Journal of Visualized Experiments

Establishment and Maintenance of Gnotobiotic American Cockroaches (Periplaneta Americana)

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61316R1
Full Title:	Establishment and Maintenance of Gnotobiotic American Cockroaches (Periplaneta Americana)
Section/Category:	JoVE Biology
Keywords:	gnotobiotic; germ-free; cockroach; periplaneta; americana; sterilize; oothecae
Corresponding Author:	Helen Dukes University System of Georgia Athens, GA UNITED STATES
Corresponding Author's Institution:	University System of Georgia
Corresponding Author E-Mail:	hd55218@uga.edu
Order of Authors:	Helen E. Dukes
	Josey E. Dyer
	Elizabeth A. Ottesen
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$4,200)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Athens, Georgia, USA

Powered by Editorial Manager® and ProduXion Manager® from Aries Systems Corporation

Manuscript
Click here to access/download;Manuscript;61316_R1_RE.docx

TITLE:

Establishment and Maintenance of Gnotobiotic American Cockroaches (*Periplaneta Americana*)

AUTHORS AND AFFILIATIONS:

Helen E. Dukes[1], Josey E. Dyer[1], Elizabeth A. Ottesen[1]

[1]Department of Microbiology, University of Georgia, Athens, GA, USA

Corresponding Author:

Helen E. Dukes (helen.dukes@uga.edu)

Email Addresses of Co-Authors:

Josey E. Dyer (Josey.Dyer@uga.edu)

Elizabeth A. Ottesen (ottesen@uga.edu)

KEYWORDS:

gnotobiotic, germ-free, cockroach, periplaneta, americana, sterilize, oothecae

SUMMARY:

This protocol is used in establishing and maintaining gnotobiotic American cockroaches (*Periplaneta americana*) by surface sterilizing the egg cases (oothecae) prior to hatching. These gnotobiotic insects contain their vertically transmitted *Blattabacterium* endosymbionts but have axenic guts.

ABSTRACT:

Gnotobiotic animals are a powerful tool for the study of controls on microbiome structure and function. Presented here is a protocol for the establishment and maintenance of gnotobiotic American cockroaches (*Periplaneta americana*). This approach includes built-in sterility checks for ongoing quality control. Gnotobiotic insects are defined here as cockroaches that still contain their vertically transmitted endosymbiont (*Blattabacterium*) but lack other microbes that normally reside on their surface and in their digestive tract. For this protocol, egg cases (oothecae) are removed from a (nonsterile) stock colony and surface sterilized. Once collected and sterilized, the oothecae are incubated at 30 °C for approximately 4–6 weeks on brain-heart infusion (BHI) agar until they hatch or are removed due to contamination. Hatched nymphs are transferred to an Erlenmeyer flask containing a BHI floor, sterile water, and sterile rat food. To ensure that the nymphs are not housing microbes that are unable to grow on BHI in the given conditions, an additional quality control measure uses restriction fragment-length polymorphism (RFLP) to test for nonendosymbiotic microbes. Gnotobiotic nymphs generated using this approach can be inoculated with simple or complex microbial communities and used as a tool in gut microbiome studies.

INTRODUCTION:

Gnotobiotic animals have proven to be invaluable tools for microbiome studies[1-3]. Germ-free and defined-flora animals have allowed elucidation of host-microbe interactions, including host immunological responses, gut epithelial maturation, and host metabolism[1,4-7]. Gnotobiotic animals inoculated with a simplified community have also assisted in a more complete understanding of microbe-microbe interactions in a gut community, specifically in unraveling cross-feeding and antagonistic relationships[8-11]. The current preferred model system for studies in the mammalian gut microbiome is the murine model. While this system has been vital in the discoveries outlined above, a key shortcoming is the cost involved. Specialized equipment and highly trained technicians are necessary to establish and maintain a gnotobiotic facility. This, in combination with extra care that must be given to every aspect of gnotobiotic animal maintenance, causes the a gnotobiotic animal to cost ten to twenty times more to breed than a standard animal model[12]. Due to high costs, many researchers may be unable to afford a gnotobiotic murine model. Additionally, while murine models may be the most widely accepted choice for studies looking to translate to human health, there are still many physiological and morphological differences between human and mouse guts[13]. Clearly no singular model is enough to answer the ever-increasing number of questions regarding the many aspects of the gut microbiome.

Insect models are a cheaper alternative due to their lower cost-of-maintenance in comparison to mammalian species. Extensive germ-free and gnotobiotic research in a variety of insect species has led to the development of multiple commonly used models. Mosquitos and *Drosophila* are common models for germ-free work due to their relevance to global diseases and genetic tractability[14,15]. Another emerging model system is that of the honey bee (*Apis mellifera*), given its importance in pollination and sociality research[16]. However, many of these commonly used insects lack the taxonomic complexity seen in mammalian gut communities[17], limiting their ability to model higher order interactions. Not only is the total diversity of microbes found in the gut of American cockroaches more similar to mammals, but many of the microbes present in the cockroach gut belong to families and phyla that are commonly found in the gut microbiota of mammals and humans[18]. The hindgut of the cockroach is also functionally analogous to the large intestine of mammals, as it is a fermentation chamber densely packed with bacteria to assist in extraction of nutrients[19,20]. Finally, the omnivorous nature of cockroaches allows for a diversity of diet regimes that would not be possible with dietary specialists.

American cockroaches can be a useful model system for understanding gut microbial communities in higher organisms, but the cockroach's status as a pest also makes this system relevant for pest control[21]. Leveraging fundamental knowledge of the gut community's influence on cockroach health and physiology assists in developing new techniques for pest management.

The goal of this method is to outline a comprehensive description of the establishment and maintenance of gnotobiotic American cockroaches (*Periplaneta americana*), but this protocol could be used to generate nymphs of any oviparous cockroach. It includes a method for efficient, noninvasive collection of mature oothecae, and a nondestructive technique to monitor gnotobiotic status of the insects[22-24]. While previous methods of achieving and maintaining gnotobiotic cockroaches describe ootheca collection[23-27], ootheca maturity is either interpreted

in terms of species-specific cues (in *Blattella germanica*[22,24,25]), or not explicitly described[27,28], making implementation difficult for those unfamiliar with the system. Since the method described here uses naturally dropped oothecae, the error of removing eggs prematurely is absent. This protocol contains both culture-dependent and culture-independent methods of quality control, and the culture-dependent method does not require sacrificing insects. Finally, this method brings together information from multiple gnotobiotic cockroach studies to create one, comprehensive protocol with all necessary information for achieving and maintaining gnotobiotic cockroaches.

PROTOCOL:

1. Preparation of materials

1.1. Maintenance of stock cockroach cultures

NOTE: There are many ways to rear these robust insects. The specifics on providing shelter and water can be different depending on accessible materials (i.e., egg cartons instead of cardboard tubes). The following sterilization protocol will work for any stock tank setup.

1.1.1. Spread enough woodchip bedding in a 37.85 L (10 gallon) fish tank to cover the bottom of the tank with approximately 1 inch of bedding. Prepare housing by cutting (flat) cardboard to 2 in x 4 in pieces. Insert cardboard pieces into cardboard tubes (e.g., toilet paper tubes) and stack tubes at one end of the tank (**Figure 1**).

1.1.2. Smear a thin layer of petroleum jelly on the top two inches of the inside of the tank to prevent insect escape.

NOTE: Be sure to properly coat the inside of the corners of the tank.

1.1.3. Add cockroaches by transferring (occupied) cardboard tubes from a previous stock tank shaking them to release their inhabitants. For each transfer, move 100−200 mixed-age, mixed-sex cockroaches. Add dog food (20−30 pieces), monitor the amount of dog food in the tank and refill when low.

1.1.4. Set up a water dish.

1.1.4.1. Fill a small plastic reusable food container with double-distilled water (ddH_2O). Cut cellulose sponges and holes in the lid of the food container to approximately the same size.

NOTE: The cellulose sponges prevent cockroaches from drowning in the water dish.

1.1.5. Insert sponges into holes in the lid and place the lid on the filled container. Place the container in the tank and refill when low. Cover the tank with cotton cloth and secure it in place with an elastic band.

1.1.6. As tanks begin to accumulate excessive quantities of frass and insect carcasses, set up new tanks and transfer cockroaches.

NOTE: Tanks are typically transferred every 6 months. Any remaining cockroaches/eggs in decommissioned stock tanks are euthanized by freezing at -20 °C for 1 h and the contents of the stock tank are then transferred to an autoclave bag and autoclaved (1 h, gravity cycle) prior to disposal. Stock tanks are sterilized with 2% bleach between uses.

1.2. Disinfect a secondary container.

NOTE: This container does not include a filter, but instead allows free air exchange.

1.2.1. Spray the inside of both the lid and bottom with 2% bleach and allow them to soak for 10 min. Wipe out the bleach with a clean paper towel.

1.2.2. Spray the inside of the lid and bottom with 70% ethanol and wipe dry with a clean paper towel. Replace the lid until use.

1.3. Make BHI slants and flasks for incubating eggs and housing nymphs.

1.3.1. Prepare BHI according to package instructions, adding 2% agar. Boil the BHI-agar solution until clarified.

1.3.2. For slants, transfer 5 mL aliquots to 18 mm x 150 mm glass test tubes and cap. Sterilize via autoclave (sterilization time = 20 min, liquid cycle). Place autoclaved tubes at a 45° angle to cool into slants. Once solidified, refrigerate until use to prevent drying out.

1.3.3. For flasks, transfer 10 mL aliquots of boiled BHI-agar solution into 250 mL Erlenmeyer flasks to completely cover the bottom of the flask. Cover the flask with foil and sterilize via autoclave (20 min, liquid cycle). Allow autoclaved flasks to cool and refrigerate until use to prevent drying out.

NOTE: No air filter is required for this setup. The foil cover is sufficient to allow gas exchange while preventing contamination from open air flow.

1.4. Sterilize via autoclave: autoclavable rat chow broken into half-sizes (~1/2 inch pieces) in a foil-covered beaker (sterilization time = 1 h, gravity cycle), ddH_2O in a capped bottle (sterilization time = 20 min, liquid cycle), and forceps in a foil-covered beaker (sterilization time = 20 min, gravity cycle).

NOTE: Do not overfill the rat chow beaker. Pellets will swell in the autoclave.

