

High-Throughput PCR Assays To Monitor *Wolbachia* Infection in the Dengue Mosquito (*Aedes aegypti*) and *Drosophila simulans*

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We have developed and validated two new fluorescence-based PCR assays to detect the *Wolbachia* wMel strain in *Aedes aegypti* and the wRi and wAu strains in *Drosophila simulans*. The new assays are accurate, informative, and cost-efficient for large-scale *Wolbachia* screening.

The intracellular bacterium *Wolbachia pipientis* parasitizes and spreads in many arthropod hosts (13, 14). One classic example is the rapid sweep of the *Wolbachia* Riverside strain (wRi) across populations of *Drosophila simulans* in California (11). This evolutionarily optimized mechanism has inspired the use of *Wolbachia* as a driver to alter insect population structure (10). The recent establishment of wMel-infected, dengue virus-suppressing *Aedes aegypti* populations in Australia paves the way for similar programs in other countries (5, 12). In future release operations, rapid monitoring of wMel in *Ae. aegypti* will remain an ongoing requirement.

Current molecular methods to detect *Wolbachia* in *Ae. aegypti* are based on PCR followed by electrophoresis (2, 3, 5, 7–9, 15). These assays might be adequate for routine applications, but they are not ideal for large-scale field experiments. Under field conditions, mosquito specimens often comprise a mixture of *Wolbachia*-infected and uninfected individuals. One potential problem is the amplification of trace amounts of exogenous *Wolbachia* DNA from *Wolbachia*-negative samples. It is therefore desirable to develop a robust screening assay that can simultaneously detect wMel infection and quantify wMel density.

Three sets of primers were developed for the *Ae. aegypti* assay: (i) *Aedes* universal primer pair *mRpS6_F* (5'-AGTTGAACGTATCGTTTCCCGCTAC) and *mRpS6_R* (5'-GAAGTGACGCAGCTGTGGTTCGTC), which target the conserved region of the *RpS6* gene, to detect the presence of *Aedes* DNA (Fig. 1a); (ii) *Ae. aegypti* primers *aRpS6_F* (5'-ATCAAGAAGCGCCGTGTCG) and *aRpS6_R* (5'-CAGGTGCAGGATCTTCATGTATTCG), which target the *Ae. aegypti*-specific polymorphisms within the variable region of *RpS6*, to distinguish *Ae. aegypti* from non-*Ae. aegypti* specimens (Fig. 1a); (iii) *Wolbachia*-specific primers *w1_F*, (5'-AAATCTTTGTGAAGAGGTGATCTGC) and *w1_R* (5'-GCACTGGGATGACAGGAAAAGG), to detect the presence of *Wolbachia* DNA (Fig. 1b).

PCR was carried out using the Roche LightCycler 480 system in a 384-well format (see the supplemental material). *Aedes aegypti* mosquitoes infected with *Wolbachia* (wMel) produced robust amplification for all three markers (Fig. 2a). *Ae. aegypti* mosquitoes that were *Wolbachia* negative supported amplification of *mRpS6* and *aRpS6*, but not *w1* (Fig. 2b). *Aedes notoscriptus* mosquitoes showed strong amplification for the generic mosquito marker (*mRpS6*) but failed to support amplification of the *Ae. aegypti*-specific marker (*aRpS6*) and the *Wolbachia* marker (*w1*) (Fig. 2c). These results indicate that the assay is able to simultaneously distinguish (i) between *Ae. aegypti* and *Ae. notoscriptus* and (ii) between *Wolbachia*-infected and uninfected

Ae. aegypti mosquitoes. We have termed this new genotyping method the RT/HRM (real-time PCR/high-resolution melt) assay. The RT/HRM assay results were consistent with the two traditional PCR/electrophoresis-based assays, namely, the Braig assay (2) and the Caragata assay (3).

The ability to quantify wMel in *Ae. aegypti* is important for preventing detection of false positives in field samples. Primer efficiencies were not significantly different from 100% based on standard curve analysis of four wMel⁺ genomic DNA dilutions (0.1×, 0.05×, 0.025×, and 0.0125×). We used the crossing point (Cp) difference between the *aRpS6* and *w1* markers to estimate *Wolbachia* load. The average density, estimated as $2^{[(Cp\ of\ aRpS6) - (Cp\ of\ w1)]}$, was ~6 copies of wMel per copy of *RpS6* of the host genome. We also subjected the same DNA dilutions to the traditional electrophoresis-based PCR method. All dilutions produced a single expected PCR product of similar intensity (see Fig. S3 in the supplemental material). This indicates that the RT/HRM method is able to detect and quantify wMel at low concentrations, whereas the traditional assay is less quantitative.

