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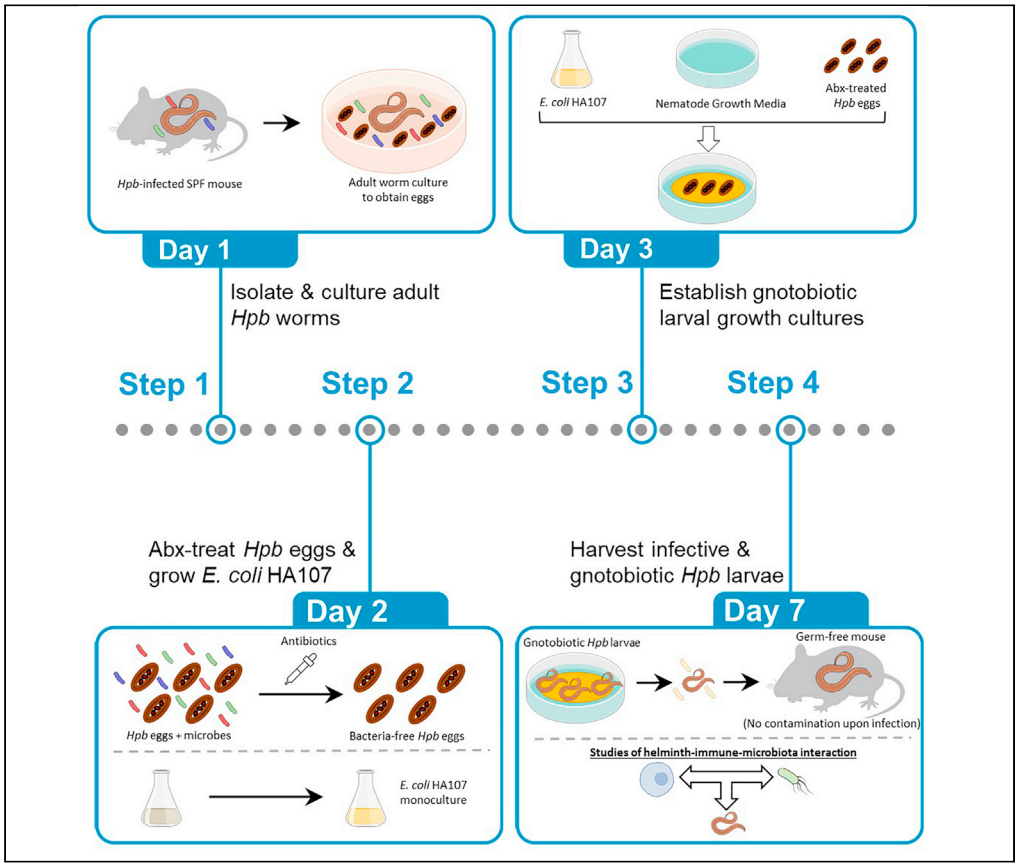
# A protocol for generating germ-free *Heligmosomoides polygyrus bakeri* larvae for gnotobiotic helminth infection studies




The microbes indigenous to helminth species are a major obstacle to deciphering host-parasite interactions. Repurposing a system of reversible bacterial colonization, we have generated germ-free *Heligomosomoides polygyrus bakeri* (*Hpb*) larvae that maintain the sterility of axenic mice upon infection. This protocol provides a valuable tool for controlled studies of helminth-microbiota-immune interactions.

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## Highlights

Protocol for rearing viable germ-free *Hpb* larvae

Larvae maintain infectivity and immunogenicity in specific pathogen-free mice

Larvae do not contaminate germ-free mice upon infection

Experimental tool to parse helminth-immune-microbiota interactions

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# A protocol for generating germ-free *Heligmosomoides polygyrus bakeri* larvae for gnotobiotic helminth infection studies

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## SUMMARY

**The microbes indigenous to helminth species are a major obstacle to deciphering host-parasite interactions. Repurposing a system of reversible bacterial colonization, we have generated germ-free *Heligomosomoides polygyrus bakeri (Hpb)* larvae that maintain the sterility of axenic mice upon infection. This protocol provides a valuable tool for controlled studies of helminth-microbiota-immune interactions.**

## BEFORE YOU BEGIN

The natural murine parasitic roundworm *Heligmosomoides polygyrus bakeri* (*Hpb*) is a widely used model of chronic helminth infection (Reynolds et al., 2012). Although it has been shown that helminths can profoundly alter gut commensal composition and function (Rapin et al., 2020; Walk et al., 2010; Zaiss et al., 2015; Ramanan et al., 2016; Rausch et al., 2018), controlled germ-free and gnotobiotic studies of enteric *Hpb* infection are limited by the presence of the parasite's own indigenous microbiota. These parasite-associated microbes create a unique dilemma as a confounding source of contamination, yet are indispensable for certain parasitic developmental stages. Indeed, progression from egg to infective *Hpb* (L3) larvae requires microbe-rich feces for development, and fecal cultures are the primary method used for generating L3 larvae in a laboratory setting (Johnston et al., 2015).

A recent study examining helminth-microbiota interactions reported a method for growing bacteriologically sterile *Hpb* larvae (Zaiss et al., 2015). However, neither details regarding the fitness or immunogenicity of these larvae nor ability of such larvae to maintain the microbe-free status of germ-free mice was provided. Here we provide a detailed description of a methodology adapted from this original report for the growth of gnotobiotic *Hpb* larvae suitable for the infection of germ-free mice. To this end, a genetically-modified strain of auxotrophic *E. coli* (strain HA107) was repurposed to facilitate the growth of axenic *Hpb* larvae. Originally engineered to reversibly colonize germ-free mice (Hapfelmeier et al., 2010), HA107 requires exogenous D-Alanine (D-Ala) and 2,6-Diaminopimelic acid (m-DAP) to grow. Since these metabolites are not produced by germ-free mice, this auxotroph is unable to contaminate or persist in these hosts. Here, after isolation and antibiotic-treatment of *Hpb* eggs to ensure no contaminant microbes are present, *E. coli*

HA107 is supplied as the sole food source for developing *Hpb* larvae, resulting in the development of gnotobiotic L3 larvae that do not contaminate germ-free hosts upon infection.

### *Hpb* infection to obtain egg-producing adult worms

Timing: 7–12 weeks

In preparation for this protocol, regular (fecal-grown) *Hpb* larvae should be generated and used to infect specific pathogen-free (SPF) wild-type C57BL/6 mice. These infected SPF mice are used as a source of adult *Hpb* worms which will lay the eggs from which gnotobiotic *Hpb* can be grown. All animal studies were performed using protocols approved by the McGill University Health Centre – Research Institute Animal Resource Division.