1.5. Set up a “maternity ward” tank.

NOTE: This tank contains the same materials as the stock tanks (cardboard tubes, water dishes with sponges, dog food, woodchip bedding; see **Figure 1**) and should be empty of cockroaches unless transferred for oothecae collection (see section 2).

1.6. Humidify an incubator by preparing a beaker full of supersaturated sodium chloride (NaCl) solution. Prepare this solution by adding 37 g of NaCl per 100 mL of ddH_2O and stirring until dissolved.

NOTE: Typically, 500 mL of saturated salt solution typically humidifies an incubator with chamber dimensions 51 cm x 46 cm x 46 cm (H x W x D) for approximately a month before more water must be added.

2. Collection of oothecae

2.1. Transfer females (in any number as appropriate for planned experiments) carrying oothecae (**Figure 2**) from the stock tank to the "maternity ward" by using forceps to move cardboard tubes that contain gravid females.

2.1.1. If a carboard tube contains multiple insects in addition to the gravid female, first shake out the tube into an additional plastic container ringed with petroleum jelly, and then encourage the target insect to climb back into the cardboard tube alone.

2.2. Transfer females back to the stock tank once they have dropped their oothecae. Retrieve the oothecae from the litter in the tank with forceps.

NOTE: Oothecae are often dropped within 24 h of the female being transferred.

3. Cleaning of oothecae

3.1. Add oothecae to a 5 mL centrifuge tube containing 3 mL of sodium dodecyl sulfate (SDS). Vortex for 10 s. Repeat for a second wash step with a centrifuge tube containing fresh SDS.

NOTE: Up to five oothecae may be used per 3 mL of SDS.

3.2. Using a delicate task wipe, gently scrub the surface of each ootheca to remove any debris. More SDS may be added to assist in thorough cleaning. Place cleaned oothecae in a weighing boat until ready for sterilization.

NOTE: Protocol may be paused here, but leaving the oothecae in low humidity environments for extended periods of time (days to weeks) will cause them to dehydrate and lose viability.

4. Sterilization and incubation of oothecae

4.1. Aliquot sterile water for poststerilization rinse. For every ootheca to be sterilized, fill two 1.5 mL centrifuge tubes with 1 mL of sterile water.

4.2. Add 10 µL of concentrated (32%) peracetic acid stock solution to 3.2 mL of ddH_2O in a 5 mL centrifuge tube to create a 0.1% solution for sterilization. Cap and invert several times to mix.

CAUTION: Peracetic acid is harmful in contact with skin or lungs. Dilute in a fume hood.

NOTE: This must be done the same day as sterilization. If diluted in advance, the solution will quickly decompose and therefore not properly sterilize. As many as five cleaned oothecae may be sterilized in 3.2 mL of dilute acid.

4.3. Place (up to five) cleaned oothecae in the 0.1% peracetic acid solution for 5 min. Invert the tube several times every 60 s.

4.4. In a laminar flow hood, use sterile forceps to transfer each ootheca to its own centrifuge tube with aliquoted sterile rinse water (step 4.1). Invert several times to mix. Repeat for a second rinse, then transfer each rinsed ootheca to its own BHI slant using sterile forceps.

4.5. Place slants in the sterilized secondary container. Move the container into the humidified incubator at 30 °C for 4−5 weeks until hatched.

NOTE: Slants may be held upright by a small test tube rack or a medium/small beaker.

4.6. Check slants regularly (1−2x per week). If fungal or bacterial colony growth appears on the agar, remove the contaminated slant. When the four-week timepoint approaches, check slants every day.

NOTE: Once hatched, nymphs can survive for up to several weeks on BHI alone but will not grow optimally.

5. Maintenance of gnotobiotic nymphs

5.1. In a laminar flow hood, aseptically transfer a pellet of sterilized rat chow to a prepared BHI flask (from step 1.3.3) with sterile forceps. As a sterility check, place the flask in the secondary container in a 30 °C incubator for 24 h, and do not use if growth appears.

5.2. Add nymphs to the BHI flask with sterile food pellet. Shake them out of their BHI slant and let them fall into the flask in a laminar flow hood.

NOTE: The nymphs do not have traction on the glass walls of the test tube. Shaking the tube to knock them off of the slant and then tipping the tube to allow them to slide down the glass into the flask is effective. While nymphs can be transferred using forceps, the risk of fatal injury is high.

5.3. Water nymphs with 300 µL of sterile water once per week in a laminar flow hood by pipetting directly onto the BHI floor of the flask.

5.4. As nymph feces begin to cover the BHI floor, transfer to a new BHI flask, adding the sterilized rat chow 24 h in advance (to verify sterility) as in step 5.1.

6. Quality control of sterility

6.1. Remove one nymph from the BHI flask to sacrifice for a culture-independent quality control check of gnotobiotic status via restriction fragment-length polymorphism (RFLP). To do this, pour the nymph into a sterile centrifuge tube (similar to nymphal transfer from step 5.1) or place a sterile wooden applicator into the flask and wait for a nymph to begin climbing it, then transfer it to the centrifuge tube.

6.2. Add 0.5 mL of 1x phosphate buffered saline (PBS) to the nymph in the centrifuge tube and homogenize with a sterile micropestle until all large pieces are broken up. Vortex well.

6.3. Extract DNA from nymph homogenate using a bacterial DNA extraction kit (**Table of Materials**) as follows.

6.3.1. Centrifuge the homogenized nymph for 10 min at 5,000 x *g* and remove the supernatant. Preheat a thermal shaker to 37 °C.

6.3.2. Add 100 µL of 1x Tris-EDTA, and vortex to completely resuspend the pellet. Add 10 µL of lysozyme and mix, followed by a 30 min (no shaking) incubation in the preheated 37 °C thermal shaker.

6.3.3. Add 25 mg of glass beads to sample and vortex at maximum speed for 5 min. Preheat the thermal shaker to 55 °C.

6.3.4. Allow the beads to settle before transferring the supernatant to a new 1.5 mL centrifuge tube with 100 µL of proteinase K buffer and 20 µL of proteinase K. Vortex to mix thoroughly.

6.3.5. Incubate, with shaking at 600 rpm, in a 55 °C thermal shaker for 60 min. Centrifuge at 10,000 x *g* for 2 min, and transfer supernatant to a new 1.5 mL centrifuge tube. Preheat the thermal shaker to 65 °C. Begin preheating the elution buffer in a 65 °C hybridization oven.

6.3.6. Add 220 µL of 100% ethanol. Vortex at maximum speed for 20 s. Break up any visible precipitate by pipetting up and down 10x.

6.3.7. Insert a DNA column into a 2 mL collection tube, and transfer the sample into the column, including any precipitate that may have formed. Centrifuge at 10,000 x *g* for 1 min, discard filtrate from the collection tube, and replace the collection tube.

6.3.8. Add 500 µL of binding buffer to the column, and centrifuge at 10,000 x *g* for 1 min. Discard filtrate from the collection tube and replace the collection tube.

6.3.9. Add 700 µL of DNA wash buffer to the column, and centrifuge at 10,000 x *g* for 1 min. Discard filtrate from the collection tube and replace the collection tube. Repeat for a second wash step.

6.3.10. Centrifuge empty column for 2 min to dry it, transferring the column to a new 1.5 mL centrifuge tube afterward. Add 50 µL of preheated elution buffer directly to the DNA column matrix, and incubate at 65 °C for 5 min.

6.3.11. Centrifuge at 10,000 x *g* for 1 min to elute. Quantify the extracted DNA in the filtrate via spectrophotometry or fluorometry.

6.4. Amplify and digest whole 16S gene. Visualize fragments using gel electrophoresis.

6.4.1. Use 12.5 µL of 2x master mix, 0.5 µL of each primer 1492R (5'-GGTTACCTTGTTACGACTT) and 27F (5'-AGAGTTTGATCCTGGCTCAG), 5 ng of DNA, and molecular-grade water for a total reaction volume of 25 µL.

6.4.2. Run the following thermocycler program: 94 °C for 60 s; followed by 35 cycles of 94 °C for 30 s, 50 °C for 45 s, 68 °C for 90 s; followed by 68 °C for 5 min.

6.4.3. Purify the polymerase chain reaction (PCR) product using a DNA purification kit (**Table of Materials**).

6.4.3.1. Add 120 µL of purifying buffer to the PCR product and vortex to mix. Briefly centrifuge to collect droplets inside the lid. Insert a DNA column into a 2 mL collection tube, transfer liquid to the prepared column and centrifuge at ≥13,000 x *g* for 1 min.

6.4.3.2. Discard the filtrate and replace the collection tube. Add 700 µL of DNA wash buffer and centrifuge at ≥13,000 x *g* for 1 min. Discard the filtrate and replace the collection tube. Repeat for a second wash step.

6.4.3.3. Centrifuge the empty column for 2 min to dry and transferring the column to a new 1.5 mL centrifuge tube. Add 50 µL of preheated elution buffer directly to the DNA column matrix, and incubate at room temperature for 2 min.

6.4.3.4. Centrifuge at 10,000 x *g* for 1 min to elute. Quantify the extracted DNA in filtrate via spectrophotometry or fluorometry.

6.4.4. Add 1 µg of purified PCR product to 5 µL of digestion buffer, 10 units RsaI, and molecular-grade water for a total reaction volume of 50 µL. Mix by pipetting up and down and incubate at 37 °C for 60 min.

6.4.5. Separate the digested product via gel electrophoresis by running 20 µL of digested DNA on a 2% agarose gel. Visualize the gel to confirm gnotobiotic status.

NOTE: Gnotobiotic insects should only result in bands at 402 bp, 201 bp, and a smear from 163 to 148 bp, based on the 16S rDNA sequence of the endosymbiont, *Blattabacterium*. Any extra bands seen in the gel are indicative of contaminating microbial species.

7. Aseptic tracking of nymphal growth

7.1. Record the body length to track nymphal growth by measuring the insects through the translucent BHI floor of the flask.

NOTE: Nymphs may be placed at 4 °C for 15 min to slow their movement, thereby making them easier to measure.