Unlike in *Ae. aegypti*, in which *Wolbachia* has been artificially introduced, some populations of *D. simulans* in Australia are naturally infected with *Wolbachia*. One such strain is wAu, which does not induce host cytoplasmic incompatibility (4). The distribution of wAu in Australia has been documented, and the infection is generally found at low frequencies in populations of *D. simulans* on the east coast of Australia (4). Recently, sequencing of the *Wolbachia* *wsp* gene from *D. simulans* isofemale lines collected at Coffs Harbour in 2008 suggested that the wRi strain (11) might be present in this population (A. R. Weeks, unpublished data).

To confirm the presence of *Wolbachia* wRi and wAu strains in *Drosophila simulans*, we developed a new assay (Fig. 1c and d). We designed a pair of *RpS6* primers (*Dsim_RpS6_F*, 5'-CCAGATCGCTTCCAAGGAGGCTGCT-3'; *Dsim_RpS6_R*, 5'-GCCTCCTCGCGCTTGGCCTTAGAT-3') to check for successful DNA isolation (Fig. 1c). To detect and differentiate *Wolbachia* wRi and wAu infection in *D. simulans*, we designed a set of *Wolbachia*-specific

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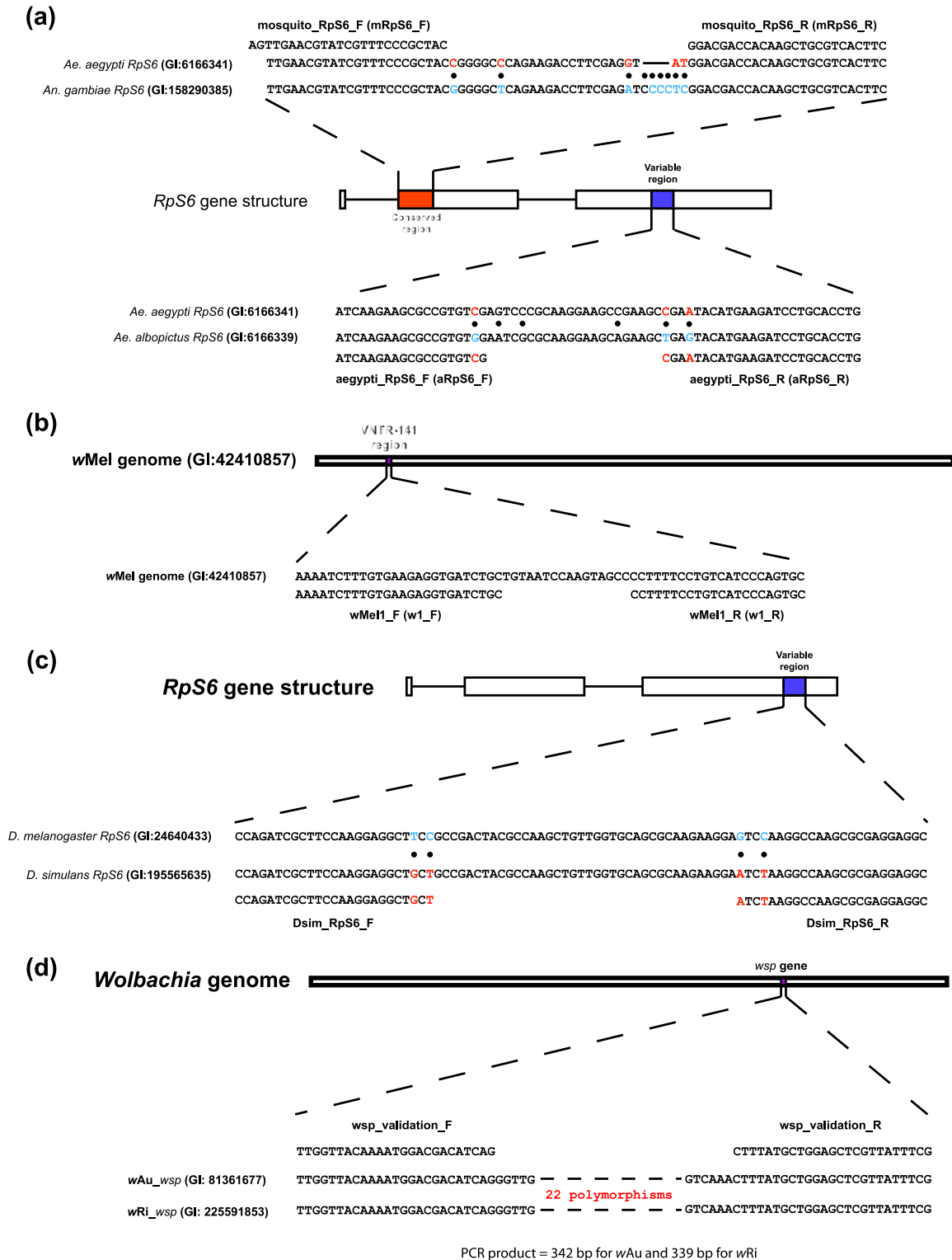