1. Generate infective L3 stage *Hpb* larvae by standard fecal-culture methods.
   a. Collect fecal pellets from C57BL/6J mice infected for 4–12 weeks with 250 L3 *Hpb* larvae. Between 20 and 40 fecal pellets is recommended; higher quantity will yield more larvae.
   b. Under a biological safety cabinet, spray the pellets with room temperature (20°C–25°C) sterile water.
   c. Place 2 Whatman filters into a sterile 150 × 15mm Petri dish and wet them with room temperature (20°C–25°C) sterile water.
   d. Using a 1 mL syringe plunger, mash the fecal pellets into a paste, and spread onto the center of the top piece of filter paper. Keep the feces moist, adding more sterile water if necessary.
   e. Close the lid of the 150 × 15 mm Petri dish and place in a box (Styrofoam or other). On top of the fecal-culture dish, place another 150 × 15 mm Petri dish. Fill this dish with sterile water and leave with no cover.
   f. Close the box and leave the fecal cultures for 10–14 days.
   g. To collect L3 larvae after 10–14 days, decant any liquid contents of the fecal culture-containing Petri dish into a 50 mL tube.
   h. Grab the top filter paper and spray the underside with sterile water to wash off L3 larvae down into the dish. Perform this step with the underside of the second piece of filter paper. Transfer the contents washed off into the 50 mL tube.
   i. Centrifuge the tube at 300*g* for 3 min at 4°C, then remove supernatant until 5 mL remains.
   j. Count the number of viable L3 *Hpb* larvae simply by pipetting 3–5 aliquots onto a slide and observing under a brightfield microscope.
   k. Store fecal-grown *Hpb* larvae in water for up to 6 months at 4°C.
   l. Using *Hpb* larvae generated by fecal-culture method, infect 1–5 C57BL/6J wild-type mice bred under SPF conditions by gavage with 400 L3. These mice will be used as a source of adult *Hpb* worms from which pure eggs (used to generate gnotobiotic larvae) are collected.

⚠ CRITICAL: For step 1l, do not infect mice more than 4 weeks in advance of the expected start date for gnotobiotic *Hpb* growth, as older worms will impact egg output and eventual larval fitness.

***Note:*** This protocol was developed using 4get.KN2 mice bred on a C57BL/6J background (Mohrs et al., 2005). Since these mice simply serve as a stock of adult *Hpb* worms, alternative genotypes can be used. However, it should be ensured the genotype used does not impair *Hpb* fitness (MyD88−/− mice, for example, display accelerated *Hpb* expulsion and are an example of a genotype that should be avoided) (Reynolds et al., 2014).

### Preparation of liquid and agar media

Timing: 4 hours

Various media preparations (both liquid and agar) should be prepared in advance – these will be used to 1) test for contamination throughout the protocol, 2) grow *E. coli* HA107, and 3) serve as a platform on which to grow gnotobiotic larvae.

2. Prepare 250 mL of Luria broth (LB) media (see Materials and equipment).
3. Prepare 250 mL of supplemented Luria broth (LB Supp) media (see Materials and equipment).
4. Prepare LB, LB Supp, Yeast extract peptone dextrose (YPD), and Nematode growth media (NGM) agar plates (see Materials and equipment). Typically, no more than 5 plates of each variety are used each time the protocol is executed. These plates can be made in bulk and stored at 4°C, however, so recipes are described at higher quantities.

### Streaking single *E.coli* HA107 colonies

Timing: overnight

**Caution:** *E. coli* HA107 derives from strain K-12, and as such is a biosafety level 1 pathogen suitable for bench-side use. Use appropriate personal protective equipment while handling. For the purposes of this protocol, handling of the bacteria is best done under a biological safety cabinet to ensure sterility.

5. From a glycerol stock, streak *E. coli* HA107 onto an LB Supp agar plate and incubate at 37°C overnight.
6. Streak *E. coli* HA107 onto a regular LB plate as a negative control and incubate at 37°C overnight.
7. After overnight growth at 37°C, colony-containing plates can be kept parafilm-wrapped for 4 weeks at 4°C.

⚠ CRITICAL: Re-streak *E. coli* HA107 if colony plates surpass 4 weeks old.

## KEY RESOURCES TABLE

| Key Resource | Vendor/Source | Catalogue # |
|---|---|---|
| Chemicals, peptides and recombinant proteins | | |
| Agar A | Bio Basic | FB0010 |
| Ampicillin, sodium salt United States Pharmacopeia (USP) | Bio Basic | AB0028 |
| Tryptone Powder | Bio Basic | TG217 |
| Yeast Extract | Bio Basic | G0961 |
| D-Alanine | Sigma-Aldrich | A7377-5G |
| Amphotericin B solution | Sigma-Aldrich | A2942-20ML |
| Ampicillin trihydrate | Sigma-Aldrich | A1593-25G |
| 2,6-Diaminopimelic acid (m-DAP) | Sigma-Aldrich | 33240-5G |
| Gentamicin sulfate | Sigma-Aldrich | G1914-25G |
| Metronidazole | Sigma-Aldrich | M3761-25G |
| Neomycin trisulfate salt hydrate | Sigma-Aldrich | N5285-25G |
| Cholesterol | Sigma-Aldrich | C3045-5G |
| Magnesium sulfate ($MgSO_4$) | Sigma-Aldrich | M7506-500G |
| Calcium chloride ($CaCl_2$) | Sigma-Aldrich | C1016-500G |
| Vancomycin hydrochloride | Sigma-Aldrich | 94747-1G |
| Bacto Peptone | WISENT BIOPRODUCTS | 800-157-LG |
| Penicillin-Streptomycin Solution | WISENT BIOPRODUCTS | 450-201-EL |
| Roswell Park Memorial Institute (RPMI) 1640 Medium | WISENT BIOPRODUCTS | 350-000-CL |
| Dulbecco's Phosphate Buffered Saline 1× (PBS) | WISENT BIOPRODUCTS | 311-425-CL |
| Fetal Bovine Serum (FBS) | WISENT BIOPRODUCTS | 090105 |
| D-Glucose (Anhydrous) | WISENT BIOPRODUCTS | 600-035-LG |

(*Continued on next page*)

| *Continued* | | |
|---|---|---|
| Key Resource | Vendor/Source | Catalogue # |
| Sodium Chloride, Anhydrous (NaCl) | WISENT BIOPRODUCTS | 600-0820-IK |
| Potassium Phosphate Monobasic | Fisher Scientific | BP363-500 |
| Potassium Phosphate Dibasic | Fisher Scientific | P285-3 |
| Dextrose | Fisher Scientific | BP350-1 |
| SYTOX Green Nucleic Acid Stain | Fisher Scientific | S7020 |
| Ethylenediaminetetraacetic acid disodium salt (EDTA) | VWR, BDH | n/a |
| Experimental Models: Organisms/strains | | |
| *Heligmosomoides polygyrus bakeri* | King Laboratory | n/a |
| *Escherichia coli* (strain: HA107) | A.J. Macpherson Laboratory | n/a |
| Mouse: 4get/KN2 mice, C57BL/6 background | Bred in-house | Female, 6–12 wks |
| Antibodies | | |
| Rat monoclonal anti-mouse CD4-BUV395 (GK1.5; 1:200) | BD Biosciences | 563790 |
| Rat monoclonal anti-mouse B220-AF700 (RA3-6B2; 1:100) | Thermo Fisher Scientific | 56-0452-82 |
| Rat monoclonal anti-mouse CD44-BUV786 (IM7; 1:400) | BD Biosciences | 563736 |
| Rat monoclonal anti-mouse CD62L-BV711 (MEL-14; 1:400) | BioLegend | 104445 |
| Mouse monoclonal anti-human CD2-PE (RPA-2.10; 1:50) | BD Biosciences | 555327 |
| Rat monoclonal anti-mouse IgG1-Biotin (SB77E; 1:5000) | SouthernBiotech | 1144–08 |
| Rat monoclonal anti-mouse IgE-Biotin (23G3; 1:1000) | Thermo Fisher Scientific | 13-5992-82 |
| Oligonucleotides | | |
| 16S V6 (Forward) | 5′- aggattagataccctggta – 3′ | n/a |
| 16S V6 (Reverse) | 5′ – cttcacgagctgacgac – 3′ | n/a |
| Other | | |
| Spectrophotometer (GENESYS 10uv Scanning) | Thermo Fisher Scientific | n/a |
| Sorvall Legend X1R centrifuge (w/ TX-400 rotor) | Thermo Fisher Scientific | n/a |
| 1300 series A2 Biosafety cabinet | Thermo Fisher Scientific | n/a |
| Heracell 150i $CO_2$ Incubator | Thermo Fisher Scientific | n/a |
| 3D Nutating Shaker | Crystal Technology & Industries, Inc. | n/a |
| Infors-HT Multitron Pro Incubator | n/a | n/a |
| Bacterial Incubator | Fisher Scientific | n/a |
| 0.2 µm filters (Filtropur S) | SARSTEDT | 83.1826.001 |
| Petri dish (150 × 15 mm) | Falcon | 351058 |
| Petri dish (100 × 15 mm) | Fisherbrand | FB0875712 |
| Whatman Filter Papers (125 mm diameter circles) | GE Healthcare | 1440–125 |
| Microscope slides (5 × 75 × 1.0 mm) | Fisherbrand | 12-550-17 |
| Sterile Sampling Bags with Flat-Wire Closures | Fisherbrand | 14-955-187 |