REPRESENTATIVE RESULTS:

Stock tanks are set up as depicted in **Figure 1**. "Pregnant" females are identified by the ootheca attached to the posterior abdomen, as pictured in **Figure 2**. Incubation of oothecae on BHI agar allows for gnotobiotic quality control in a nondestructive fashion. In some cases, sterilization is unsuccessful, and growth appears around the oothecae as in **Figure 3B**. These oothecae should be removed and discarded. In our hands, a 10% average failure rate was observed for sterilization (n = 51). The oothecae hatch an average of 34 days after sterilization without growth on the medium, as seen in **Figure 3A**. We have observed typical hatch rates of 41% (n = 46) for sterilized, noncontaminated oothecae, with an average of 11 nymphs per ootheca. **Figure 4** displays the rearing setup. The foil covering the flasks prevent contamination, while the secondary container allows air exchange. RFLP of the 16S rDNA from a homogenized nymph is used to confirm gnotobiotic status. **Figure 5** displays results from successfully gnotobiotic insects as well as standard (nonsterile) nymphs.

While this test has not yet identified contamination in the absence of a positive culture result, this step has been carried out routinely during critical experiments to rule out the presence of contaminating oxygen-sensitive or fastidious microbes. Slower growth has been observed in the gnotobiotic cockroaches when compared to standard/nonsterile insects.

FIGURE LEGENDS:

Figure 1: Cockroach stock culture setup. Cardboard tubes can be seen stacked in the far end of the tank. Food and water are both near the front of the tank. Cotton cloth cover and elastic band have been removed for visibility.

Figure 2: A "pregnant" American cockroach. The arrow indicates the ootheca.

Figure 3: Images of successfully gnotobiotic nymphs hatched and unsuccessfully sterilized oothecae on BHI slants. Oothecae were sterilized and incubated as described in this protocol. (**A**) The lack of microbial growth on the BHI slant indicates that the insects are free of culturable organisms. (**B**) Oothecae on slants that result in colony formation should be discarded as contaminated.

Figure 4: Gnotobiotic rearing apparatus. Insects are kept in sterile flasks covered with a foil lid to prevent contamination. The secondary container (green lid) is sterilized with 2% bleach followed by 70% ethanol. Air flow is not restricted in the secondary container.

Figure 5: A representative gel image of RFLP results for quality control. Whole-16S gene amplicons were digested with RsaI. DNA for PCR was extracted from nymphs homogenized in 1x PBS. "G nymph" lanes correspond to gnotobiotic nymphs, while "conv nymph" lanes correspond to conventional, nonsterile counterparts. Based on virtual restriction digest, the endosymbiont (*Blattabacterium*) is expected to have bands at the sizes 402 bp, 206 bp, and 163 bp, with a smear of bands between 163 bp and 148 bp. A gnotobiotic insect should show only the *Blattabacterium* banding pattern. A mixed bacterial community is expected to have a smear of bands with varying sizes, labeled here "other bacterial 16S fragments".

DISCUSSION:

Other methods describing generation of gnotobiotic cockroaches either did not describe oothecae collection or used benchmarks specific to other cockroach species to indicate when the oothecae could be removed from the mother[23,25,26]. Originally, oothecae were collected from the woodchip bedding in the stock tanks, resulting in very low hatch rates (~10%) compared to nonsterilized oothecae (47%)[29]. This is likely due to the fact that unhatched oothecae accumulate over time in the stock cage, and there is no way of verifying ootheca age or viability. Implementation of the "maternity ward" approach allows collection of freshly deposited oothecae of known age. This further facilitates experimental planning, as the researcher can anticipate likely hatch times for individual oothecae. Another modification from initial and published protocols includes the incubation of oothecae and nymphs in semi-sealed chambers also containing a supersaturated sodium chloride solution. The presence of the solution maintains a relative humidity of approximately 75%[30]. Oothecae is routinely incubated at 30 °C, which has been shown to minimize the number of days required for incubation while also maximizing the embryos' viability and number of nymphs produced per ootheca[31]. After hatching, gnotobiotic nymphs are routinely cultured on the benchtop at laboratory room temperature and ambient conditions, although humidity-controlled chambers are again utilized for critical experiments. After establishment of these changes to ootheca collection and incubation, hatch rates increased to approximately 41% (n = 51), not including oothecae removed due to contamination. A potential route to further optimization of hatch rates may include extending the time between ootheca collection and sterilization. The cuticle of the egg case may not be fully tanned on initial release[32], and therefore may be permeable to solutions used during sterilization within 24 h of being dropped.

The sterilization protocol using 0.1% peracetic acid was adapted from Doll et al.[25]. Other studies have documented alternative techniques for sterilizing oothecae[23,26]. Contamination rates are based on the nondestructive method of incubating the oothecae on a BHI slant. This approach is highly advantageous as it allows for quick identification and removal of contaminated oothecae. Most previous protocols test for culturable organisms by plating feces or nymph homogenate on bacteriological media and checking for growth[22,23,25,27,28,33]. In at least one case, the method for testing gnotobiotic status was not fully described[26]. Except Clayton who added a small slab of sterility testing medium to rearing bottles[24], previous methods[22,23] housed gnotobiotic insects on bacteriological media only for short periods of time to initially evaluate the sterilization protocol.

Continued housing of the resulting nymphs on BHI medium as a built-in quality-control measure allows their gnotobiotic status to be monitored in semi-real-time—a technique not seen in most previous methods[22,23]. This is especially useful for long-term experiments that require gnotobiotic nymphs to be accessed. If the BHI floor under nymphs appears contaminated with bacterial or fungal growth, the flask should be discarded. This type of contamination typically occurs when uncovering the flasks to water nymphs, but it may also arise from the feces in the case of insufficiently sterilized oothecae or food. The use of a laminar flow hood when watering improves the contamination rate caused by uncovering flasks.

As not all contaminating organisms may grow aerobically on BHI medium, an additional culture-independent method of sterility testing is required. One potential approach is microscopy[27], but this approach can be labor-intensive. Other protocols use sequence-based techniques to detect organisms that may escape culture[14,23,27,28]. However, such approaches are often expensive and hard to interpret, as the results of high-throughput sequencing approaches can easily be impacted by low-level contamination of reagents[34] and barcode hopping[35]. Instead, a new approach has been developed which uses PCR amplification of the 16S rRNA gene in combination with restriction fragment-length polymorphism to visualize both the endosymbiont and any contaminating gut symbionts. This technique includes an internal PCR control, since *Blattabacterium*'s 16S gene has been sequenced, and its banding pattern should be present in both gnotobiotic and nonsterile insects. As the endosymbiont's restriction pattern can be predicted from its genome sequence[36], there is no need to sequence the amplicons or the restriction fragments, unless identification of any contaminant is desired. The current version of this protocol calls for a nymph to be sacrificed for PCR/RFLP, but this technique could also be used on feces as a nondestructive measure. However, it will not include a built-in control, as the feces should not contain much *Blattabacterium*.

An additional easily modifiable yet critical component of rearing gnotobiotic animals is diet. While BHI agar can serve as a temporary food source for the insects, it has been found that it results in substantial growth deficits among nymphs when used as the sole food source for extended periods. While diverse diets have been tried, autoclavable rat chow is recommended as a routine diet for the maintenance of gnotobiotic insects. Diets not specifically formulated for sterilization were often difficult to render fully sterile, and many sterile or autoclavable laboratory animal diets were found to exhibit rapid fungal growth under nonsterile conditions. This tendency to degrade under nonsterile conditions rendered them unsuitable for use in

experiments directly comparing gnotobiotic and nongnotobiotic insects. The recommended diet allows for the use of a consistent diet between gnotobiotic and standard nymphs, facilitating comparison of characteristics—such as growth rates—between the two groups.

As others have observed[27], the gnotobiotic nymphs grow more slowly than their nonsterile counterparts. A comparison between body lengths of gnotobiotic (n = 105) and nonsterile nymphs (n = 50) fed the same, autoclaved rodent diet and kept at room temperature reveals that nonsterile nymphs grow an average of 0.059 mm/day, while gnotobiotic nymphs grow 0.028 mm/day ($p < 0.0001$). The presence of gut microbiota in *P. americana* has been shown to alter the insects' metabolic rate[37], and gut communities in general are thought to affect nutrient absorption[38,39]. These reasons support the observed differences in growth rate of gnotobiotic and nonsterile nymphs.

A possible limitation to this technique is that gnotobiotic nymphs may not reach sexual maturity, as the oldest sterile cohorts are more than 10 months old and have reached only the seventh instar (out of 10; 11 being adulthood) as approximated by body length[40]. These oldest cohorts are not on the autoclaved rat diet but instead eat irradiated rat chow, a diet that contains too much moisture to feed to nonsterile cohorts without excessive mold growth. Nonsterile nymphs on a nonsterilized dog food diet were found to reach adulthood after 9−10 months under laboratory conditions (room temperature and humidity). Cohorts of gnotobiotic and nonsterile nymphs on the shared, autoclaved rat chow are currently less than 7 months old, nonsterile insects are estimated to be seventh instar (average: 16.7 mm) while sterile insects are estimated to be fifth instar (average: 11.2 mm). As a result, we cannot, as of yet, verify whether our gnotobiotic cockroaches can successfully reproduce. However, given the ease with which new gnotobiotic cohorts can be established using this approach, this method shows great promise even in the absence of proven reproduction of gnotobiotic insects.

In conclusion, this protocol provides a versatile tool that allows microbiome researchers to operate their own, low-cost gnotobiotic "facility" using common laboratory materials. This approach may be used to generate gnotobiotic cockroaches for experiments examining the role of the microbiota in shaping host behavior, immunity, development, and stress responses[21,26,27]. These gnotobiotic insects may also be inoculated with either synthetic or xenobiotic communities and subsequently used as subjects for gut microbiome studies[23,28]. Further, elements of this approach, including the use of bacteriological media-lined incubation chambers as a built-in sterility check, are generalizable to other model systems and can facilitate routine maintenance of gnotobiotic animals in smaller-scale facilities.

ACKNOWLEDGMENTS:

This publication was supported by the National Institute of General Medical Sciences of the National Institutes of Health under award number R35GM133789. The content is solely the responsibility of the authors and does not necessarily represent the official view of the National Institutes of Health. The authors would like to acknowledge Josey Dyer for tracking sterilization rates, hatch rates, and growth rates of the gnotobiotic cockroaches.

DISCLOSURES:

The authors have no conflicts of interest to disclose.

