



## Note

## A simple protocol to obtain highly pure *Wolbachia* endosymbiont DNA for genome sequencing

Iñaki Iturbe-Ormaetxe, Megan Woolfit, Edwige Rancès, Anne Duploux<sup>1</sup>, Scott L. O'Neill\*

School of Biological Sciences, The University of Queensland, Brisbane, Qld 4072, Australia

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## ABSTRACT

Most genome sequencing projects using intracellular bacteria face difficulties in obtaining sufficient bacterial DNA free of host contamination. We have developed a simple and rapid protocol to isolate endosymbiont DNA virtually free from fly and mosquito host DNA. We purified DNA from six *Wolbachia* strains in preparation for genome sequencing using this method, and achieved up to 97% pure *Wolbachia* sequence, even after using frozen insects. This is a significant improvement for future *Wolbachia* and other endosymbiont genome projects.

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The  $\alpha$ -proteobacterium *Wolbachia pipientis* is probably the most common endosymbiont in the biosphere, infecting an estimated 25–76% of all insect species, as well as many other arthropod and filarial nematode species (Hilgenboecker et al., 2008; Jeyaprakash and Hoy, 2000). *Wolbachia* is able to induce a range of reproductive abnormalities in its hosts that promote its spread by favoring the reproduction of infected females (Werren et al., 2008). It can also confer fitness benefits on its host (Brownlie et al., 2009), and increase host resistance to infection with a range of pathogens (Bian et al., 2010; Hedges et al., 2008; Kambris et al., 2009; Moreira et al., 2009; Teixeira et al., 2008). *Wolbachia*'s remarkable biology and its potential application for control of vector-borne diseases such as dengue fever, malaria or filariasis (Moreira et al., 2009; Sinkins and O'Neill, 2000) have led to numerous genome sequencing projects aimed at understanding *Wolbachia*'s evolution and the genetic basis of the phenotypes it induces.

Because *Wolbachia* are fastidious bacteria that cannot be cultured outside host cells, a major hurdle for the sequencing of *Wolbachia* genomes has been obtaining enough endosymbiont DNA free from contaminating host mitochondrial and nuclear DNA. Endosymbiont DNA isolation from insects usually requires large amounts of starting material, laborious collection of embryos, density gradients and/or numerous DNA enrichment steps. For example, the Sanger sequencing

of the first *Wolbachia* genome (wMel) involved numerous rounds of DNA purification followed by pulse gel electrophoresis (Wu et al., 2004); material for the wPip sequence was obtained from more than 25,000 preblastoderm mosquito embryos pooled from 50 different extractions from fresh material (Klasson et al., 2008); and wRi was purified from *Drosophila* embryos in renografin density gradients and then the DNA treated in agarose plugs to remove host contamination (Klasson et al., 2009). Even after these measures, host contamination made up approximately 16–40% of the sequence data for these projects.

In response to these issues, we have developed a simple and rapid purification method to obtain *Wolbachia* DNA using relatively small numbers of infected adult flies and mosquitoes as the starting material. We used this method to sequence the genomes of six *Wolbachia* isolates, using next-generation sequencing technologies (Illumina and 454). The sequences produced by our method were highly enriched for *Wolbachia*, typically producing data sets in which 90–97% of the raw reads are *Wolbachia* sequence, an extremely high purity for an intracellular bacteria DNA preparation.

The *Wolbachia* strains (Table 1) were maintained in transinfected *Aedes aegypti* mosquitoes (McMeniman et al., 2008) or in infected *Drosophila* stocks using standard insect rearing conditions. For each purification, approximately 2000–5000 adult flies (approximately 10–25 mL in volume) or 400 mosquitoes were collected, surface-sterilized for 3 min in 50% bleach, rinsed in double distilled water and then further sterilized for 3 min in 70% ethanol followed by sterile water. The insects were homogenized in a glass Dounce homogenizer using a 40 mL cold SPG buffer (218 mM sucrose, 3.8 mM KH<sub>2</sub>PO<sub>4</sub>, 7.2 mM K<sub>2</sub>HPO<sub>4</sub>, 4.9 mM L-glutamate, pH 7.2). The extract was split into 4 Falcon tubes containing another 20 mL SPG buffer each and

\* Corresponding author. School of Biological Sciences, The University of Queensland, St Lucia, Brisbane, Qld 4072, Australia. Tel.: +61 7 3365 2471; fax: +61 7 3346 9213. E-mail address: [scott.oneill@uq.edu.au](mailto:scott.oneill@uq.edu.au) (S.L. O'Neill).