## MATERIALS AND EQUIPMENT

| **LB or LB Supp Liquid Media** | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| NaCl | 5 mg/mL | 1.25 g |
| Yeast Extract | 5 mg/mL | 1.25 g |
| Tryptone | 10 mg/mL | 2.5 g |
| *D-Ala (100 mg/mL) | 0.2 mg/mL (500X) | 0.5 mL |
| **m-DAP (50 mg/mL) | 0.05 mg/mL (1000X) | 0.25 mL |
| Water (de-ionized) | n/a | 250 mL |
| **Total** | **n/a** | **250 mL** |

Autoclave media. Store at room temperature (20°C–25°C) after making and use within 72 h.

*For LB Supp only. Dissolve D-Ala in de-ionized water by vortexing. Filter-sterilize and add to autoclaved cooled media.

**For LB Supp only. Add m-DAP to de-ionized water at 1/3$^{rd}$ of the total volume (e.g., If making 250 mL media, 0.25 mL of m-DAP will be added, so 1/3$^{rd}$ of the total volume would be 0.083 mL). Then add another 1/3$^{rd}$ volume of 1M HCl. Dissolve the m-DAP fully by vortexing, then add the final 1/3$^{rd}$ volume as de-ionized water to achieve the final concentration. Filter-sterilize and add to autoclaved cooled media.

| LB or LB Supp Agar | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| NaCl | 5 mg/mL | 1.25 g |
| Yeast Extract | 5 mg/mL | 1.25 g |
| Tryptone | 10 mg/mL | 2.5 g |
| Agar A | 15 mg/mL | 3.75 g |
| *D-Ala (100 mg/mL) | 0.4 mg/mL (250X) | 1 mL |
| **m-DAP (50 mg/mL) | 0.1 mg/mL (500X) | 0.5 mL |
| Water (de-ionized) | n/a | 250 mL |
| **Total** | **n/a** | **250 mL** |

Autoclave media. After making, store plates at 4°C and use within 2 months.
Recipe makes enough for ~15 plates (15 mL per plate).
*For LB Supp only. Dissolve D-Ala in de-ionized water by vortexing. Filter-sterilize and add to autoclaved cooled agar media.
**For LB Supp only. Add m-DAP to de-ionized water at 1/3$^{rd}$ of the total volume (e.g.,: If making 250 mL agar media, 0.5 mL of m-DAP will be added, so 1/3$^{rd}$ of the total volume would be 0.167 mL).Then add another 1/3$^{rd}$ volume of 1M HCl. Dissolve the m-DAP fully by vortexing, then add the final 1/3$^{rd}$ volume as de-ionized water to achieve the final concentration. Filter-sterilize and add to autoclaved cooled agar media.

| YPD Agar | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Yeast Extract | 10 mg/mL | 2.5 g |
| Bacto Peptone | 20 mg/mL | 5.0 g |
| Dextrose | 20 mg/mL | 5.0 g |
| Agar | 20 mg/mL | 5.0 g |
| Water (de-ionized) | n/a | 250 mL |
| **Total** | **n/a** | **250 mL** |

Autoclave media. After making, store plates at 4°C and use within 2 months.
Recipe makes enough for ~15 plates (15 mL per plate).

| NGM Agar | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| NaCl | 3 mg/mL | 3.0 g |
| Bacto Peptone | 2.5 mg/mL | 2.5 g |
| Agar A | 17 mg/mL | 17 g |
| $MgSO_4$* (1 M) | 1 μM | 1 mL |
| $CaCl_2$* (1 M) | 1 μM | 1 mL |
| Cholesterol (5 mg/mL)** | 0.05 μg/mL | 1 mL |
| Potassium phosphate buffer (PPB)*** | n/a | 25 mL |
| Water (de-ionized) | n/a | 972 mL |
| **Total** | **n/a** | **1,000 mL** |

Autoclave media. After making, store plates at 4°C and use within 2 months.
Recipe makes enough for ~60 plates (15 mL per plate).
*Filter sterilize. Add 1 mL per L agar after autoclaving and cooling.
**Dissolve cholesterol to 5mg/mL in ethanol. Filter sterilize. Add 1 mL per L agar after autoclaving and cooling.
*** Mix 132 mL of 1M $K_2HPO_4$ with 868 mL of 1M $KH_2PO4_4$ to make PPB. Filter sterilize and add after autoclaving and cooling.

| Low Antibiotic Media | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Metronidazole | 0.1 mg/mL | 5 mg |
| Ampicillin | 0.1 mg/mL | 5 mg |
| Vancomycin | 0.05 mg/mL | 2.5 mg |

(*Continued on next page*)

| *Continued* | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Neomycin | 0.1 mg/mL | 5 mg |
| Penicillin & Streptomycin | 100 IU/mL & 0.1 mg/mL | 5000 IU & 5 mg |
| Gentamycin | 1 mg/mL | 50 mg |
| Amphotericin B | 2.5 μg/mL | 125 μg |
| D-Glucose | 0.01 g/mL | 0.5 g |
| FBS | 10% | 5 mL |
| RPMI 1640 | n/a | 45 mL |
| **Total** | **n/a** | **50 mL** |
| Filter-sterilize and store at 4°C. Use within 24 h. | | |

| **High Antibiotic Media** | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Metronidazole | 0.2 mg/mL | 5 mg |
| Ampicillin | 0.2 mg/mL | 5 mg |
| Vancomycin | 0.1 mg/mL | 2.5 mg |
| Neomycin | 0.2 mg/mL | 5 mg |
| Penicillin & Streptomycin | 200 IU/mL & 0.2 mg/mL | 5000 IU & 5 mg |
| Gentamycin | 2 mg/mL | 50 mg |
| Amphotericin B | 5 μg/mL | 125 μg |
| RPMI 1640 | n/a | 25 mL |
| **Total** | **n/a** | **25 mL** |
| Filter-sterilize and store at 4°C. Use within 48 h. | | |

## STEP-BY-STEP METHOD DETAILS

### Preparation of pure *Hpb* eggs & *E. coli* HA107 monoculture

Timing: 2 days

In this step, egg-laying adult *Hpb* worms are isolated from the duodenum of infected mice, washed, and incubated overnight in antibiotic-containing media. The following day, the eggs produced by these worms are isolated, washed, and incubated at a higher concentration of antibiotics overnight. During this second overnight incubation, a pure monoculture of *E. coli* HA107 is grown – this will serve as the food source for *Hpb* hatched from these sterilized eggs.