REFERENCES:

1. Yi, P., Li, L. The germfree murine animal: An important animal model for research on the relationship between gut microbiota and the host. *Veterinary Microbiology.* **157** (1-2), 1-7 (2012).
2. Laying better plans for mice. *Nature Biotechnology.* **31** (4), 263-263 (2013).
3. Nicklas, W., Keubler, L., Bleich, A. Maintaining and Monitoring the Defined Microbiota Status of Gnotobiotic Rodents. *ILAR Journal.* **56** (2), 241-249 (2015).
4. Faith, J. J., Ahern, P. P., Ridaura, V. K., Cheng, J., Gordon, J. I. Identifying Gut Microbe-Host Phenotype Relationships Using Combinatorial Communities in Gnotobiotic Mice. *Science Translational Medicine.* **6** (220), 220ra11 (2014).
5. Moon, C. et al. Vertically transmitted faecal IgA levels determine extra-chromosomal phenotypic variation. *Nature*. **521** (7550), 90-93 (2015).
6. Eun, C. S. et al. Induction of Bacterial Antigen-Specific Colitis by a Simplified Human Microbiota Consortium in Gnotobiotic Interleukin-10-/- Mice. *Infection and Immunity*. **82** (6), 2239-2246 (2014).
7. Cherbuy, C. et al. Microbiota matures colonic epithelium through a coordinated induction of cell cycle-related proteins in gnotobiotic rat. *American Journal of Physiology: Gastrointestinal and Liver Physiolog*y. **299** (2), G348-G357 (2010).
8. Van Den Abbeele, P. et al. Arabinoxylans and inulin differentially modulate the mucosal and luminal gut microbiota and mucin-degradation in humanized rats. *Environmental Microbiology.* **13** (10), 2667-2680 (2011).
9. Stecher, B., Berry, D., Loy, A. Colonization resistance and microbial ecophysiology: using gnotobiotic mouse models and single-cell technology to explore the intestinal jungle. *FEMS Microbiology Reviews.* **37** (5), 793-829 (2013).
10. Sugahara, H. et al. Probiotic Bifidobacterium longum alters gut luminal metabolism through modification of the gut microbial community. *Scientific Reports.* **5** (1), 13548 (2015).
11. Martín, R., Bermúdez-Humarán, L. G., Langella, P. Gnotobiotic Rodents: An In Vivo Model for the Study of Microbe–Microbe Interactions. *Frontiers in Microbiology*. **7**, 409 (2016).
12. Mallapaty, S. Gnotobiotics: getting a grip on the microbiome boom. *Lab Animal.* **46** (10), 373-377 (2017).
13. Nguyen, T. L. A., Vieira-Silva, S., Liston, A., Raes, J. How informative is the mouse for human gut microbiota research? *Disease Models & Mechanisms.* **8** (1), 1-16 (2015).
14. Correa, M. A., Matusovsky, B., Brackney, D. E., Steven, B. Generation of axenic Aedes aegypti demonstrate live bacteria are not required for mosquito development. *Nature Communications.* **9** (1), 4464 (2018).
15. Trinder, M., Daisley, B. A., Dube, J. S., Reid, G. Drosophila melanogaster as a High-Throughput Model for Host–Microbiota Interactions. *Frontiers in Microbiology.* **8**, 751 (2017).
16. Zheng, H., Steele, M. I., Leonard, S. P., Motta, E. V. S., Moran, N. A. Honey bees as models for gut microbiota research. *Lab animal.* **47** (11), 317-325 (2018).
17. Engel, P., Moran, N. A. The gut microbiota of insects – diversity in structure and function. *FeMS Microbiology Reviews.* **37** (5), 699-735 (2013).

18. Tinker, K. A., Ottesen, E. A. The Core Gut Microbiome of the American Cockroach, Periplaneta americana, Is Stable and Resilient to Dietary Shifts. *Applied and Environmental Microbiology.* **82** (22), 6603-6610 (2016).
19. Zurek, L., Keddie, B. A. Contribution of the colon and colonic bacterial flora to metabolism and development of the american cockroach Periplaneta americana L. *Journal of Insect Physiology.* **42** (8), 743-748 (1996).
20. Cruden, D. L., Markovetz, A. J. Microbial aspects of the cockroach hindgut. *Archives of Microbiology.* **138**, 131-139 (1984).
21. Pietri, J. E., Tiffany, C., Liang, D. Disruption of the microbiota affects physiological and evolutionary aspects of insecticide resistance in the German cockroach, an important urban pest. *PLoS ONE.* **13** (12), e0207985 (2018).
22. Benschoter, C., Wrenn, R. Germfree techniques for establishment and maintenance of a colony of aseptic German cockroaches. *Annals of the Entomological Society of America.* **65** (3), 641-644 (1972).
23. Tegtmeier, D., Thompson, C. L., Schauer, C., Brune, A. Oxygen Affects Gut Bacterial Colonization and Metabolic Activities in a Gnotobiotic Cockroach Model. *Applied and Environmental Microbiology.* **82** (4), 1080-1089 (2016).
24. Clayton, R. A simplified method for the culture of *Blattella germanica* under aseptic conditions. *Nature.* **184** (4693), 1166-1167 (1959).
25. Doll, J. P., Trexler, P. C., Reynolds, L. I., Bernard, G. R. The Use of Peracetic Acid to Obtain Germfree Invertebrate Eggs for Gnotobiotic Studies. *American Midland Naturalist.* **69** (1), 231 (1963).
26. Wada-Katsumata, A. et al. Gut bacteria mediate aggregation in the German cockroach. *Proceedings of the National Academy of Science.* **112**, 15678–15683 (2015).
27. Jahnes, B. C., Herrmann, M., Sabree, Z. L. Conspecific coprophagy stimulates normal development in a germ-free model invertebrate. *PeerJ.* **7**, e6914 (2019).
28. Mikaelyan, A., Thompson, C. L., Hofer, M. J., Brune, A. Deterministic Assembly of Complex Bacterial Communities in Guts of Germ-Free Cockroaches. *Applied Environmental Microbiology.* **82** (4), 1256-1263 (2016).
29. Katoh, K. et al. Group-housed females promote production of asexual ootheca in American cockroaches. *Zoological Letters.* **3**, 3 (2017).
30. Greenspan, L. Humidity fixed points of binary saturated aqueous solutions. *Journal of Research of the National Bureau of Standards.* **81A** (1), 89-96 (1976).
31. Bressan-Nascimento, S., Oliveira, D. M. P., Fox, E. G. P. Thermal requirements for the embryonic development of Periplaneta americana (L.) (Dictyoptera: Blattidae) with potential application in mass-rearing of egg parasitoids. *Biological Control.* **47** (3), 268-272 (2008).
32. Nation, J. L. *Insect Physiology and Biochemistry, Second Edition*. Taylor & Francis. Philadelphia, PA (2008).
33. House, H. L. Nutritional studies with *Blattella germanica* (L.) reared under aseptic conditions I. Equipment and technique. *The Canadian Entomologist.* **81** (5), 94-100 (1949).
34. Stinson, L. F., Keelan, J. A., Payne, M. S. Identification and removal of contaminating microbial DNA from PCR reagents: impact on low-biomass microbiome analyses. *Letters in Applied Microbiology.* **68** (1), 2-8 (2018).

35. Kircher, M., Sawyer, S., Meyer, M. Double indexing overcomes inaccuracies in multiplex sequencing on the Illumina platform. *Nucleic Acids Research.* **40**, e3 (2011).
36. Sabree, Z. L., Kambhampati, S., Moran, N. A. Nitrogen recycling and nutritional provisioning by Blattabacterium, the cockroach endosymbiont. *Proceedings of the National Academy of Sciences of the United States of the America.* **106** (46), 19521-19526 (2009).
37. Ayayee, P. A., Ondrejech, A., Keeney, G., Muñoz-Garcia, A. The role of gut microbiota in the regulation of standard metabolic rate in female Periplaneta americana. *PeerJ.* **6**, e4717-e4717 (2018).
38. Krajmalnik-Brown, R., Ilhan, Z.-E., Kang, D.-W., DiBaise, J. K. Effects of gut microbes on nutrient absorption and energy regulation. *Nutrition in clinical practice : official publication of the American Society for Parenteral and Enteral Nutrition.* **27** (2), 201-214 (2012).
39. Rowland, I. et al. Gut microbiota functions: metabolism of nutrients and other food components. *European Journal of Nutrition.* **57** (1), 1-24 (2018).
40. Gier, H. T. Growth rate in the cockroach *Periplaneta americana* (Linn). *Annals of the Entomological Society of America.* **40**, 303-317 (1947).

