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PCR-based cytotyping to detect *Wolbachia* in insect tissues

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Shropshire JD, Conner WR, Vanderpool D, Hoffmann AA, Turelli M, Cooper BS. (2024) Rapid host switching of *Wolbachia* and even more rapid turnover of their phages and incompatibility-causing loci. bioRxiv. 12.04.569981. PMCID: PMC10723362

Shropshire JD, Hamant E, Conner WR, Cooper BS. (2022) *cifB*-transcript levels largely explain cytoplasmic incompatibility variation across divergent *Wolbachia*. PNAS Nexus. 1(3):pgac099. PMCID: PMC9364212

Hague MTJ, Shropshire JD, Caldwell CN, Statz JP, Stanek KA, Conner WR, Cooper BS. (2022) Temperature effects on cellular host-microbe interactions explain continent-wide endosymbiont prevalence. Curr Biol. 32(4):878. PMCID: PMC8891084

Shropshire JD, Hamant E, Cooper BS. (2021) Male age and *Wolbachia* dynamics: investigating how fast and why bacterial densities and cytoplasmic incompatibility strengths vary. mBio. 12(6):e02998-21. PMCID: PMC8686834

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We use this protocol and it's working

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Abstract

This protocol details a PCR-based method for detecting *Wolbachia* in insect tissues. By amplifying the *wsp* gene, specific to *Wolbachia*, and the host 28s rDNA gene as a control, this technique allows for accurate identification of *Wolbachia*-bearing and *Wolbachia*-free samples. Proper interpretation of control samples is crucial to ensure reliable results.

Guidelines

Unless otherwise specified, steps are performed at room temperature in a 1.5 mL PCR-clean Eppendorf tube containing the appropriate tissue. Precautions are taken throughout to protect samples against contamination. All centrifuge steps are at 12,000 x g.

Materials

Materials

- Agarose, electrophoresis grade (Fisher Scientific, BP-160)
- Centrifuge tubes, safe-lock, PCR clean, 1.5 mL (Eppendorf, 022363212)
- Ceramic beads, 2.8mm (VWR, 19-646-3)
- DNA ladder (Thermo Fisher Scientific, SM1552)
- EDTA (PR1MA, KCE57020-250)
- Erlenmeyer flask, 250 mL (any)
- Ethidium bromide (Thermo Fisher Scientific, 15585011)
- GoTaq Green (Promega, M7123)
- Graduated cylinder (any)
- LB Buffer, 20x (Faster Better Media LLC, LB20-4)
- NaCl (Apex BioResearch, DB0483)
- Pipette tips, 0.1-10 µL (Eppendorf, 0030078500)
- Pipette tips, 2-100 µL (Eppendorf, 0030078543)
- Pipette tips, 50-1000 µL (Eppendorf, 0030078578)
- PCR tubes/plates and caps/seals (any)
- Primers (wsp & 28s)
- Proteinase K (Thermo Fisher Scientific, 25530049, ThermoFisher)
- Screw-top jar, Glass, 500 mL (any)
- Tris-HCl (1M, pH = 8.0) (Thermo Fisher Scientific, J22638-AE)
- Water, distilled (PR1MA, KCW20525-10000)
- Water, molecular biology grade (MidSci, 786-73C)

Equipment

- Analytical balance (any)
- Autoclave
- Bead-mill homogenizer (Benchmark, IPD9600)
- Centrifuge (Eppendorf, 5420)
- Electrophoresis power supply (Thermo Fisher Scientific, PS0600)
- Electrophoresis system (Thermo Fisher Scientific, OWL)
- Freezer (any)
- Fridge (any)
- Gel imager (any)
- Microwave (any)
- Pipette, 10 µL (Eppendorf, 4861000708)
- Pipette, 100 µL (Eppendorf, 4861000716)
- Pipette, 1000 µL (Eppendorf, 4861000732)
- Thermocycler (Bio-Rad, C1000)
- Thermomixer (Eppendorf, F2.0)

Safety warnings

This protocol involves the use of various chemicals and reagents that require careful handling and strict adherence to safety guidelines to ensure safe laboratory practices. Please review the Material Safety Data Sheets (MSDS) for each reagent before beginning the protocol and take appropriate precautions. All steps should be performed while wearing gloves, a lab coat, and eye protection.

Before start

Sterilize the working surface with 10% bleach and 70% ethanol.

Prepare squish buffer

1 Mix the following in a 500 mL glass screw-top jar. Use a graduated cylinder to measure liquids and an analytical balance to measure solids.

