relative *Wolbachia* density

estimation. A total of 16 *Wolbachia*-infected mosquito samples were extracted using Chelex[®] resin and then diluted ten times before real-time PCR.

Results and discussion

Diagnostic primer design

In this study, we developed a diagnostic primer pair, *wMwA*, that can detect and distinguish between the *wMel* and *wAlbB* infections in *Aedes aegypti* (Fig 1), which is important in simplifying current approaches for *Wolbachia* identification. In the initial test for the specificity of this primer pair, all uninfected samples were negative, and all *Wolbachia*-

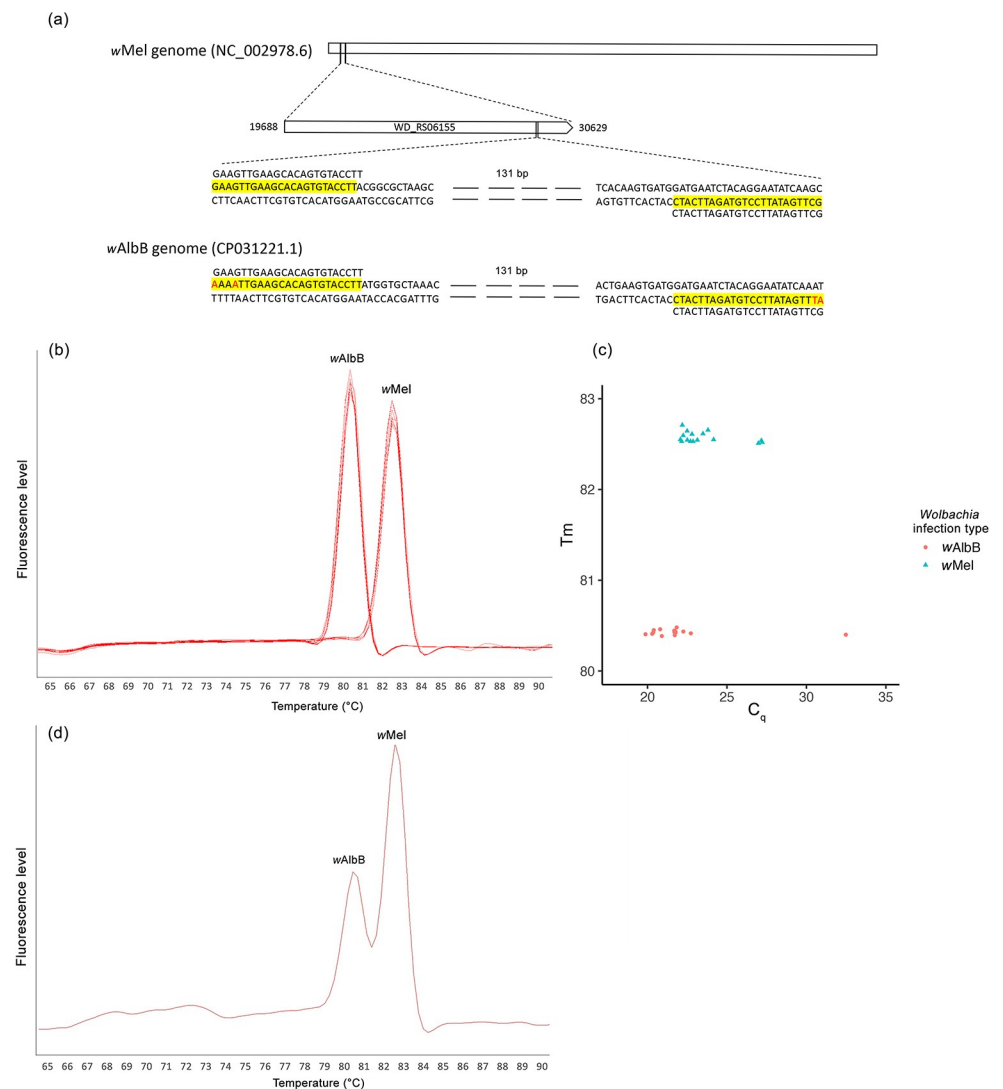


Fig 1. Development of primers to detect *Wolbachia wMel* and *wAlbB* infection in *Aedes aegypti*. (a) The new primer pair *wMwA* aligns to a region in gene *WD_RS06155* of *wMel*, and also aligns to its analogue in the *wAlbB* genome which has two base-pair mismatches at the 3'-end; (b) the *wMwA* primers showed distinct T_m peaks for *Wolbachia wMel* and *wAlbB*. (c) the *wMwA* primers showed distinct T_m values for *Wolbachia wMel* ($82.6 \pm 0.03^\circ\text{C}$) and *wAlbB* ($80.4 \pm 0.02^\circ\text{C}$), the x axis represents the quantification cycle (C_q) and the y axis represents the amplicon melting temperature; (d) the *wMwA* primers showed two T_m peaks when mixing DNA templates of *wMel* and *wAlbB*-infected *Ae. aegypti*.