Figure 1

Click here to access/download;Figure;Fig1.ai

lick here to access/download;Figure;Fig2.ai

Figure 2

Figure 4

Click here to access/download;Figure;Fig4.ai

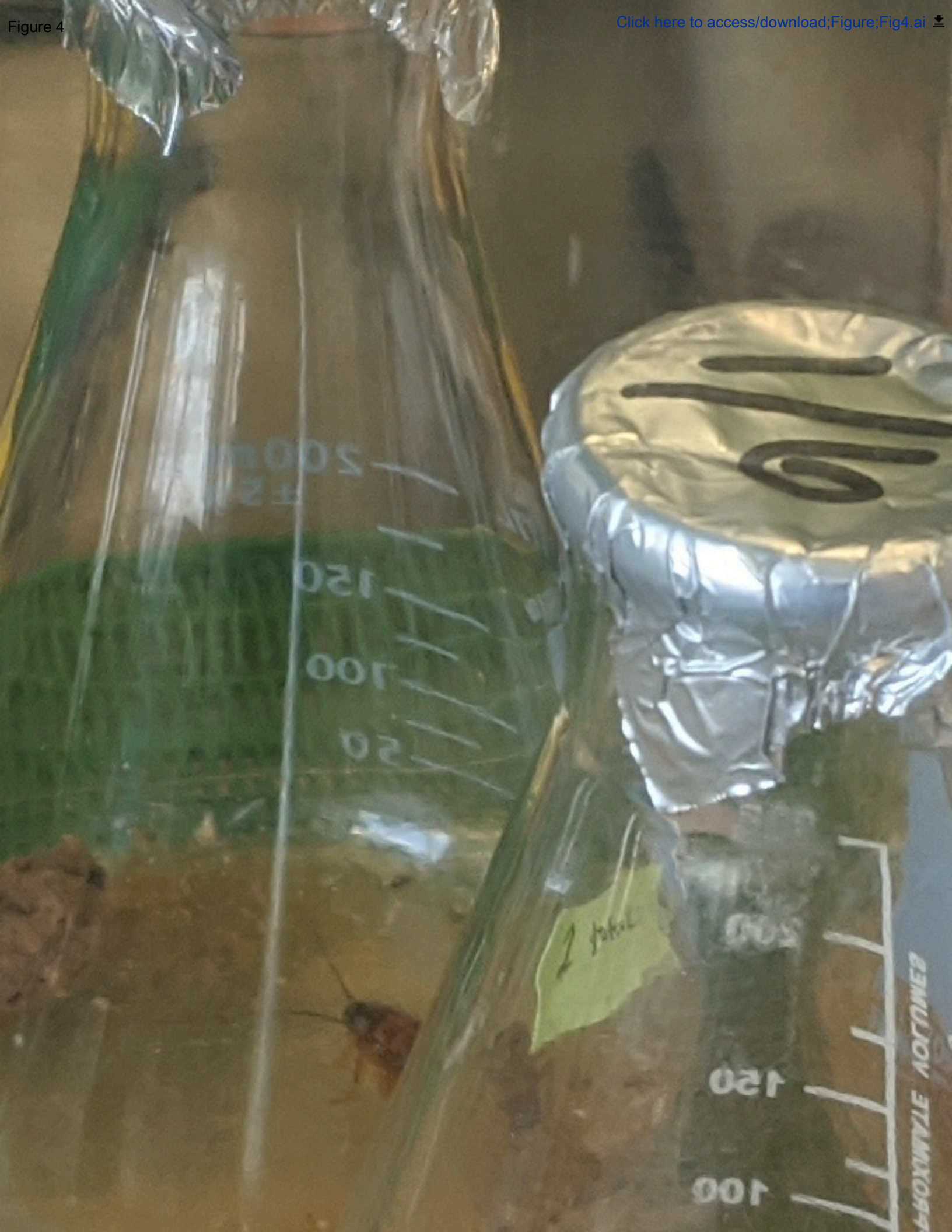

Figure 5

Click here to access/download;Figure;Fig5.ai

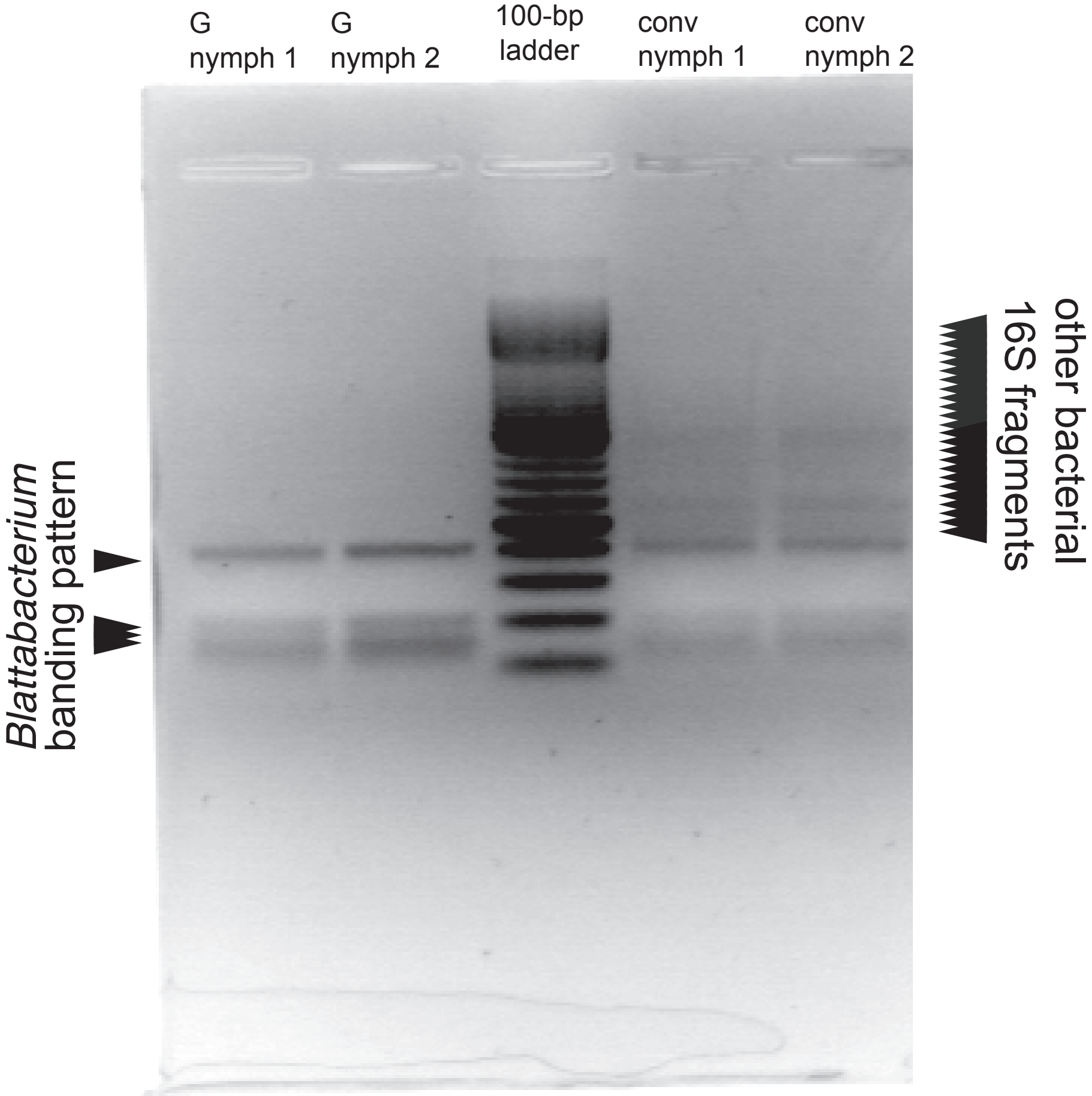

Figure 3

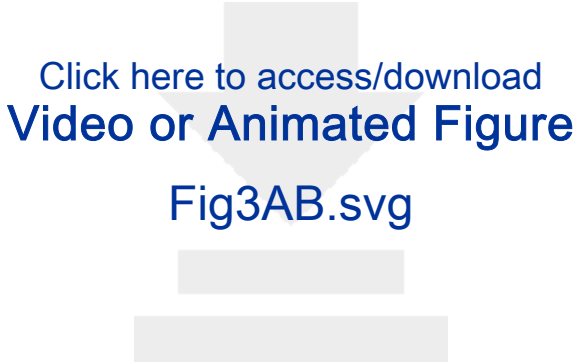
Click here to access/download
Video or Animated Figure
Fig3AB.svg

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials.xls

Name of Material/Equipment	Company	Catalog Number	Comments/Description
2X master mix	New England BioLabs	M0482	OneTaq MasterMix
Autoclavable rat chow	Zeigler	NIH-31 Modified Auto	
Bacterial DNA extraction kit	Omega Bio-Tek	D-3350	E.Z.N.A. Bacterial DNA kit; includes lysozyme, glass beads, proteinase K, buffers (proteinase K, binding, wash, elution), DNA columns, 2-mL collection tube
binding buffer	Omega Bio-Tek	PD099	included in Omega Biotek's bacterial DNA extraction kit ("HBC" buffer)
brain-heart infusion (BHI) broth	Research Products International	B11000	
Delicate task wipes	KimWipe	JS-KCC-34155-PK	KimWipes
DNA purification kit	Omega Bio-Tek	D6492	E.Z.N.A. Cycle Pure kit; D6493 may also be used; includes buffers (purifying ,wash, elution)
elution buffer	Omega Bio-Tek	PDR048	included in Omega Biotek's bacterial DNA extraction kit
glass beads	Omega Bio-Tek	n/a	included in Omega Biotek's bacterial DNA extraction kit
Hybridization oven	UVP	95-0330-01	we use a hybridization oven for preheating elution buffer, but a water bath could probably also be used
Laminar flow biological safety cabinet	NuAire, Inc.	NU-425-400	Protocol refers to this as "laminar flow hood" for brevity
lysozyme	Omega Bio-Tek	n/a	included in Omega Biotek's bacterial DNA extraction kit
peracetic acid stock solution (32%)	Sigma-Aldrich	269336	
Petroleum jelly	Vaseline	n/a	
proteinase K buffer	Omega Bio-Tek	PD061	included in Omega Biotek's bacterial DNA extraction kit ("TL buffer")

purifying buffer	Omega Bio-Tek	PDR042	included in Omega Biotek's CyclePure kit ("CP" buffer)
RsaI	New England BioLabs	R0167	Includes CutSmart (digestion) buffer
Secondary container	n/a	n/a	a plastic container with a lid (such as a Kritter Keeper) works well for this (25cm long x 15cm wide x 22cm high); it should be large enough to fit BHI slants and test tubes
spectrophotometer	ThermoFisher	ND-2000	Catalog info is for NanoDrop2000
thermal shaker	Eppendorf	EP5386000028	Thermomixer R
Tris-EDTA	Fisher	BP1338-1	10 nm Tris, 1 mM EDTA, pH 8
wash buffer	Omega Bio-Tek	PDR044	included in Omega Biotek's bacterial DNA extraction kit ("DNA wash" buffer)
Woodchip bedding	P.J. Murphy Forest Products	Sani-Chips	

Rebuttal Letter
Click here to access/download;Rebuttal Letter;Rebuttal_FINAL.docx

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
> Proofread completed!

2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Kimwipe, Omega Bio-Tek, NanoDrop, Qubit, OneTaq, E.Z.N.A. Cycle Pure, CutSmart, etc.
> These commercial products were renamed with generic terms.

3. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.
> Details added to corresponding lines

4. 1.1.4: Please specify the age, male/female number/ratio of cockroaches added.
> The text now reads:"Add cockroaches, typically 100-250 mixed-age, mixed-sex cockroaches. Add dog food (20-30 pieces). Monitor the amount of dog food in the tank and refill when low"

5. 2: How do you identify females carrying oothecae? How many females are transferred? How do you transfer them?
> As many females as have eggs may be transferred. The text now reads: "Transfer any number of females carrying oothecae (see Figure 2) from the stock tank to the "maternity ward" using forceps to move cardboard tubes that contain gravid females."

6. 2.1: How do you pick up the oothecae? With what tools?
> The text now reads: "Transfer females back to the stock tank once they have dropped their oothecae, and retrieve the oothecae from the litter in the tank with forceps."