- 20 mL Tris-HCl ([M] 1 Mass Percent , pH = 8.0)
- 0.0744 g EDTA
- 0.2922 g NaCl
- 180 mL diH2O

2 Autoclave 00:30:00 on fluid cycle to sterilize. Make sure the screw top is loose. Allow to cool to room temperature. 30m

3 Add 300 µL of Proteinase K. Mix by gently shaking the bottle.

4 Create 15 mL aliquots labeled with the date.

5 Store at 4°C until use.

Sample collection, homogenization, and lysis

6 Collect samples by adding three ceramic beads (2.8 mm) and flies to a 1.5 mL centrifuge tube

7 Homogenize flies for 00:02:00 at 1500 rpm using a bead-mill homogenizer. 2m

8 Add 50 µL of squish buffer per *Drosophila* fruit fly.

9 Incubate at 65 °C for 00:45:00 at 300 rpm to lyse cells using a ThermoMixer. 45m

10 Incubate at 94 °C for 00:04:00 at 300 rpm to inactivate Proteinase K. 4m

Debris precipitation, storage, and use

11 Centrifuge for 00:02:00 to pellet cellular debris. 2m

12 Store squish-extracted DNA at -20 °C for 2 weeks or at 4 °C for 24 hours.

Note

Considerations for use:
1. Centrifuge the DNA before use to bring any protein/fly pieces to the bottom of the tube.
2. When pipetting, take care to only collect the liquid on the top of the mixture.

Polymerase chain reaction (PCR)

13 Create two PCR master mixes with a final reaction volume of 10 µL with 2 µL of DNA template. Each sample should be tested with *wsp* primers (for *Wolbachia*) and 28s (for the host).

- wsp_F: 5'-TGGTCCAATAAGTGATGAAGAAAC-3'
- wsp_R: 5'-AAAAATTAAACGCTACTCCA-3'
- 28s_F: 5'-TACCGTGAGGGAAAGTTGAAA-3'
- 28s_R: 5'-AGACTCCTTGGTCCGTGTTT-3'

Note

Plan for the inclusion of a positive control known to have *Wolbachia*, a negative control without *Wolbachia*, and a no template control where water is added in place of a DNA template.

14 Add 8 µL of the master mix to each well.

15 Add 2 µL of the DNA template to each well.

16 Cap or seal wells. Centrifuge for 00:00:10 at 12000 rcf . Transfer to a thermocycler. 10s

17 Perform PCR following the relevant program.
- *wsp* program: 94°C for 5 min, (94°C for 30 sec, 55°C for 30 sec, and 72°C for 75 sec)x40, 72°C for 5 min, hold at 4°C
- 28s program: 94°C for 2 min, (94°C for 30 sec, 50°C for 30 sec, and 72°C for 60 sec)x40, 72°C for 8 min, hold at 4°C

Make 1% LB Gel

18 Seal gel tray in preparation of adding liquid agarose.

19 Add 100 mL 1x LB Buffer to a 250 mL Erlenmeyer flask. Add 1 g agarose to the flask. Swirl to mix.

20 Microwave until just boiling. Stop the microwave. Swirl the flask to mix. Look for flakes of unmelted agarose. Continue until fully dissolved.

Note

Caution: The glass will be hot, and the agarose can boil over if not observant.

21 Run tap water over the flask for 00:00:30 to cool. Swirl the flask while cooling. 30s

22 Add 2 drops of ethidium bromide to the flask and mix well by swirling. Pour liquid agarose gel into the gel tray and add comb(s). Be careful not to create bubbles in the gel with the comb.

23 Wait 00:20:00 for the gel to solidify 20m

24 Carefully remove the combs and unseal the gel tray.

Perform gel electrophoresis with PCR products

25 Load gel and tray into gel box. Add 1x LB Buffer until the fill line.

26 Add 3 µL of the ladder into the furthest left well on each row.

27 Add 3 µL of each PCR sample into each well.

Note

- Work quickly or the samples will start to disperse in the wells.
- Be careful not to poke the pipet tip into the side or bottom of the well.

28 Cover the gel box and attach it to a power supply. Run the gel at 300 Volts. Monitor the migration of the loading dyes to determine when to stop.

29 Image the gel.

30 Interpret the control samples. If the controls are as expected, proceed to interpret the experimental samples.

Interpreting no-template control (NTC):

- Expected result: No bands for *wsp* or 28s.
- Unexpected result: Any band indicates reagent contamination. Cytotyping inconclusive.

Interpreting *Wolbachia*-free DNA (negative control):

- Expected result: No *wsp* band, clear 28s band.
- Unexpected result: *wsp* band indicates sample contamination during collection or processing. No 28s band indicates poor DNA quality, PCR issues, or errors in sample loading. Cytotyping inconclusive.

Interpreting *Wolbachia*-containing DNA (positive control):

- Expected result: Clear bands for both *wsp* and 28s.
- Unexpected result: No bands indicate poor DNA quality, PCR issues, or errors in sample loading. Cytotyping inconclusive.

Interpreting test samples:

- + *wsp*, + 28s: *Wolbachia*-positive
- - *wsp*, + 28s: *Wolbachia*-negative
- + *wsp*, - 28s: Potentially *Wolbachia*-positive, but technical or human errors may have affected the 28s reaction.

- - *wsp*, - 28s: Inconclusive. Technical or human errors likely impacted both reactions.

Protocol references

Oligos reported in this protocol originate from Braig et al. 1998 and Nice et al. 2009.

Braig HR, Zhou WG, Dobson SL, O'Neill SL. (1998) Cloning and characterization of a gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia pipientis*. J Bacteriol. 180(9):2373–2378. PMID: 9573188

Nice CC, Gompert Z, Forister ML, Fordyce JA. (2009) An unseen foe in arthropod conservation efforts: The case of *Wolbachia* infections in the Karner blue butterfly. Biol Conserv. 142(12):3137–3146.