<https://doi.org/10.1371/journal.pone.0257781.g001>

infected samples were positive with distinctive T_m values from *Wolbachia* *wMel* ($82.6 \pm 0.03^\circ\text{C}$) and *wAlbB* ($80.4 \pm 0.02^\circ\text{C}$) screening (Fig 1C). The high-resolution melt produces two joined peaks when the template contains both *Wolbachia* *wMel* and *wAlbB* DNA (Fig 1D).

Primer efficiency test

We tested the efficiency of each of the primers for screening *Wolbachia* in *Ae. aegypti* by using a threefold dilution series. When template DNA was extracted in Chelex[®] 100 Resin solution, the efficiencies of all primers ranged from 86.4% to 104.9%, (Table 2 and Fig 2) and the efficiency curves all showed an R^2 valued greater than 0.99.

However, we found the amplification curve increase showed inhibition at the first dilution (Fig 3) for each of the primers, particularly when DNA was first diluted six times instead of ten times, resulting in outliers (S1 and S2 Figs and S2 Table). These results highlight a potential risk of lowering the relative density estimate in *Wolbachia* screening when using a highly concentrated Chelex[®]-extracted DNA solution. We also found differences between primer efficiency when a different DNA extraction method was used, with changes ranging from -22.2% to 29% (S3 Table). Different DNA extraction methods may affect DNA yield and quality, and/or change PCR inhibitors and their effects, which can increase variation between host and parasite DNA [20–22]. It is, therefore, worth noting that new standard curves should be run when changing to a different DNA extraction method, given that the efficiency of primers can deviate substantially from recommendations (90% - 110%) [23, 24] to prevent an inaccurate estimate of relative density being made.

C_q value comparisons in Chelex[®] 100 Resin

We noticed that primers had different C_q values even when screening the same individual organism/endosymbiont (*Ae. aegypti*, *wMel* or *wAlbB*) and using the same DNA concentration, despite the efficiency of these primers all falling within 85% - 110%. We therefore tested the C_q ranges of the primers and correlated them with *wsp*. We found variation between these primers (Fig 2), which would be expected to result in differences in relative density estimates. The relationship between C_q values of different primers all fit into a linear relationship, with R^2 greater than 0.97, whereas the coefficient varies from 0.83 to 1.05 (Fig 4). For the newly-designed primer pair *wMwA*, the coefficients for *wMel* and *wAlbB* are similar (0.97 for *wMel* and 1.04 for *wAlbB*).

Table 2. Primer efficiency for primer pairs used in detection of *Wolbachia* strains and estimation of density.

Colony	Primers	Slope of graph	R^2	Efficiency	DNA concentration* (ng/ μL)	Efficiency curve
Uninfected	<i>mos</i>	-1.566	0.999	101.659%	5.12	Fig 2A
Uninfected	<i>aeg</i>	-1.531	0.999	104.946%	5.12	Fig 2B
<i>wMel</i>	<i>wI</i>	-1.644	0.999	95.109%	5.24	Fig 2C
<i>wMel</i>	<i>wM</i>	-1.677	0.998	92.559%	5.24	Fig 2D
<i>wMel</i>	<i>wsp</i>	-1.767	0.999	86.195%	5.24	Fig 2E
<i>wMel</i>	<i>wMwA</i>	-1.769	0.999	86.107%	5.24	Fig 2F
<i>wAlbB</i>	<i>wA</i>	-1.741	0.993	87.953%	6.87	Fig 2G
<i>wAlbB</i>	<i>wsp</i>	-1.755	0.999	87.032%	6.87	Fig 2H
<i>wAlbB</i>	<i>wMwA</i>	-1.730	0.997	88.732%	6.87	Fig 2I

*Template DNA was extracted in 250 μL 5% Chelex[®] 100 Resin and then diluted ten times before making a three-fold dilution series. Concentration was measured before dilution.