7. 5.2: How many oothecae per tube?
> The text now reads: "Place (up to five) cleaned oothecae in the 0.1% peracetic acid solution for 5 min. Invert the tube several times every 60 seconds."

8. 5.5: Please specify the temperature of the incubator.
> Temperature added. Now reads: "Place slants in sterilized secondary container. Move container into humidified incubator at 30°C for 4-5 weeks until hatched"

9. 6.1: Growth of what?
> Now reads: "fungal or bacterial colony growth"

10. 7.4.2 and substeps: Please ensure that the protocol here can stand alone. As currently written, users must refer to another protocol and refer back and forth in order to complete this protocol. Please remove the references to the specific steps of the other manual.
> The references to the kit protocols were replaced with stand-alone descriptions (lines 464-542, 553-629)

11. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.
> Steps were combined throughout the whole protocol (lines 129-647)

12. After you have made all the recommended changes to your protocol section (listed above), please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text.
> Highlighted lines: 288-452

13. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the highlighting.
> This was adjusted. See highlighted lines 288-452

14. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.
> Highlighting was altered to follow these directions (lines 288-452)

15. Please reference Figure 1 and Figure 2 in the protocol section.
> Added references to these figures in lines 165, 276, and 287

16. References: Please do not abbreviate journal titles; use full journal name.
> The references were changed to include the full journal name.

17. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the materials alphabetically by material name.
> This was updated to include all relevant materials and sorted by alphabetical order.

Reviewers' comments:
Reviewer #1:

Manuscript Summary:
This manuscript describes a protocol for producing and maintaining American cockroaches that have axenic guts. Although several previous studies have generated gnotobiotics of several cockroach species, including P. americana, the methods for doing so have not been thoroughly described. In addition, the particular protocol presented in this manuscript contains several key advances over previously used methods, most notably in the extensive and ongoing monitoring of sterility using agar and PCR-RFLP. Therefore, this manuscript will be quite important and useful for the field. Overall the paper is well-written and contains detailed descriptions which, along with video, should enable use by others. This is a neat piece of work, a great fit for JOVE, and I do not have any major concerns about the paper, but some items would be good to address prior to publication.

Major Concerns:
None

Minor Concerns:
Abstract:
Lines 32-33: This wording is a bit confusing. Based on reading the full manuscript I am assuming the meaning is they are incubated until they hatch, which takes 4 to 6 weeks, but this could also be interpreted as an either-or situation (i.e. because some oothecae don't hatch and are discarded after 4-6 weeks). Please clarify.
> Wording changed to: "Once collected and sterilized, the oothecae are incubated at 30□C for approximately 4-6 weeks on Brain-Heart Infusion agar (BHI) until they hatch or are removed due to contamination"

Introduction:
This section is adequate, but I do have several minor suggestions for improvement.
-The first two paragraphs of the introduction focus on the application of gnotobiotic cockroaches as a model for higher organisms, which is no doubt important. However, there is also increasing interest in understanding the effects of the microbiota on the fundamental physiology of insect pests, which can have many implications for pest control and management. There is no mention of the significance of gnotobiotic cockroaches from this perspective, which I believe merits at least a few sentences in the introduction.
> A short paragraph was added to include this perspective: "American cockroaches can be a useful model system for understanding gut microbial communities in higher organisms, but the cockroach's status as a pest also makes this system relevant for pest control21. Leveraging fundamental knowledge of the gut community's influence on cockroach health and physiology assists in developing new techniques for pest management."

-The last paragraph of the introduction accurately mentions some issues with previous publications that have generated gnotobiotic cockroaches. However, I note that the fairly recent Jahnes et al. 2019 paper is not mentioned here, though it is cited later in the discussion as reference 27. Since the Jahnes et al. paper also involved American cockroaches and did include some quality control measures and descriptions of their protocol, it would be helpful to also discuss differences/advances over this paper in the introduction as well.

> This section was heavily edited to improve the description of previous protocols: "The goal of this method is to outline a comprehensive description of the establishment and maintenance of gnotobiotic American cockroaches (Periplaneta americana), but this protocol could be used to generate nymphs of any oviparous cockroach. It includes a method for efficient, non-invasive collection of mature oothecae, and a non-destructive technique to monitor gnotobiotic status of the insects[22-24]. While previous methods of achieving and maintaining gnotobiotic cockroaches describe oothecae collection[23-27], ootheca maturity is either interpreted in terms of species-specific cues (in Blattella germanica), or not at all[27], increasing the likelihood that oothecae are removed and sterilized prematurely, thereby lowering hatch rate. Since the method described here uses naturally dropped oothecae, the error of removing eggs prematurely is absent.
Most previous protocols test for culturable organisms by plating feces or nymph homogenate bacteriological media and checking for growth[22,23,25,27-29]. In at least one case, the method for testing gnotobiotic status was not fully described[26]. Except Clayton—who added a small slab of sterility testing medium to rearing bottles[24]—previous methods[22,23] housed gnotobiotic insects on bacteriological media only for short periods of time to initially evaluate the sterilization protocol. The following method, however, maintains the hatched nymphs on BHI long-term for a continuous method of monitoring.
As for culture-independent sterility testing, microscopy is one option [27]. Jahnes et al. used a DAPI stain and microscopic examination of the gut. While other protocols use sequence-based techniques to detect organisms that may escape culture[14,23,27,28], the use of whole-16S PCR in combination with a restriction digest (RFLP) to visualize both the endosymbiont and any contaminating gut symbionts is new. This technique includes a PCR control, since Blattabacterium's 16S gene has been sequenced, and its known banding pattern should be present in both gnotobiotic and non-sterile insects. There is no need to sequence the amplicons nor fragments, unless the contaminant's identity is necessary. The current version of this protocol calls for a nymph to be sacrificed for PCR/RFLP, but this technique could be used on feces as a non-destructive measure—however it will not include a built-in control, as the feces should not contain much Blattabacterium. This protocol contains both culture-dependent and -independent methods of quality control, and the culture-dependent method does not require sacrificing insects. Finally, this method brings together information from multiple gnotobiotic cockroach studies to create one, comprehensive protocol with all necessary information for achieving and maintaining gnotobiotic cockroaches."

Protocol:
Line 148: What standard precautions are taken to ensure sterility during storage? Just kept covered with foil? This should be sufficient but non-microbiologists may want/need more information.
> Yes, just foil. A few lines were added to explicitly state that no filter is used for air exchange: "Sterilize secondary container. This container does not include a filter but instead allows free air exchange." and "NOTE: There is no air filter for this setup. The foil is sufficient in preventing contamination from open air flow."

Line 152: Are rat chow pelleted autoclaved whole, or broken up at all?
> We typically break up the rat chow, since the insects don't need very much of it. This was

added in the protocol: "Sterilize via autoclave: rat chow broken into half-sizes (approx. 1/2-inch pieces) in a foil-covered beaker"

Line 153: typo? "will" instead of "with"
> Typo corrected.

Line 222: Is the secondary container ventilated/filtered in any special way, or is just keeping the tubes covered with foil in the secondary container in the incubator sufficient to prevent contamination?
> (see above comment from line 148)

Lines 313-314: This is consistent with results in Jahnes et al, which supports the success of the protocol. I would suggest citing that study here.
> A citation for Jahnes et al. was added instead in the discussion: "As others have observed[27], the gnotobiotic nymphs grow more slowly than their non-sterile counterparts."

Discussion:
I have a few inquiries that could perhaps be address in the discussion or detailed elsewhere as the authors see fit.
- Regarding the deposition of feces in the agar, have the authors noted any microbial growth from feces at all? Similarly, have the authors tried their PCR assay on feces?
> We have not noted growth that we can say came from the feces, but if growth (of any origin) is found in the flask, that flask is removed and the cohort discarded. We do not typically use the PCR assay on feces, but that is a good, non-destructive use of that assay! These concerns were addressed in the introduction: "While other protocols use sequence-based techniques to detect organisms that may escape culture[14,23,27,28], the use of whole-16S PCR in combination with a restriction digest (RFLP) to visualize both the endosymbiont and any contaminating gut symbionts is new. This technique includes a PCR control, since Blattabacterium's 16S gene has been sequenced, and its known banding pattern should be present in both gnotobiotic and non-sterile insects. There is no need to sequence the amplicons nor fragments, unless the contaminant's identity is necessary. The current version of this protocol calls for a nymph to be sacrificed for PCR/RFLP, but this technique could be used on feces as a non-destructive measure—however it will not include a built-in control, as the feces should not contain much Blattabacterium." and discussion: "Housing the resulting nymphs on the medium as a built-in quality-control measure also allows their gnotobiotic status can be monitored in semi-real-time—a technique not seen in previous methods[23]. This is especially useful for long-term experiments that require gnotobiotic nymphs to be accessed. If the BHI floor under nymphs appears contaminated with bacterial or fungal growth, the flask should be discarded. This type of contamination typically occurs when uncovering the flasks to water nymphs, but it may also arise from the feces in the case of insufficiently sterilized oothecae. The laminar flow hood improves the contamination rate caused by uncovering flasks."

-An aspect of the manuscript that is somewhat lacking is the description of the origin and verification of the PCR RFLP assay. While the purpose of this assay is clear and the protocol is adequately described, a few things are unclear. Did the authors design this assay

themselves or was this adapted from others? Have the resulting fragments been sequenced at all to verify identities?
> We designed this assay ourselves. Fragments were not sequenced, as they followed the banding pattern as expected based on the published Blattabacterium *whole-16S sequence. Fragments from contaminating bacteria have also not been sequenced.*

Reviewer #2:
Manuscript Summary:
This is a timely and very useful contribution. It nicely integrates procedures from disparate sources into a unified protocol.