⚠ CRITICAL: All steps in this protocol, where possible, should be performed under a biological safety cabinet. All utensils and media should be autoclaved, and all non-autoclavable liquids should be filter-sterilized.

1. Extract adult worms
   a. Fill a sterile 100 × 15 mm Petri dish with 20 mL of Low Antibiotic Media (see Materials and equipment).
   b. Excise duodenums from C57BL/6J mice infected 2–4 weeks with *Hpb*.
   c. Using sterile scissors and forceps, open the infected duodenum (Figure 1A), and carefully pick out adult *Hpb* worms, placing them into the 20 mL of prepared media. Worms can be extracted in clumps, and do not need to be picked individually.

⚠ CRITICAL: Avoid transferring large quantities of mucus or luminal debris. Additionally, take care to preserve worm viability during extraction by not crushing adult worms with the forceps during extraction.

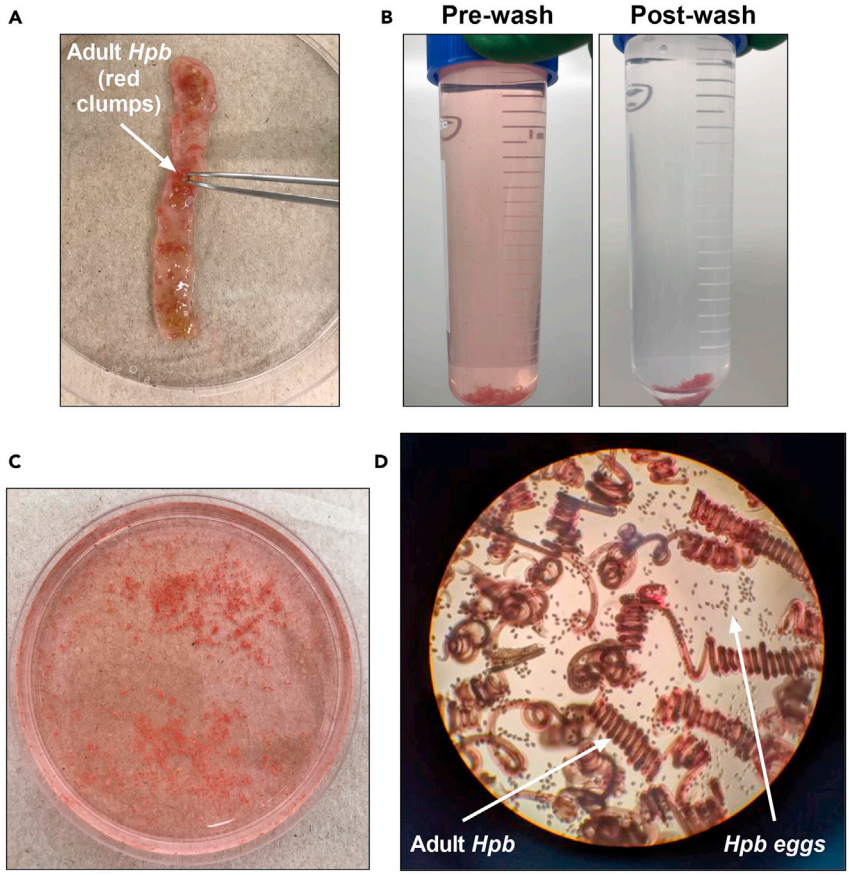


**Figure 1. Depiction of adult *Hpb* worm isolation steps**

(A) The duodenum of a mouse infected 2–4 weeks with fecal-grown *Hpb*, cut open longitudinally to reveal clumps of egg-producing adult worms in red, which can be removed with sterile forceps.

(B) Between crude washes of isolated adult *Hpb* worms, allow the worms to sediment to the bottom of the tube (as shown) by gravity. Photo demonstrates the approximate clarity of media before and after all washes.

(C) Depiction of overnight adult *Hpb* incubation setup, where isolated and washed adult worms are incubated in ~30 mL of antibiotic-containing RPMI (Low Antibiotic Media) and left overnight.

(D) Example of adult *Hpb* worm cultures after 18 h of incubation, photographed through a Brightfield microscope at 10× resolution. *Hpb* eggs can be seen surrounding adult worms in the dish.

2. Wash and incubate adult worms
   a. Pipette the worms and media into a sterile 50 mL tube, then complete the volume of the tube to 50 mL using sterile room temperature (20°C–25°C) PBS.
   b. Cap and invert the tube several times, then allow the worms to sediment by gravity for roughly 30 s, or until most worms have collected at the bottom of the tube.
   c. Use a sterile pipette to aspirate off the supernatant above the worms, then complete the volume of the tube to 50 mL with sterile PBS once again. Perform this wash step a minimum of 8 times. The PBS should become increasingly clear as crude luminal debris is washed away (Figure 1B).
   d. After the final wash, pipette off all but 5 mL of media.
   e. Prepare a new sterile 100 × 15 mm Petri Dish with 25 mL of Low Antibiotic Media (see Materials and equipment).
   f. Pipette the worms into the dish (Figure 1C), and incubate overnight at 37°C , 5% $CO_2$ for 18 h.
3. Isolation and Incubation of *Hpb* Eggs

a. After overnight incubation, eggs should be visible alongside adult *Hpb* worms (Figure 1D). Filter the contents of the Petri dish through a 70μm strainer into a sterile 50 mL tube. Adult *Hpb* worms should be removed from the egg-containing flow-through by size filtration.
b. Centrifuge the flow-through at 1000*g* for 4 min at 4°C, discard supernatant. Pellet will contain *Hpb* eggs.
c. Resuspend the eggs in 5 mL of sterile 4°C de-ionized water and transfer to a sterile 15 mL tube.
d. Centrifuge eggs at 700*g* for 2 min at 4°C. Wash with de-ionized water 2 additional times.
e. After the final wash, resuspend the egg pellet in 14 mL of High Antibiotic Media (see Materials and equipment). Incubate the eggs for 24 h at 4°C. To ensure even antibiotic treatment, place the eggs on an orbital shaker, or orient the 15 mL tube horizontally to prevent the eggs from pooling at the bottom due to gravity.

4. Overnight culture of *E. coli* HA107
   a. Under a biological safety cabinet, fill 1 sterile Erlenmeyer flask with 100 mL of LB Media, and two sterile Erlenmeyer flasks with 100 mL each of LB Supp Media (see Materials and equipment).
   b. From a pre-streaked plate of *E. coli* HA107, split a single colony, inoculating one half in 100 mL of LB Media, and the other half in 100 mL of LB Supp Media. This can be done using a sterile pipette tip to scrape the half-colony and dropping it into the media (Figure 2A). Use the third flask of LB Supp Media as a negative control, containing a pipette tip (with no bacteria).
   c. Seal the Erlenmeyer flasks with autoclaved aluminum foil, and shake overnight (14–18 h) in a bacterial incubator at 250rpm, 37°C.
   d. Set aside two separate 10 mL aliquots of sterile LB Supp Media in 50 mL tubes, and leave at room temperature (20°C–25°C) overnight. These will be needed for the dilution of the overnight culture when measuring the optical density at 600nm (OD600) (done at a benchside spectrophotometer – will become non-sterile). The other will be used for the final dilution of overnight cultures.

   **Alternative:** Use any sterile means to appropriately inoculate the *E. coli* HA107 (e.g.: inoculating loop).

   *Note:* If using autoclaved pipette tips for colony inoculation, handle the tips with a pair of autoclaved forceps, not gloves.