<https://doi.org/10.1371/journal.pone.0257781.t002>

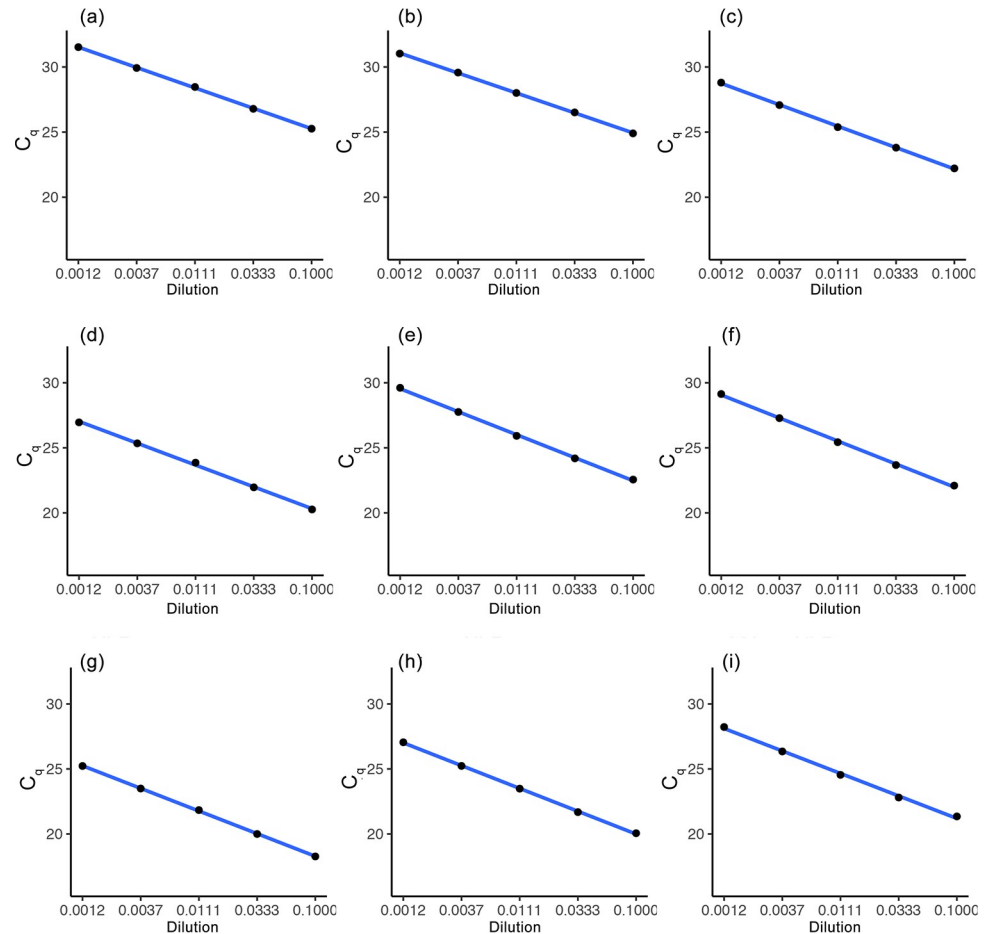


Fig 2. Primer efficiency for detection of *Wolbachia* strains and estimation of density. DNA was extracted in 250 μ L 5% Chelex[®] 100 Resin and then diluted ten times before making a three-fold dilution series. The primer names are defined in Table 2.

<https://doi.org/10.1371/journal.pone.0257781.g002>

These primer differences could not be explained fully by pipetting error and PCR inhibition [25, 26]. Inhibition effects on DNA amplification can vary when using different primers, and/or when the DNA concentration varies. Intercepts of these C_q values ranged from -1.52 to 1.37 though all primers used in this study only have one copy based on their genomic sequences. However, it is possible that there may be different copies of *Wolbachia* genes inside mosquito cells [27, 28], such as is documented for the octomom region [29, 30] which can be variable under different environmental conditions [31, 32]. As a result, care is needed when choosing primers for assessing the relative concentration of *Wolbachia*.

In our study, the *wsp* primers represent a useful pair of universal primers for amplifying the *Wolbachia* surface protein gene which has been applied as a *Wolbachia* diagnostic for decades [14]. Given potential variation between *Wolbachia* primers, comparisons with universal *Wolbachia* primers should be undertaken before using the newly-designed primers in *Wolbachia* density calculations. Our newly-designed primer pair, *wMwA*, correlated with density estimates based on *wsp*, with coefficients for both *wMel* and *wAlbB* close to 1. Thus, this new primer pair has the potential to be accurate and efficient for large-scale *Wolbachia* detection and relatively density estimate.