Minor Concerns:
1. To extend the utility of this approach, it would be useful to add a statement that this procedure can be used for all oviparous cockroaches (those that drop the ootheca soon after it is formed). For Blattella germanica things are a bit more complicated because the sterilization procedure must be done just before hatching, about 3 weeks after the egg case is formed.
*> Great idea! Now reads: "The goal of this method is to outline a comprehensive description of the establishment and maintenance of gnotobiotic American cockroaches (*Periplaneta americana*), but this protocol could be used to generate nymphs of any oviparous cockroach."*

2. L71: "pickier eaters" is rather non-scientific. Perhaps "diet specialists"?
> Corrected as suggested

3. L81-82: The statement "In at least one case, the gnotobiotic status was not tested" seems out of context. That study concentrated on sterility of feces and confirmed that feces of axenic cockroaches were sterile: "Although aerobic bacteria were found in the control feces, none were detected in the feces of the axenic cockroaches."
> This statement was tempered to say "not fully described" rather than "not tested", as the method of detection for aerobic bacteria was not stated by [26] (line 104)

4. L93+: It might be useful to note that there are many ways to rear cockroaches without compromising the effectiveness of the following procedures. For example, toilet paper tubes are not easy to come by and egg cartons can serve just as well or even better because all insects are readily visible and reachable. Likewise, water can be provided in test tubes stoppered with absorbent cotton.
> A note was added for this: "There are many ways to rear these robust insects. The specifics on providing shelter and water can be different depending on accessible materials (i.e. egg cartons instead of cardboard tubes). The following sterilization protocol will work for any stock tank setup."

5. L152: In my experience, autoclaving rat chow drastically diminishes the nutritional quality of the diet. Perhaps this explains why the gnotobiotic insects have slower development (L313-314).

> Yes, a comparison of growth rates between gnotobiotic and non-sterile insects was expanded in the discussion to remove the difference in nutritional quality of autoclaved rat chow: "As others have observed[27], the gnotobiotic nymphs grow more slowly than their non-sterile counterparts. A comparison between body lengths of gnotobiotic (n = 105) and non-sterile nymphs (n = 50) fed the same, autoclaved rodent diet and kept at room temperature reveals that non-sterile nymphs grow an average of 0.059 mm/day, while gnotobiotic nymphs grow 0.028 mm/day ($p < 0.0001$). The presence of gut microbiota in P. americana has been shown to alter the insects' metabolic rate[33], and gut communities in general are thought to affect nutrient absorption[34,35]. These reasons support our observed differences in growth rate of gnotobiotic and non-sterile nymphs.
A possible limitation to this technique is that gnotobiotic nymphs may not reach adulthood, as our oldest sterile cohorts are 10+ months old and have reached the seventh instar as approximated by body length[36]. These oldest cohorts are not on the autoclaved rat diet but instead eat irradiated rat chow, a diet that contains too much moisture to feed to non-sterile cohorts without excessive mold growth. Non-sterile nymphs on a non-sterilized dog food diet were found to reach adulthood after 9-10 months. Cohorts of gnotobiotic and non-sterile nymphs on the shared, autoclaved rat chow are currently less than 7 months old, non-sterile insects are estimated to be seventh instar (average: 16.7 mm) while sterile insects are estimated to be fifth instar (average: 11.2 mm). As a result, we cannot, as of yet, verify whether our gnotobiotic cockroaches can successfully reproduce. However, given the ease with which new gnotobiotic cohorts can be established using this approach, we feel that this method shows great promise even in the absence of proven reproduction of gnotobiotic insects."

6. L215: Remove "at" or add temperature setting.
> Inserted temperature setting (line 386)

7. L308: "per ootheca"
> Corrected (line 659)

8. L346+: The protocol sterilizes newly dropped oothecae and results in acceptable but rather low success rate (10% failed sterilization, 41% hatching success of the remaining 90%). Since the oothecae are aged from day 0 (drop day), I wonder if success rate will be higher if the sterilization was delayed, say 5 days. Freshly dropped egg cases may still not be fully tanned (the case itself is made of secreted proteins), and thus adversely affected by the SDS and acid treatments.
> Good tip! This thought was added to the discussion: "A potential route to further optimization of hatch rates may include extending the time between ootheca collection and sterilization. The cuticle of the egg case may not be fully tanned on initial release[32], and therefore may be permeable to solutions used during sterilization within 24 hours of being dropped."

9. L382: "sexual maturity" is different from "adult stage". Cockroaches reach the adult stage and then undergo several days of sexual maturation.
> This was overlooked; thank you for pointing out the difference. "Sexual maturity" was replaced with "adult stage".

10. L383: The 1942 paper on nymphal development likely overestimated nymphal development compared to more recent papers. No adults after 7+ months at 30C does indeed suggests slower development. However, it is not clear from this write-up how the control group was treated. The control cockroaches should be maintained on the autoclaved diet to account for deficiencies in the diet due to autoclaving, which will then reveal the effects of lacking the gut microbiome. A second control group on normal diet would reveal whether the autoclaved diet is deficient.
> More details were added in this section: "As others have observed[27], the gnotobiotic nymphs grow more slowly than their non-sterile counterparts. A comparison between body lengths of gnotobiotic (n = 105) and non-sterile nymphs (n = 50) fed the same, autoclaved rodent diet and kept at room temperature reveals that non-sterile nymphs grow an average of 0.059 mm/day, while gnotobiotic nymphs grow 0.028 mm/day ($p < 0.0001$). The presence of gut microbiota in P. americana has been shown to alter the insects' metabolic rate[33], and gut communities in general are thought to affect nutrient absorption[34,35]. These reasons support our observed differences in growth rate of gnotobiotic and non-sterile nymphs.
A possible limitation to this technique is that gnotobiotic nymphs may not reach adulthood, as our oldest sterile cohorts are 10+ months old and have reached the seventh instar as approximated by body length[36]. These oldest cohorts are not on the autoclaved rat diet but instead eat irradiated rat chow, a diet that contains too much moisture to feed to non-sterile cohorts without excessive mold growth. Non-sterile nymphs on a non-sterilized dog food diet were found to reach adulthood after 9-10 months. Cohorts of gnotobiotic and non-sterile nymphs on the shared, autoclaved rat chow are currently less than 7 months old, non-sterile insects are estimated to be seventh instar (average: 16.7 mm) while sterile insects are estimated to be fifth instar (average: 11.2 mm). As a result, we cannot, as of yet, verify whether our gnotobiotic cockroaches can successfully reproduce. However, given the ease with which new gnotobiotic cohorts can be established using this approach, we feel that this method shows great promise even in the absence of proven reproduction of gnotobiotic insects."

Reviewer #3:
Manuscript Summary:
The authors present a manuscript detailing a procedure for rearing and validating gnotobiotic Periplaneta americana. The use of gnotobiotic animals is extremely important for a better understanding of this topic and to facilitate, cheapen and improve this technique is very important for the field. While the authors should more exhaustively cite historical research on the subject, their modifications to existing procedures for incubation of ootheca and cockroach rearing on an agar bed is a valuable contribution to the work of germ-free and gnotobiotic insect rearing.

Major Concerns:
Lines 28,29: While use of the term gnotobiotic is technically correct here, as germ-free animals are considered to be gnotobiotic, it may be clearer to use the terms "germ-free" or "axenic" to describe the cockroaches generated in this work. In common usage, gnotobiotic is often employed in reference to artificially colonized animals and germ-free is used to

indicate the axenic or baseline state of the same organism. In the case of cockroaches, Blattabacterium and the cockroach are indivisible, neither existing permanently on its own, and may be considered an integral feature of the cockroach fat body, not a horizontally transmissible bacterium. As such, the cockroach plus Blattabacterium is the baseline state for the organism. "Aposymbiotic cockroach" describes Blattabacterium free cockroaches. So, a useful descriptive continuum would be aposymbiotic (no bacteria/microbes), germ-free (no free-living bacteria/microbes), and gnotobiotic (artificially introduced and defined gut microbial community).

> In preparing this manuscript, we extensively debated the appropriate terminology for our cockroaches. In the end, as insect endosymbionts are routinely discussed as obligately symbiotic but separate organisms, we were uncomfortable referring to the insects generated by our protocol as axenic, and felt that the term 'germ-free' was overly vague. Instead, we felt the appropriate approach was to use the term 'gnotobiotic', but to explicitly define our usage of it prominently in the article summary and abstract to minimize reader confusion.

Lines 62-64: The authors made no explicit reference at any time to studies using honeybees, mosquitoes, fruit flies (a major model insect used in geerm-free work) or other insect models where similar methods for quality control have been applied.

> References to other common model insects were added to the introduction: "Insect models are cheaper alternative due to their lower cost-of-maintenance in comparison to mammalian species. Extensive germ-free and gnotobiotic research in a variety of insect species has led to a vast collection of knowledge available on different sterilization and rearing methods. The different species used depend on the application of the given research. Mosquitos and Drosophila are common models for germ-free work due to their relevance to global diseases and their genetic tractability[14,15]. Another emerging model system is that of the honey bee (Apis mellifera), given its importance in pollination and sociality research[16]. However, many of these commonly used insects lack the taxonomic complexity seen in mammalian gut communities[17], limiting their ability to model higher order interactions"

Lines 74,75: The novelty of incubating ootheca on bacterial growth media needs to be tempered and qualified. Benschoter and Wrenn (1972) detail a procedure for rearing aseptic Blattella germanica which includes incubation of ootheca and rearing nymphs for up to two weeks on trypticase soy agar to ensure asepsis. The authors of this manuscript should be careful not to overlook this historical achievement.

> Thank you for calling this to our attention!. The introduction and discussion were edited to more thoroughly describe historic work: "The goal of this method is to outline a comprehensive description of the establishment and maintenance of gnotobiotic American cockroaches (Periplaneta americana), but this protocol could be used to generate nymphs of any oviparous cockroach. It includes a method for efficient, non-invasive collection of mature oothecae, and a non-destructive technique to monitor gnotobiotic status of the insects[22-24]. While previous methods of achieving and maintaining gnotobiotic cockroaches describe oothecae collection[23-27], ootheca maturity is either interpreted in terms of species-specific cues (in Blattella germanica), or not at all[27], increasing the likelihood that oothecae are removed and sterilized prematurely, thereby lowering hatch rate. Since the method described here uses naturally dropped oothecae, the error of removing eggs prematurely is absent.