FIG 1 Development of gene markers to detect and quantify *Wolbachia* infection in *Ae. aegypti* and *D. simulans*. (a) Coding sequences of the *RpS6* gene from *Ae. aegypti* and *Anopheles (An.) gambiae* were aligned; a conserved region was selected to place the universal *Aedes* primers *mRpS6_F* and *mRpS6_R*. Nucleotide alignments of the coding sequences from *Ae. aegypti* and *Ae. albopictus* *RpS6* were used to identify a variable region in which to position a pair of *Ae. aegypti*-specific primers (*aRpS6_F* and *aRpS6_R*), with 1 to 2 diagnostic nucleotides at the 3' end of each primer. (b) The complete genome sequence of *Wolbachia wMel* was used to design a *Wolbachia*-specific marker at the VNTR-141 locus (see reference 9). The GenBank identifiers (GI) of the source sequences are given. Solid circles indicate polymorphic sites. (c) The variable region of the coding sequences in the *RpS6* gene between *Drosophila melanogaster* and *D. simulans* is shown. Primers were designed to amplify *D. simulans* but not *D. melanogaster*. Solid circles indicate polymorphic sites. (d) Universal primers were placed at the conserved regions between the *wRi* and the *wAu* strains. These primers flank an ~290-bp highly variable region that contains 22 polymorphic sites. The GI numbers of the source sequences are given. Note: reverse primers (*mRpS6_R*, *aRpS6_R* *w1_R*, *Dsim_RpS6_R*, and *w1_R*) are illustrated in the sense direction; their 5'-to-3' sequences are described in the text.