<sup>1</sup> Current address: Metapopulation Research Group, Department of Biological and Environmental Sciences, PO Box 65 (Viikinkaari 1) FI-00014, The University of Helsinki, 00790 Helsinki, Finland.

**Table 1**

*Wolbachia* strains sequenced, and the percentage of reads for each data set derived from *Wolbachia*, host mitochondrion or nucleus. PE = paired end sequencing.

Strain	Source	Sequencing method	Number of reads	% <i>Wolbachia</i>	% Host mitochondria	% Host nuclear	Remainder
wMelCS	<i>D. melanogaster</i> Canton-S (Riegler et al., 2005)	Illumina PE 36/75 bp <sup>a</sup>	61,790,230	61.10	4.05	29.44	5.41
wMelPop	<i>D. melanogaster</i> W <sup>1118</sup> (Min and Benzer, 1997)	454 Titanium PE	271,728	96.72	0.63	0.48	2.17
wMelPop-CLA	<i>A. aegypti</i> <sup>b</sup> PGYP1 (McMeniman et al., 2008, 2009)	454 GS-FLX PE	960,440	95.30	0.31	0.55	3.84
wRi-Riv88	<i>D. simulans</i> (Weeks et al., 2007)	Illumina PE 47 bp	4,903,570	90.29	0.19	7.70	1.82
wRi-IR2	<i>D. simulans</i> (Weeks et al., 2007)	Illumina PE 47 bp	6,037,698	96.84	0.25	1.48	1.43
wRi-IR33	<i>D. simulans</i> (Weeks et al., 2007)	Illumina PE 47 bp	5,742,320	96.71	0.08	1.99	1.22

<sup>a</sup> Values combined for two Illumina runs from the same purification.

<sup>b</sup> Transfected with wMelPop-CLA (McMeniman et al., 2008).

centrifuged at 3200 ×g for 15 min, twice. The supernatant was sequentially filtered through 5, 2.7 and 1.2 μm syringe filters, and *Wolbachia* were pelleted at 18,000 ×g for 20 min in an Oakridge tube and resuspended in 4 × 750 μL cold SPG buffer in eppendorf tubes. Intact *Wolbachia* in SPG were treated with 20 μL DNaseI (Roche) (30 μg/mL) for 30 min at 37 °C to remove host DNA contamination without disrupting the cells. After treating the samples with 5 μL RNase (Fermentas) for 15 min at 37 °C, *Wolbachia* cells were lysed by incubation with 10 μL proteinase K (Amresco; 20 mg/mL) at 56 °C for 60 min. DNA was purified using 2 phenol/chloroform/isoamyl alcohol (IAA) and one chloroform/IAA extraction, precipitated overnight at –20 °C using sodium acetate/ethanol, washed in 70% ethanol, and resuspended in 80 μL TE or milli Q water (Millipore).

Total DNA was quantified using a nanodrop spectrophotometer. The A260/280 ratio of the DNA was typically 1.7–1.9. The phenol/chloroform extractions of DNA generally yielded brownish-colored pellets, but this did not seem to affect the quality of the libraries generated and the sequence data. The yield for the wMelPop strains was 100–200 μg based on nanodrop absorbance readings, although fluorimeter readings gave approximately 10 times lower concentration values. For the wRi and wMelCS genomes, we further purified the DNA using a DNAeasy kit (Qiagen), following the manufacturer's instructions, which produced a clear DNA solution but reduced the yield and did not significantly improve the purity of the sample (Table 1) compared to a normal purification. An aliquot of the DNA run on a 0.8% agarose gel appeared as a single band, in some cases with some faint smear.

The Illumina and 454 sequencing of these genomes generated large amounts of sequence data (Table 1), which are currently being assembled and analyzed. To determine the percentage of reads derived from *Wolbachia*, we mapped all reads from each data set to three reference genomes in turn: the closest available *Wolbachia*, host mitochondrial and host nuclear genomes (Table 2). Mapping was performed with Maq (Li et al., 2008) for Illumina data sets and gsMapper (454 Roche) for 454 data sets. Default settings for mapping programs were used in all cases. For each data set, we then counted the number of reads that were mapped to one and only one of the reference genomes, and the number that was mapped either to multiple or to no references (these last two categories are combined as 'remainder', Table 1).