Most previous protocols test for culturable organisms by plating feces or nymph homogenate bacteriological media and checking for growth[22,23,25,27-29]. In at least one case, the method for testing gnotobiotic status was not fully described[26]. Except Clayton—who added a small slab of sterility testing medium to rearing bottles[24]—previous methods[22,23] housed gnotobiotic insects on bacteriological media only for short periods of time to initially evaluate the sterilization protocol. The following method, however, maintains the hatched nymphs on BHI long-term for a continuous method of monitoring.
As for culture-independent sterility testing, microscopy is one option [27]. Jahnes et al. used a DAPI stain and microscopic examination of the gut. While other protocols use sequence-based techniques to detect organisms that may escape culture[14,23,27,28], the use of whole-16S PCR in combination with a restriction digest (RFLP) to visualize both the endosymbiont and any contaminating gut symbionts is new. This technique includes a PCR control, since Blattabacterium's 16S gene has been sequenced, and its known banding pattern should be present in both gnotobiotic and non-sterile insects. There is no need to sequence the amplicons nor fragments, unless the contaminant's identity is necessary. The current version of this protocol calls for a nymph to be sacrificed for PCR/RFLP, but this technique could be used on feces as a non-destructive measure—however it will not include a built-in control, as the feces should not contain much Blattabacterium. This protocol contains both culture-dependent and -independent methods of quality control, and the culture-dependent method does not require sacrificing insects. Finally, this method brings together information from multiple gnotobiotic cockroach studies to create one, comprehensive protocol with all necessary information for achieving and maintaining gnotobiotic cockroaches."

Lines 77: Jahnes et al. 2019 describes, "[o]otheca were manually detached from gravid P. americana females..."
> Yes, however there is no description of how to determine when the ootheca is matured enough to detach without negative consequences. Wording was altered to reflect this detail: "While previous methods of achieving and maintaining gnotobiotic cockroaches describe oothecae collection[23-27], ootheca maturity is either interpreted in terms of species-specific cues (in Blattella germanica), or not at all[27], increasing the likelihood that oothecae are removed and sterilized prematurely, thereby lowering hatch rate."

Lines 79-81: Jahnes et al. 2019 describes using frass DNA extracts as the basis for dPCR to confirm germ-free status of the alimentary tract in a non-destructive way.
> This section was changed to more accurately represent previously published protocols: "As for culture-independent sterility testing, microscopy is one option[27]. Jahnes et al. used a DAPI stain and microscopic examination of the gut. While other protocols use sequence-based techniques to detect organisms that may escape culture[14,23,27,28], the use of whole-16S PCR in combination with a restriction digest (RFLP) to visualize both the endosymbiont and any contaminating gut symbionts is new. This technique includes a PCR control, since Blattabacterium's 16S gene has been sequenced, and its known banding pattern should be present in both gnotobiotic and non-sterile insects. There is no need to sequence the amplicons nor fragments, unless the contaminant's identity is necessary. The current version of this protocol calls for a nymph to be sacrificed for PCR/RFLP, but this technique could be used on feces as a non-destructive measure—however it will not include a built-in control, as the feces should not contain much Blattabacterium."

Lines 82-84: Jahnes et al. 2019 used frass cultivation as a non-destructive cultivation dependent method and epifluorescence microscopy of thin-sectioned DAPI stained guts as a cultivation independent method to verify their procedure.
> (see previous comment for relevant addition)

138-158 Consider grouping some steps so that the indication to autoclave the materials does not become too repetitive.
> These steps were grouped: "Sterilize via autoclave: rat chow broken into half-sizes (approx. 1/2-inch pieces) in a foil-covered beaker (sterilization time 1 hr, gravity cycle), ddH2O in a capped bottle (sterilization time = 20 min, liquid cycle), forceps in a beaker covered in foil (sterilization time = 20 min, gravity cycle)."

Line 228 The authors should include a photo of the rearing apparatus and describe how air exchange is controlled (whether it is filtered in any way).
> This was added as a figure and in several places in the protocol. (lines 189-190, 265-266, 606-608): "Sterilize secondary container. This container does not include a filter but instead allows free air exchange." and "NOTE: There is no air filter for this setup. The foil is sufficient in preventing contamination from open air flow."

305- Since a percentage of failure was described, it might be interesting to add approximately how many times this protocol was implemented, so that the reader would be aware of how many repetitions were made.
> Sample size was added for these data: "After establishment of these changes to oothecae collection and incubation, hatch rates increased to approximately 41% (n = 51),…" and "A comparison between body lengths of gnotobiotic (n = 105) and non-sterile nymphs (n = 50) fed the same, autoclaved rodent diet…"

Lines 380-383: The authors should describe to what instar is consistently attained by the described methods as this would facilitate experimental design and general expectations of using this method. Also, what would be a probable literature-based explanation for the slower growth of nymphs?
> This information was added: "A possible limitation to this technique is that gnotobiotic nymphs may not reach adulthood, as our oldest sterile cohorts are 10+ months old and have reached the seventh instar as approximated by body length[36]. These oldest cohorts are not on the autoclaved rat diet but instead eat irradiated rat chow, a diet that contains too much moisture to feed to non-sterile cohorts without excessive mold growth. Non-sterile nymphs on a non-sterilized dog food diet were found to reach adulthood after 9-10 months. Cohorts of gnotobiotic and non-sterile nymphs on the shared, autoclaved rat chow are currently less than 7 months old, non-sterile insects are estimated to be seventh instar (average: 16.7 mm) while sterile insects are estimated to be fifth instar (average: 11.2 mm)."

Minor Concerns:
Line 64: What is the relevance of mentioning termites and Cryptocercus? Neither of these insects has bacterial species diversity anywhere close to that of the omnivorous

cockroaches. Also the mention of termites 'emerging' from the Cryptocercus clade is irrelevant to this manuscript.
> This extraneous information was removed.

Line 71: Referring to insects with specialized diets as 'pickier eaters' is distractingly colloquial and incorrect.
> This was changed to "dietary specialists"

Reviewer #4:
Manuscript Summary:
The manuscript describes general husbandry of American cockroaches and the generation of germ-free individuals through the surface-sterilization of oothecae. The protocol for germ-free sterilization relies on the use of peracetic acid, first described by Doll et al. (1963) to surface-sterilize eggs from a wide range of invertebrates, and used by other recent studies (Tegtmeier et al. 2016; Mikaelyan et al. 2016). The manuscript was clear to follow, however, the significance of the work might be overstated. The protocols described are quite straightforward and do not (in my opinion) necessarily need a visual demonstration. Additionally, the manuscript is critically missing a full discussion of why the authors' protocol is better than previous well-established protocols. I have the following concerns that must be addressed before I can recommend publication.

Major Concerns:
1. Maintaining colonies of pest cockroaches like Periplaneta americana, Shelfordella lateralis, or Blatella germanica has never been a challenge - indeed there are several protocols available for this fairly easy task and I don't think it justifies a visualization. I would suggest removing this section altogether.
> This section is not included in the filmed protocol. (See yellow highlighted text in lines 288-452 for filmed steps)

2. The surface sterilization of cockroach oothecae is not technically challenging either and has been well-established for blattid cockroaches (Shelfordella lateralis - Tegtmeier et al. 2016; Periplaneta americana - Jahnes et al. 2019). Contrary to what the authors suggest, I would argue that these earlier papers have sufficiently explained the methods for collecting oothecae and surface-sterilizing them - these protocols are fairly simple: they involve physical removal of debris, followed by treatment with peracetic acid or bleach, followed by the removal of the sterilizing agent. When it comes to contamination checks, Tegtmeier et al. (2016) describe a protocol similar to the one used by the authors for checking for contamination, except they used LB agar in stead of the far richer BHI agar - I don't consider this a monumental advancement. Tegtmeier et al. 2016 also described the use of sequencing as a cultivation-independent contamination check.
> Given the time it took to develop this protocol despite the fact that other published protocols existed, we came to the conclusion that publishing this unified protocol with JoVE visualization would make this type of technique more accessible to others interested in this vein of work. Similar to Benschoter & Wrenn, Tegtmeier used an LB floor only for establishing their sterilization protocol, NOT for long-term monitoring of germ-free status (a statement

about this was added—see below). Our protocol does not use sequencing for its cultivation-independent contamination check, as high-throughput sequencing is not an ideal technique to check to sterility for a variety of reasons (contamination of reagents, barcode hopping, etc.). Instead, extracted and amplified whole-16S genes are digested and run on a gel to check for banding that varies from Blattabacterium*. However, the description of contamination checks was improved to more accurately reflect previously published protocols: "The goal of this method is to outline a comprehensive description of the establishment and maintenance of gnotobiotic American cockroaches (Periplaneta americana), but this protocol could be used to generate nymphs of any oviparous cockroach. It includes a method for efficient, non-invasive collection of mature oothecae, and a non-destructive technique to monitor gnotobiotic status of the insects[22-24]. While previous methods of achieving and maintaining gnotobiotic cockroaches describe oothecae collection[23-27], ootheca maturity is either interpreted in terms of species-specific cues (in Blattella germanica), or not at all[27], increasing the likelihood that oothecae are removed and sterilized prematurely, thereby lowering hatch rate. Since the method described here uses naturally dropped oothecae, the error of removing eggs prematurely is absent.*

Most previous protocols test for culturable organisms by plating feces or nymph homogenate bacteriological media and checking for growth[22,23,25,27-29]. In at least one case, the method for testing gnotobiotic status was not fully described[26]. Except Clayton—who added a small slab of sterility testing medium to rearing bottles[24]—previous methods[22,23] housed gnotobiotic insects on bacteriological media only for short periods of time to initially evaluate the sterilization protocol. The following method, however, maintains the hatched nymphs on BHI long-term for a continuous method of monitoring.

As for culture-independent sterility testing, microscopy is one option [27]. Jahnes et al. used a DAPI stain and microscopic examination of the gut. While other protocols use sequence-based techniques to detect organisms that may escape culture[14,23,27,28], the use of whole-16S PCR in combination with a restriction digest (RFLP) to visualize both the endosymbiont and any contaminating gut symbionts is new. This technique includes a PCR control, since Blattabacterium's 16S gene has been sequenced, and its known banding pattern should be present in both gnotobiotic and non-sterile insects. There is no need to sequence the amplicons nor fragments, unless the contaminant's identity is necessary. The current version of this protocol calls for a nymph to be sacrificed for PCR/RFLP, but this technique could be used on feces as a non-destructive measure—however it will not include a built-in control, as the feces should not contain much Blattabacterium. This protocol contains both culture-dependent and -independent methods of quality control, and the culture-dependent method does not require sacrificing insects. Finally, this method brings together information from multiple gnotobiotic cockroach studies to create one, comprehensive protocol with all necessary information for achieving and maintaining gnotobiotic cockroaches."

Minor Concerns:

71: "pickier eaters" is a tad informal - I think the authors mean "dietary specialists"?

> Yes! This replacement was made.

114: What is the purpose of using cellulose sponges?

> The sponges prevent the insects from drowning in their water dish.

172--242: Why is this section highlighted?
> JoVE requests that authors highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video (lines 288-452).