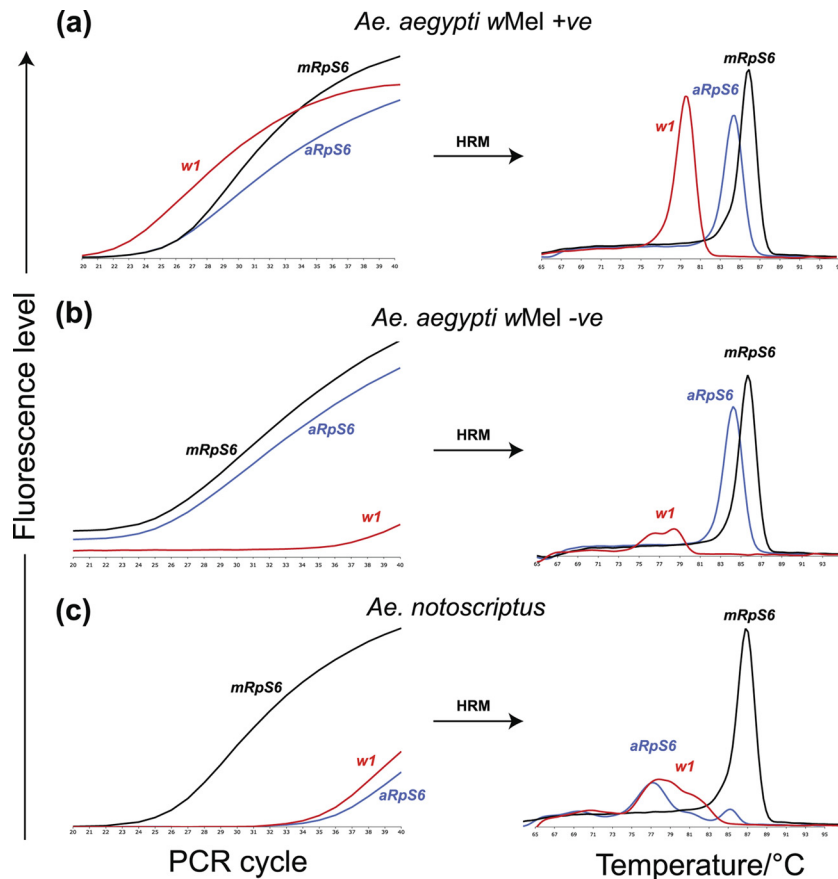


FIG 2 Performance and expected outcomes of the *Aedes* RT/HRM assay. (Left graphs) PCR amplification profile; (right graphs) amplicon-specific melting peaks (i.e., T_m). (a) *Ae. aegypti* that carries *wMel* supports robust amplification of all three markers, with average C_p values (means \pm 95% confidence intervals) of 27.86 ± 0.49 for *mRpS6*, 27.54 ± 0.49 for *aRpS6*, and 24.97 ± 0.83 for *w1*. The amplicons of these three markers had distinct T_m values: $85.65 \pm 0.03^\circ\text{C}$ for *mRpS6*, $84.33 \pm 0.02^\circ\text{C}$ for *aRpS6*, and $79.47 \pm 0.02^\circ\text{C}$ for *w1*. (b) *Ae. aegypti* that does not carry *wMel* supports robust amplification of the *mRpS6* and *aRpS6* markers from mosquito host DNA but not of the *w1* primer from the *Wolbachia* DNA. (c) An *Ae. notoscriptus* mosquito could support amplification of only the universal *Aedes* marker (*mRpS6*), and not the *Ae. aegypti*-specific (*aRpS6*) or the *wMel*-specific (*w1*) marker.

primers (*wsp_validation_F*, 5'-TTGGTTACAAAATGGACGACA TCAG-3'; *wsp_validation_R*, 5'-CGAAATAACGAGCTCCAGC ATAAAG-3'). The priming sites of the *Wolbachia* primers are located at conserved sequences flanking a variable region (22 polymorphisms) of the *wsp* gene between the *wAu* and the *wRi* sequences (Fig. 1d). Among the 28 *D. simulans* flies from Coffs Harbor successfully genotyped, 17 were *Wolbachia* positive. A closer inspection of the melting temperatures (T_m) of the *wsp* products revealed two distinct T_m clusters that differed by $\sim 0.5^\circ\text{C}$ (Fig. 3). Sequencing of amplicons confirmed that the high- T_m cluster ($\sim 82.7^\circ\text{C}$) was the *wRi* allele and the lower- T_m cluster ($\sim 82.2^\circ\text{C}$) was *wAu*. Since amplicon T_m is condition dependent, we believe that the 0.5°C T_m difference between *wRi* and *wAu* is a more useful diagnostic than their respective T_m 's. Based on this T_m -based genotyping method, there were 4 occurrences of *wRi* (14.3%) 13 of *wAu* (46.4%), and 11 flies uninfected (39.3%) in Coffs Harbor, Australia.

The *wRi* strain of *Wolbachia* has not previously been detected in Australian *D. simulans* populations (1, 4) and likely represents a new infection. The origin of the infection is unclear, but given the strong cytoplasmic incompatibility associated with this strain (6) and its incompatibility with the endemic *wAu* strain (4), it is likely

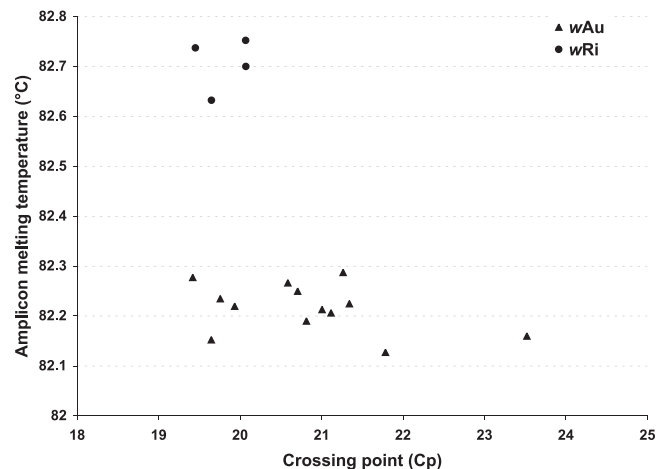


FIG 3 Classification of *Wolbachia* *wRi* and *wAu* infection status based on amplicon melting temperature differences. The graph shows a plot of melting temperature against the crossing point (C_p) of the *wsp* validation PCR amplicon in 17 *Wolbachia*-positive individuals. The proposed *Wolbachia* genotypic clusters are indicated by triangles (*wAu*) and circles (*wRi*).

that the distribution of the Australian *Wolbachia* infections in *D. simulans* populations will change over time.

While the traditional assays are sufficient for small-scale *Wolbachia* screening, the new RT/HRM assays provide high-throughput options to detect and quantify *Wolbachia* infection in *Ae. aegypti* and *D. simulans* at all life stages. We have successfully reduced the unit cost of genotyping such that large-scale field monitoring can be more feasible. Although the reagent costs have been minimized, the RT/HRM assay does require an initial capital investment in (or access to) an RT-PCR instrument capable of performing HRM analysis. In addition, the current specificity of the RT/HRM assays means that further adjustments will be needed if additional *Wolbachia* strains (e.g., *wMelpop*) are introduced into *Ae. aegypti* or *D. simulans* populations.

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