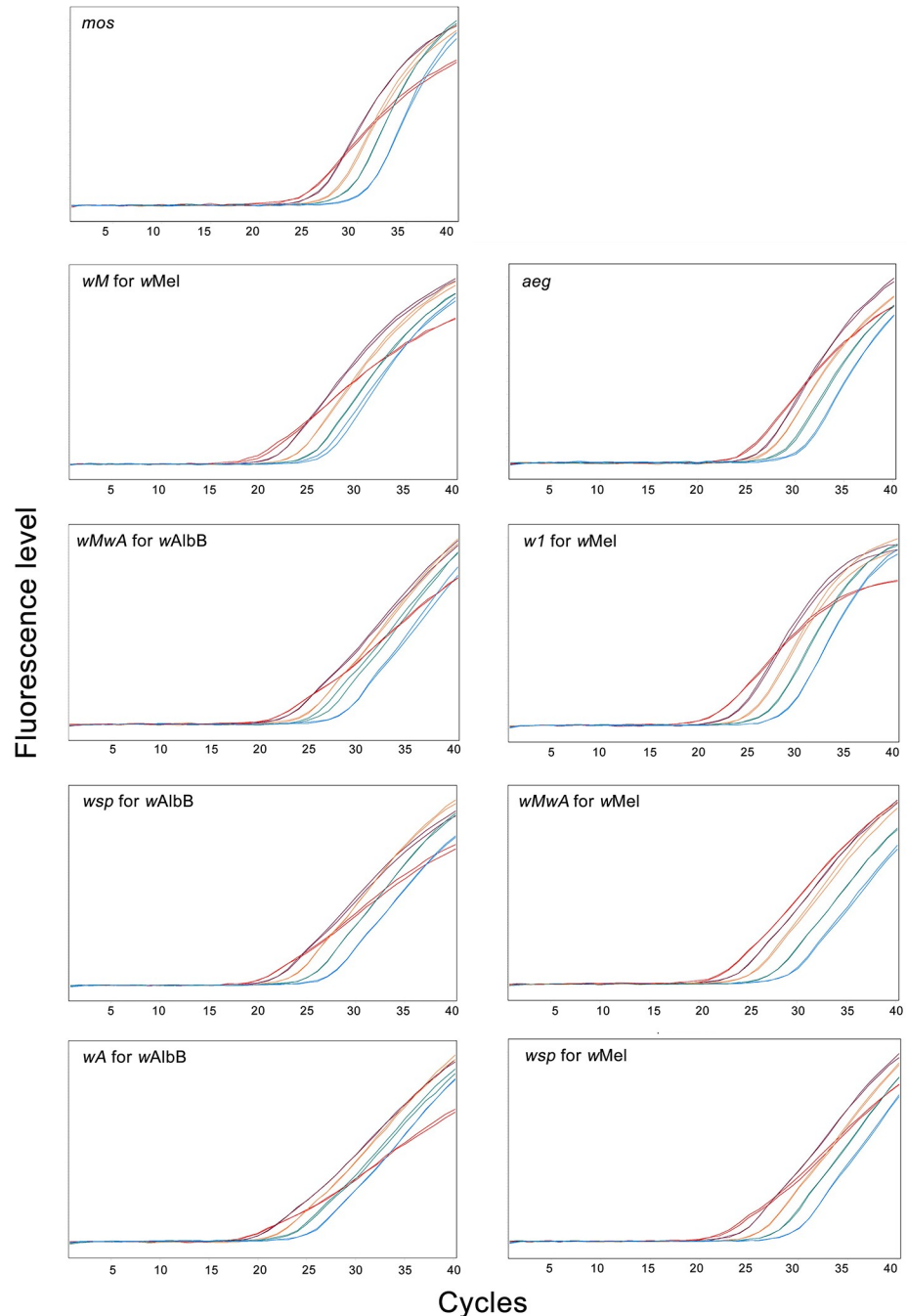


Fig 3. Variation in the shape of the PCR amplification curves. The curves from left to right represent amplification curves of 1/10, 1/30, 1/90, 1/270 and 1/810 DNA dilution from initial extraction in 250 μ L 5% Chelex[®] 100 Resin. The primers are defined in Table 2.