### Preparation and growth of gnotobiotic *Hpb* larvae

**Timing: approximately 6 days**

In this step, antibiotic-treated *Hpb* eggs are washed and incubated with pure *E. coli* HA107 overnight culture. After hatching, *Hpb* will utilize this restricted food source for growth and develop into the infective L3 stage of larvae.

5. Washing and Enumeration of *Hpb* eggs
   a. Centrifuge the 15 mL tube containing *Hpb* eggs at 1000*g* for 4 min at 4°C. Remove the supernatant and wash the eggs with a full 14 mL of sterile 4°C de-ionized water.
   b. Centrifuge the eggs at 700*g* for 2 min and repeat the wash with sterile 4°C de-ionized water a minimum of 7 more times.
   c. After the final wash, resuspend the eggs in 10 mL of sterile 4°C de-ionized water. Using a sterile pipette tip, pipette a minimum of five 10μL aliquots onto a microscope slide, and count the number of eggs per aliquot under a microscope. Calculate the total number of eggs present in the 10 mL.
   d. Centrifuge the eggs once more at 700*g* for 2 min and resuspend them to a concentration of 20 eggs/μL in sterile 4°C de-ionized water. Place at 4°C while performing step 6.

   *Note:* Though numbers may vary, the worms from one *Hpb*-infected mouse yields roughly 5,000–8,000 *Hpb* eggs.

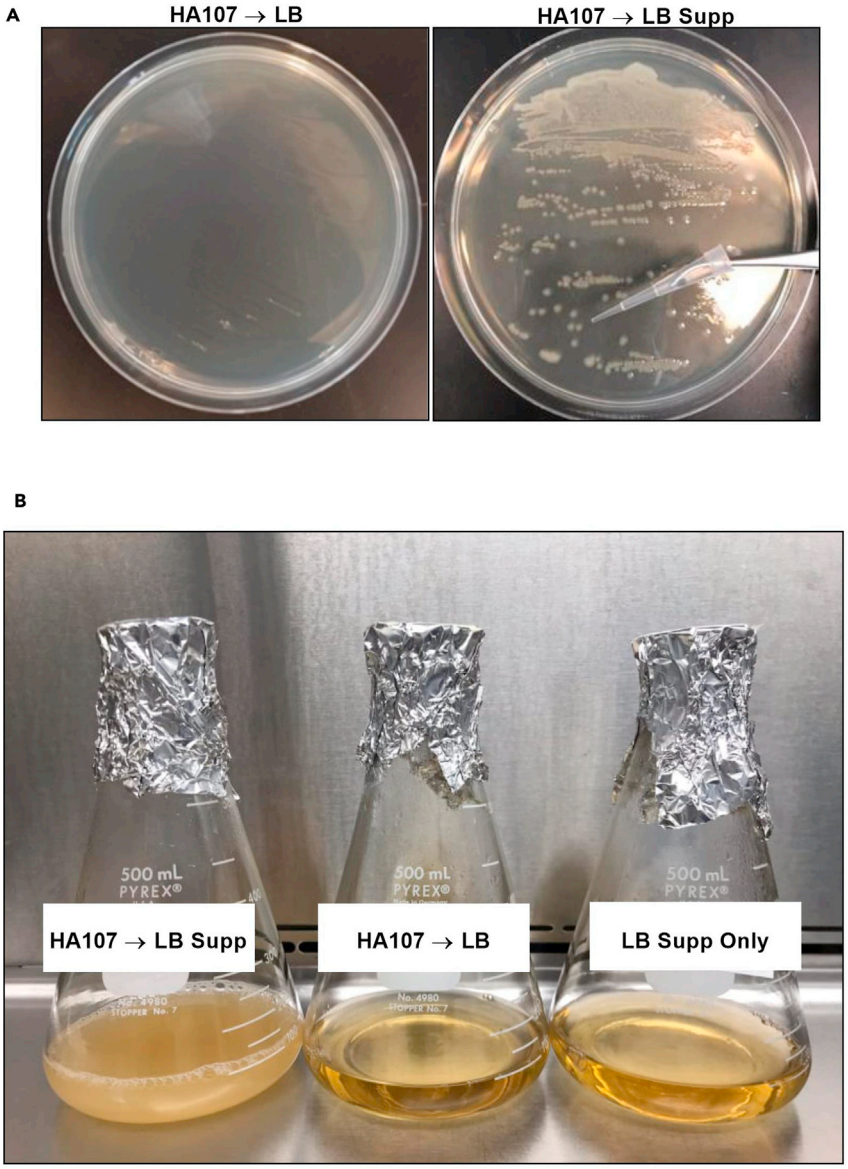


**Figure 2. Depiction of *E. coli* HA107 steps**

(A) When *E. coli* is streaked onto LB and LB Supp Agar media from a glycerol stock and incubated overnight at 37°C, bacteria should grow only on the latter media, as depicted. As is demonstrated in the right picture, *E. coli* HA107 can be inoculated by picking a single colony with an autoclaved pipette tip (handled with sterile forceps) and dropped into liquid media.

(B) HA107 should only grow in the appropriate overnight cultures.

⚠ CRITICAL: Keeping *Hpb* eggs at 4°C delays hatching, but not indefinitely. Use the *Hpb* eggs the day of to ensure the proper timing of larval growth in this protocol.

*Optional:* Plate some eggs onto LB and YPD agar and incubate at least 24 hours at 37°C to validate the effectiveness of antibiotic treatment.

6. Preparation of *E. coli* HA107
   a. Obtain the overnight cultures of *E. coli* HA107. Growth should only have occurred in the flask containing LB Supp Media inoculated with *E. coli* HA107 (Figure 2B).
   b. Using a spectrophotometer, measure the OD600 of *E. coli* HA107 grown in LB Supp Media.

i. Keep the flask under a biological safety cabinet and bring a small (~1 mL) aliquot of bacterial culture to the benchside spectrophotometer to preserve the purity of the flask bacteria.
ii. Measure and record the dilution required to achieve an OD600 between 0.7 and 0.8. To perform these bench-side dilutions, use one of the two aliquots of LB Supp Media saved from the previous day.

⚠ CRITICAL: Ensure you have 2 aliquots of LB Supp Media set aside as mentioned in step 4c, and only use one of these for the spectrophotometer-related dilutions in step 6b. This aliquot will become un-sterile once opened at the bench-side spectrophotometer and is to be discarded after OD600 values are recorded. Do NOT bring this aliquot back under the hood and use it to dilute the HA107 bacteria that will go into the gnotobiotic larvae cultures (use the second un-opened aliquot of LB Supp media for this).

7. Dilution of *E. coli* HA107 and preparation of gnotobiotic *Hpb* growth plates
   a. Using the other sterile aliquot of LB Supp Media saved from the previous day (step 4c), dilute pure *E. coli* HA107 down to an OD600 of 0.7–0.8.

   ⚠ CRITICAL: Dilutions must be done with LB Supp Media (not regular LB Media).

   b. In a sterile Eppendorf tube, combine 100µL of *E. coli* HA107 (OD600 = 0.7–0.8) with 5000 *Hpb* eggs. Refer to the dilutions tabulated at the spectrophotometer to calculate how to achieve this (e.g.,: if a D4 of the bacterial culture gave an OD600 of 0.740, then the 100µL should comprise 25µL of pure bacterial culture, and 75µL of LB Supp Media.
   c. Pipette these egg-bacterial mixture onto nematode growth media (NGM) agar plates (see Materials and equipment). Gently rotate the plates in an orbital manner to spread the mixture in a circle, leaving a gap between the liquid and edge of the dish.
   d. Place the seeded plates into sterile plastic bags and seal shut (Figure 3). Cover these plates with aluminum foil and leave them in the dark at room temperature (20°C–25°C) for 2 days
   e. After 2 days, carefully open the bags and add 1–2 mL of sterile room temperature (20°C–25°C) de-ionized water on top of the bacteria and larvae (plates will have dried noticeably).
   f. Re-bag the plates and leave in the dark for another 3 days.