The percentage of reads derived from *Wolbachia* was greater than 90% for five of the six data sets, with a single outlier of 61% (wMelCS). Host contamination was sometimes as low as 1% and was usually less than 10%. We are confident that the extremely high proportion of reads that map to *Wolbachia* represent a sequence data from our target *Wolbachia* strain, rather than from gut bacteria or other components of the insect microbiota. We tested for non-specific mapping to other bacterial taxa by mapping the wRi IR2 data set against the genomes of wBm, another *Wolbachia* strain; *Ehrlichia ruminantium*, *Anaplasma marginale* and *Rickettsia bellii*, three other members of the Rickettsiales closely related to *Wolbachia*; and *Lactobacillus brevis*, *Acetobacter pasteurianus* and *Enterobacter cloacae*, three taxonomically divergent, common gut commensals of laboratory-reared *Drosophila* (Cox and Gilmore, 2007; Ryu et al., 2008). Only 6.3% of IR2 reads mapped to the wBm genome, and fewer than 0.3% mapped to the genomes of any of the Rickettsiales or commensals. Our test for purity is therefore extremely unlikely to be affected by non-specific mapping to other bacteria of the insect microbiota.

Filtration (McMeniman et al., 2008; Rasgon et al., 2006) and DNase treatment (Frutos et al., 2006) have been used in the purification of intracellular bacteria previously, though not in the combination described here. The generally high purity obtained by our method is probably due to three main factors acting in concert. First, the final filter size (1.2 μm) through which we pass the homogenized material should exclude all but the smallest bacteria. Most species of the non-endosymbiotic microbial flora will have cell sizes too large to pass intact through this filter. Second, the DNase treatment after filtration but prior to the lysis of intact *Wolbachia* cells will destroy any fragments of host or other microbial DNA that remain in the filtrate, removing most other sources of contaminating sequence. Finally, some of the *Wolbachia* strains we used are present at high density in their hosts, which may contribute to their preponderance in our purifications. We do not have a simple explanation for the lower purity observed in the wMelCS extraction, the first we performed using this method. It is possible that it was due to lower DNase efficacy or lower *Wolbachia* density in the sample. Nonetheless, despite the lower purity, the method produced more than sufficient DNA for two lanes of Illumina sequencing.

The method we describe here is simple and quick, taking only a few hours to perform, in stark contrast to previous methods used to

**Table 2**

Reference genomes against which our sequence data sets were mapped, with their Genbank accession numbers.

Sequenced strain	<i>Wolbachia</i> reference genome	Host mitochondrial reference genome	Host nuclear reference genome
wMelPop and wMelCS	wMel, NC_002978.6	<i>D. melanogaster</i> , NC_001709.1	<i>D. melanogaster</i> , NT_033779.4, NT_033778.3, NT_037436.3, NT_033777.2, NC_004353.3, NC_004354.3
wMelPop-CLA	wMel, NC_002978.6	<i>A. aegypti</i> , NC_010241.1	<i>A. aegypti</i> , CH477186–CH479178, CH899794–CH902558
wRi (Riv88, IR2, IR33)	wRi, NC_012416.1	<i>D. simulans</i> , AF200841.1	<i>D. simulans</i> , CM000361.1–CM000366.1

obtain material for *Wolbachia* genome sequencing projects. It works equally well on frozen material: the wMelPop strain was purified from flies that were collected, frozen in liquid nitrogen and kept at  $-80^{\circ}\text{C}$  for 2 weeks prior to the extraction. It uses insects in numbers easily obtained in the laboratory, and could probably use far fewer and still produce ample material for next-generation sequencing, as the numbers of insects we used generated more DNA than we required. Although we have only tested this method for *Wolbachia*, we anticipate that it may be useful for purifying DNA for sequencing from other endosymbiont genomes that have small cell sizes, such as *Rickettsia*, *Chlamydia* and *Mycoplasma*, where advances in genomic research have also been limited compared to other free living species, partly due to the difficulty in culturing and purifying them. Finally, *Wolbachia* isolated from fresh insects using this method (prior to the proteinase K lysis step) can be used for cell culture infection (data not shown) and are therefore viable. They might also be used for insect transinfection experiments, in which the absence of mitochondrial contamination may be advantageous. In summary, the method we describe here for isolation of clean *Wolbachia* DNA should prove invaluable for future genome sequencing projects and other applications requiring large quantities of pure endosymbiont material.

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