<https://doi.org/10.1371/journal.pone.0257781.g003>

Conclusions

Chelex[®] DNA extraction and real-time PCR provide an easy and economical approach for detecting both currently-released *Wolbachia* (*wMel* and *wAlbB*) infections in *Aedes aegypti*, while other options like multiplex probe assays and the use of DNA extraction kits are likely to cost more. Here, we designed a new primer pair, *wMwA*, which not only identifies *wMel* and

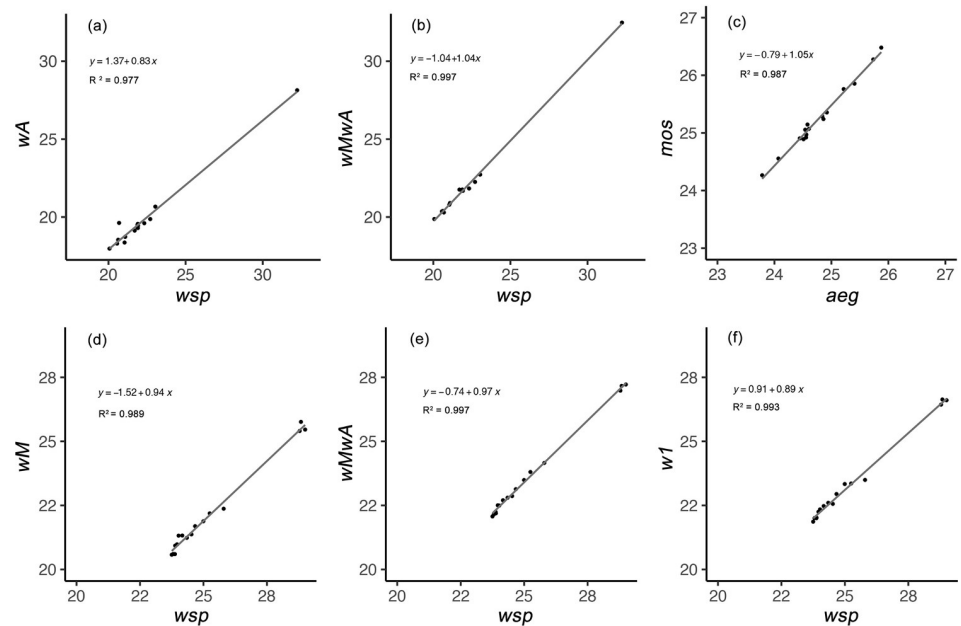


Fig 4. Variation in C_q values when using different *Wolbachia* primers for the same samples. Correlation of C_q values between (a) wA and wsp primers in *Wolbachia wAlbB* screening; (b) $wMwA$ and wsp primers in *Wolbachia wAlbB* screening; (c) mos and aeg primers in *Aedes aegypti* screening; (d) wM and wsp primers in *Wolbachia wMel* screening; (e) $wMwA$ and wsp primers in *Wolbachia wMel* screening; (f) $w1$ and wsp primers in *Wolbachia wMel* screening.

<https://doi.org/10.1371/journal.pone.0257781.g004>

$wAlbB$ at the same time, but is also correlated with density estimates based on a universal *Wolbachia* primer wsp . We demonstrated this new primer pair has the potential to be accurate and efficient for large-scale *Wolbachia* detection and relatively density estimates, especially for use in field collected *Ae. aegypti*.

Supporting information

S1 Table. Real-time PCR reagents and volume in 384-well plates with white wells.

(DOCX)

S2 Table. Primer efficiency when sample DNA was first diluted six times.

(DOCX)

S3 Table. Primer efficiency when sample DNA was extracted using different methods.

(DOCX)

S1 Fig. Primer efficiency when sample DNA was first diluted six times. DNA is extracted in 250 μ L 5% Chelex® 100 Resin and then diluted six times before making a three-fold dilution series. Outliers are marked with red colour and are excluded from the efficiency curve. The primer names are defined in S3 Table.

(PNG)

S2 Fig. Variation in the shape of the PCR amplification curves when sample DNA was first diluted six times. The curves from left to right represent amplification curves of 1/6, 1/18, 1/54, 1/162 and 1/486 DNA dilution from 250 μ L 5% Chelex® 100 Resin. The primers are defined in S2 Table.

(PNG)

Acknowledgments

We thank Perran A. Ross and Jason Axford for providing the mosquito samples. We also thank the support of the Jasper Loftus-Hills award, offered by the Faculty of Science, the University of Melbourne.

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Investigation: Meng-Jia Lau.

Methodology: Meng-Jia Lau, Nancy M. Endersby-Harshman.

Supervision: Ary A. Hoffmann, Nancy M. Endersby-Harshman.

Visualization: Meng-Jia Lau.

Writing – original draft: Meng-Jia Lau.

Writing – review & editing: Ary A. Hoffmann, Nancy M. Endersby-Harshman.

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