8. Harvest and washing of *E. coli* HA107-grown larvae
   a. Harvest *Hpb* larvae by gently pipetting sterile 4°C de-ionized water across the surface of the NGM culture plates. Collect the larvae into a sterile 15 mL tube.

   ⚠ CRITICAL: The water used to harvest HA107-grown *Hpb* must be cold (as well as sterile). Larvae must be kept on ice during harvest.

   b. Complete the tube to 14 mL with cold sterile de-ionized water, and centrifuge at 700*g* for 2 min at 4°C.
   c. Repeat this wash a minimum of 3 more times.

   ⚠ CRITICAL: Larval washes must be performed immediately upon harvest to preserve larval viability.

   d. Calculate the number of viable L3 *Hpb* larvae by manual counting of 10µL aliquots under a microscope. Dead larvae do not move and adopt an erect shape. L3 *Hpb* are distinguishable from other larval stages by long, slender morphology, the presence of a sheath/cuticle, size, as well as a distinct rapid movement pattern (Figure 4).
   e. Test the sterility of the larvae by plating 200 L3 (standard infectious dose) each on LB and YPD agar plates, and incubating these plates at least 24 h at 37°C.
   f. Store HA107-grown *Hpb* larvae at 4°C and use within 1 week of harvest.


**Figure 3. Depiction of setup for the growth of gnotobiotic larvae**

After combining 5000 *Hpb* eggs with 100µL of *E. coli* HA107 (OD600 0.7-0.8), pipette the mixture onto an NGM agar plate, and spread into a rough circle and seal individually in sterile plastic bags as shown. Keep in the dark for 2 days, water, and then harvest after a total of 5 days.

g. Prior to use, add 50µM of EDTA to larval suspensions to prevent sticking to the interior of the tube, and recount as in (a). Keep the larvae on ice during infection.

*Note:* Though numbers may vary, one *Hpb*-infected mouse yields roughly 1,000 infective L3 larvae.

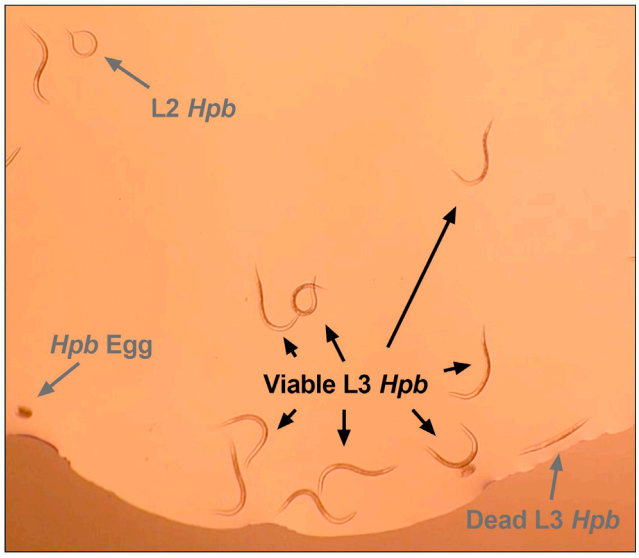


**Figure 4. Example of gnotobiotic larval growth culture results**

After 4–5 days of growth, gnotobiotic larval cultures can be harvested and washed before counting and observation. Shown is a photograph of gnotobiotic larvae at 10× magnification on a brightfield microscope. Minimal visible contamination or bacteria is present around the larvae. L3 larvae can be distinguished from non-infectious L1/2 larvae by their distinct long and slender morphology, size, and rapid movement pattern. Dead L3 *Hpb* are non-moving and erect.

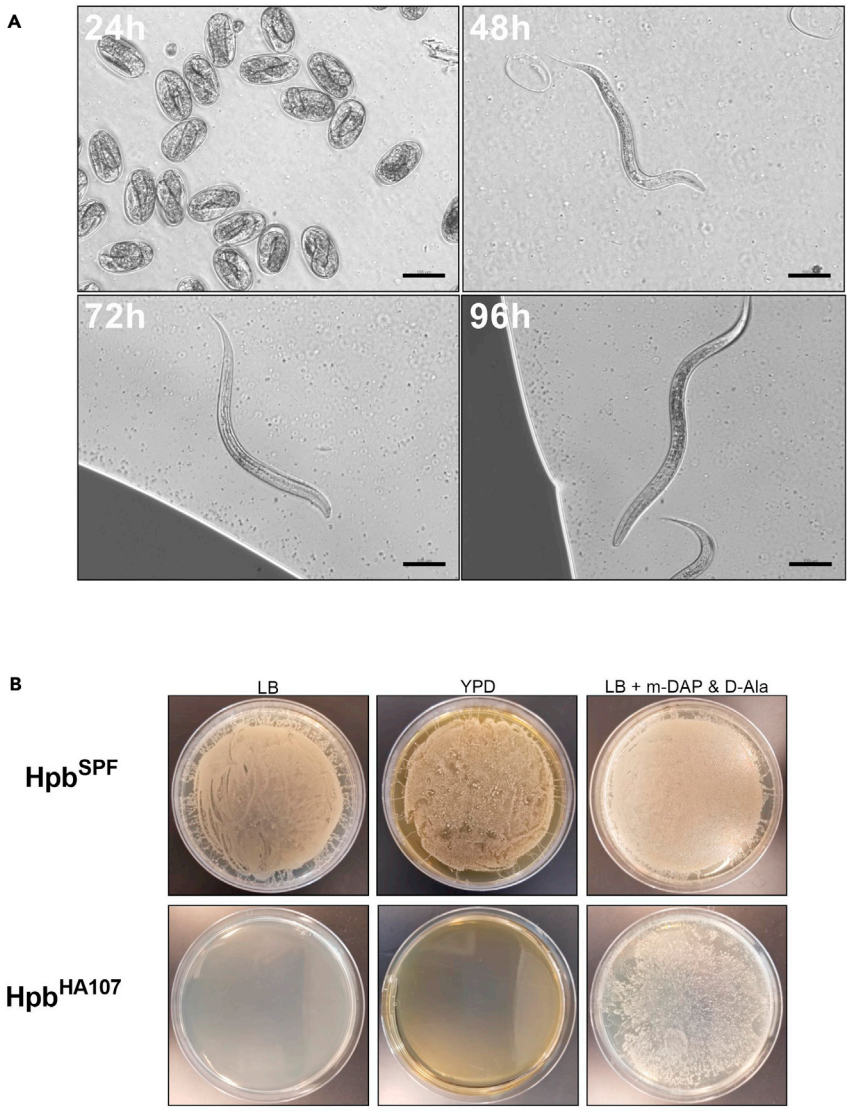


**Figure 5. HA107-grown *Hpb* develop into infective and fit L3 larvae that are devoid of contaminant bacteria**

(A) Visualization of gnotobiotic *Hpb*$^{HA107}$ growth progression from egg to L3 stage using the described protocol. Images are taken using an Olympus BX50 brightfield microscope, scale bars represent 100μm. Examples of egg, L1, L2, and L3 larvae are featured at 24h, 48h, 72h, and 96h timepoint photos, respectively.

(B) 200 *Hpb*$^{HA107}$ or *Hpb*$^{SPF}$ larvae were cultured aerobically for 48 h on agar media (either LB, YPD, or LB Supp).

## EXPECTED OUTCOMES

Over 4–5 days of culturing on NGM media, *Hpb* eggs will hatch and grow into the infective L3 stage of larvae (Figures 4 and 5A). These larvae are void of contaminant bacteria, as aerobic culture of a typical infectious dose (200 L3) on LB or YPD media results in a complete lack of microbial growth, in stark contrast to *Hpb* reared by regular fecal-culture methods (Figure 5B). Despite being grown under controlled conditions with a restricted food source solely of *E. coli* HA107, these gnotobiotic larvae are viable and infective. Indeed, when compared to regular fecal-grown larvae, HA107-grown *Hpb* display similar infection rates and fecundity (a readout of fitness) when infecting wild-type mice housed under SPF conditions (Figures 6A and 6B).

*Hpb* is often used as a model to study the immune response to chronic helminth infection (Reynolds et al., 2012). The anti-*Hpb* cellular immune response features a robust expansion of CD4+ T cells,

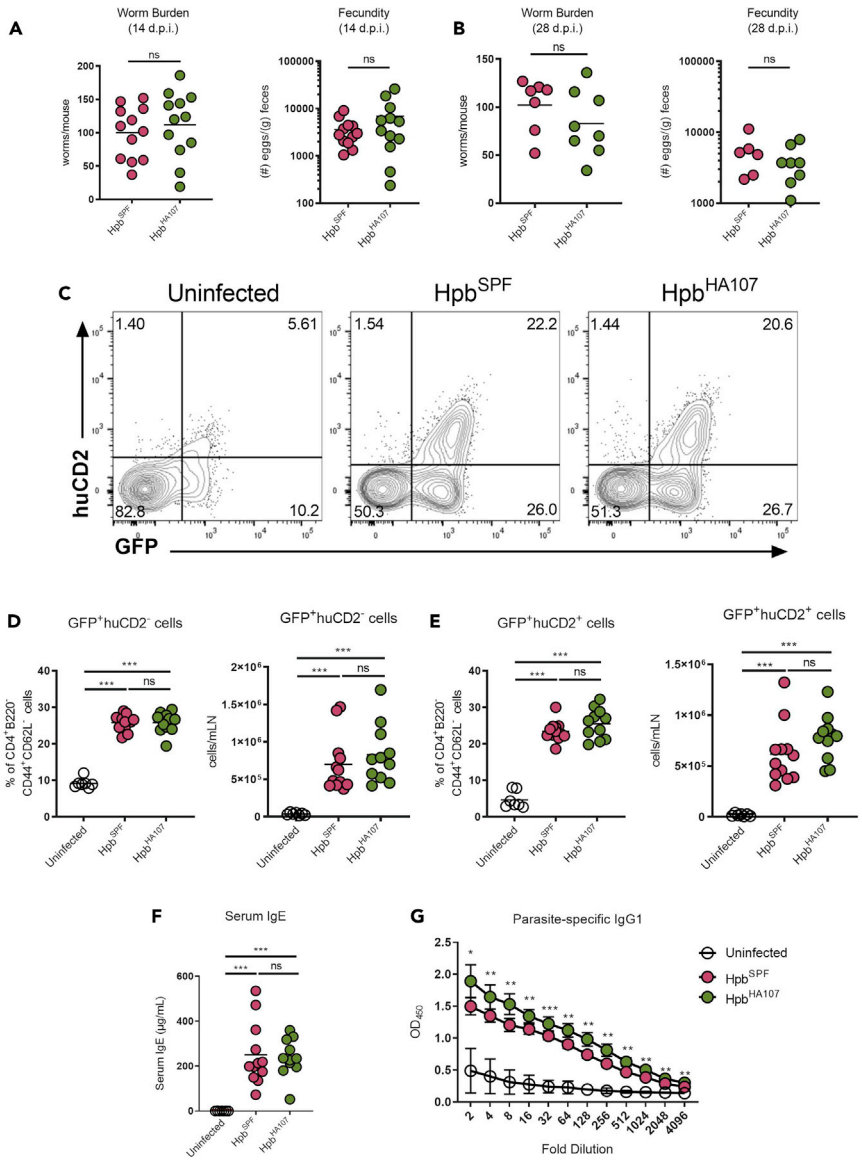


**Figure 6. HA107-grown *Hpb* display comparable infectivity and immunogenicity to fecal-grown larvae in SPF mice**

4get.KN2 mice bred on a C57BL/6J background were gavaged with 200 *Hpb* larvae, either gnotobiotic ($Hpb^{HA107}$) or regular fecal-grown ($Hpb^{SPF}$).

(A and B) After (A) 14 or (B) 28 days post-infection (d.p.i.), mice were sacrificed and the number of adult worms as well as the number of eggs per gram of feces were counted.

(C–E) In (C–E), after 14 d.p.i. Th2 and Tfh cells in the draining mesenteric lymph nodes (mLNs) were assessed by flow cytometric analysis of GFP (4get) expression (a readout of *Il4* transcription) as well as huCD2 (KN2) surface expression (a readout of IL-4 secretion) on live CD4+B220-CD44+CD62L- cells.

(F and G) Serum levels of (F) total IgE as well as (G) parasite-specific IgG1 were assessed by ELISA. Statistical analysis was performed using a t test (A and B) or a one-way ANOVA (C–G). In panel G, the statistics shown apply to both Uninfected vs. $Hpb^{SPF}$, as well as Uninfected vs. $Hpb^{HA107}$ comparisons. *p < 0.05, **p < 0.01, ***p < 0.001, ns - not significant.

including T follicular helper (Tfh) and Th2 cells (King and Mohrs, 2009). Despite being grown under highly controlled conditions, the gnotobiotic *Hpb* larvae reared using this protocol display comparable induction of Tfh and Th2 cells in the draining mesenteric lymph nodes during infection, relative to fecal-grown *Hpb* larvae, bolstering the validity of this model for immunological studies. Indeed, infection of IL-4 dual reporter '4get/KN2' mice, wherein GFP expression marks cells expressing *Il4* mRNA and surface huCD2 expression marks IL-4 protein secreting Tfh cells revealed HA107 and

fecal-grown *Hpb* larvae to induce comparable levels of IL-4 competent and producing CD4+ T cells during infection (Figures 6C–6E) (Mohrs et al., 2005). Similarly, the humoral component of the anti-*Hpb* immune response, dominated by the induction of class-switched IgE and IgG1 antibodies, was similar following fecal-grown and germ-free *Hpb* larvae (Figures 6F and 6G).

Finally, the gnotobiotic *Hpb* larvae grown using this protocol are void of contaminant bacteria and maintain the bacteriologically sterile status of germ-free mice upon infection, as determined by multiple readouts. First, germ-free mice infected with the gnotobiotic *Hpb* larvae generated by this protocol retained significantly larger ceca relative to germ-free mice challenged with conventionally-reared parasites (Figures 7A and 7B). Second, fecal samples from all germ-free mice challenged with *Hpb* HA107 were negative for bacterial contamination, at two weeks post-infection, by culture in brain heart infusion broth (BHI) for seven days in three conditions (37°C and 25°C aerobic, and 30°C anaerobic). Third, 16S DNA quantification by quantitative polymerase chain reaction (qPCR) confirmed no appreciable increase in bacterial load in the feces of germ-free mice challenged with HA107-grown *Hpb*, in contrast to the over 100-fold increase in 16S rDNA load observed in germ-free mice infected with fecal-grown larvae (Figure 7C). Finally, compared to mice infected with fecal-grown *Hpb*, SYTOX green staining of cecal contents from mice infected with HA107-grown larvae revealed no detectable live bacteria (Figure 7D). Importantly, gnotobiotic *Hpb* larvae were able to establish within germ-free mice (Figure 7E), indicating the potential for *ex vivo* experiments using germ-free adult *Hpb* worms.

## LIMITATIONS

Regardless of the culture method, some *Hpb* eggs will not reach L3 stage and will be unavoidably harvested alongside the viable L3 stage larvae. It is possible that dead eggs impact the course of infection. Nevertheless, L3 *Hpb* larvae efficiently parasitize the murine host. Since we validate that HA107-grown *Hpb* display similar infectivity, fecundity, and immunogenicity to fecal-grown larvae even in an SPF host, a single batch of germ-free larvae can be used for all infection conditions to properly control for this aspect of the protocol.

While HA107-reared *Hpb* do not contain viable bacteria or fungi, we cannot completely rule out the possibility that dead bacteria or bacteria-derived products (e.g., LPS) remaining on or in *Hpb* larvae may impact the course of and/or immune response to infection. Additional washes of the final *Hpb* larvae preparation prior to infection is advised to minimize this confounding factor. The inclusion of germ-free mice gavaged with the final media preparation without the presence of live larvae could be considered as an additional control group.

## TROUBLESHOOTING

### Problem 1

Lack of *E. coli* HA107 overnight growth in LB Supp Media (step 6a).

### Potential solution

If *E. coli* HA107 does not grow in LB Supp Media, ensure that the colonies being picked for overnight culture growth are not too old - streak fresh colonies the day before commencing the protocol. Ensure precise and equal splitting of the chosen bacterial colony between flasks. Prepare fresh liquid media (verifying the composition) and make fresh preparations of m-DAP and D-Ala metabolites. Dissolving the m-DAP as indicated is a critical step - replacing the 1/3$^{rd}$ of the total volume that is HCl with water will compromise bacterial growth in the eventual media. Only add filter-sterilized m-DAP and D-Ala (as well as HA107 inoculate) after the base LB media has been autoclaved and cooled to room temperature (20°C–25°C).

### Problem 2

Contamination of overnight culture negative controls (e.g., growth in un-supplemented LB Media – step 6a).

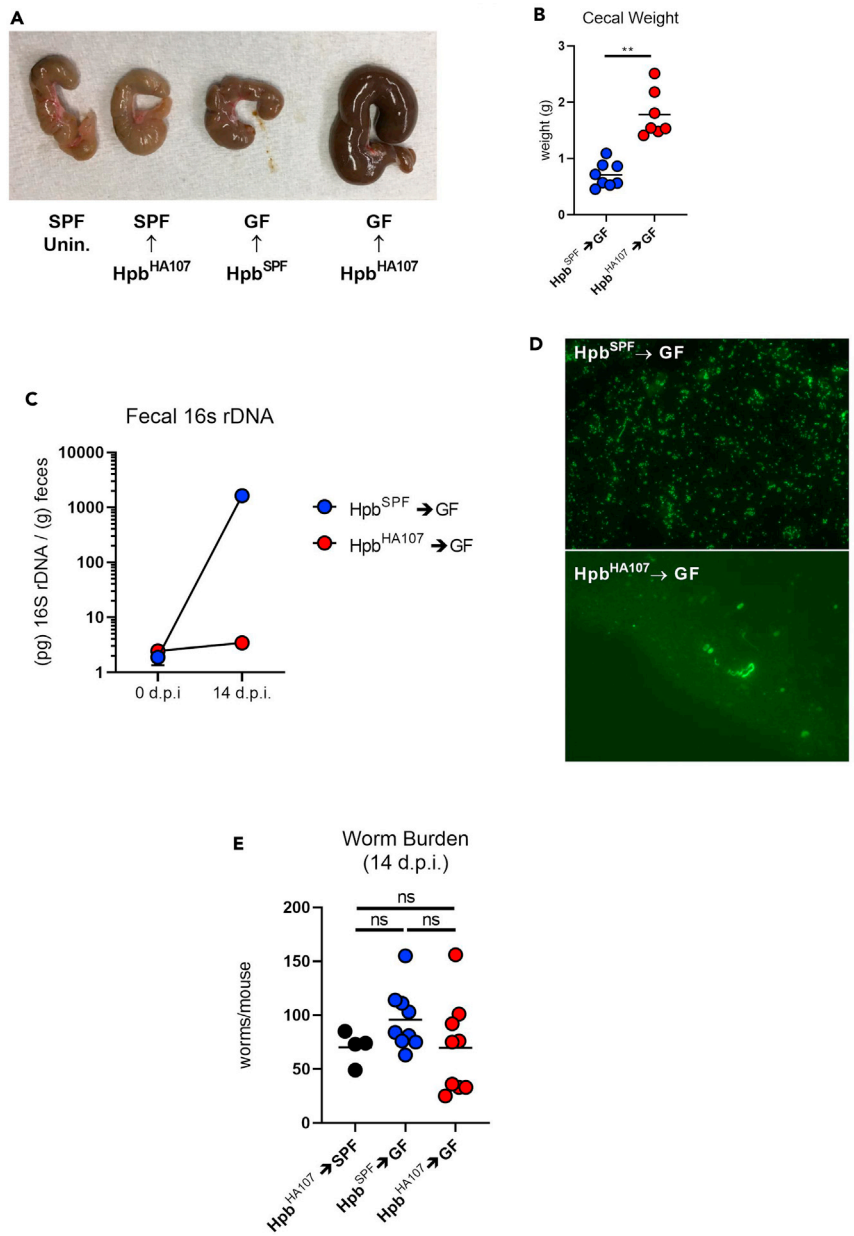


**Figure 7. HA107-grown *Hpb* infection maintains the sterility of germ-free mice**

(A–E) Germ-free (GF) C57BL/6 mice were infected with 200 HA107-reared or fecal-grown L3 *Hpb* larvae (denoted $Hpb^{HA107}$ → GF and $Hpb^{SPF}$ → GF, respectively). ($Hpb^{HA107}$ → SPF) indicates SPF C57BL/6 mice receiving 200 HA107-reared larvae. (Uninf. SPF) indicate uninfected SPF C57BL/6J mice. At 2 weeks post-infection, mice were sacrificed, and ceca were manually excised in a germ-free isolator using sterile scissors and forceps, (A) photographed and (B) weighed. (C) DNA was extracted from select fecal samples and a quantitative polymerase chain reaction (qPCR) for 16S ribosomal DNA was performed. (D) Select fresh cecal contents were stained with SYTOX Green (Thermofisher) for detection of bacterial DNA. (E) Duodenum of infected subjects were excised, opened, and using forceps adult worms were manually counted under a dissecting microscope. Statistical analysis was performed using a t test in (B) and a one-way ANOVA in (E). **$p < 0.01$, ns - not significant.

## Potential solution

Streak a fresh agar plate of HA107 colonies. Streak some HA107 glycerol stock onto an un-supplemented LB agar plate as a negative control, to ensure the stock itself does not contain non-HA107 microbes. Ensure HA107 inoculation is done carefully. If using pipette tips, ensure they are autoclaved and only handle them with autoclaved forceps if inoculating the entire pipette tip along

with the scraped bacteria. Perform all steps handling bacteria in a biological safety cabinet (not on the bench with a Bunsen burner).

### Problem 3

Contamination of *Hpb* eggs (as assessed by plating on YPD and LB agar – optional note after step 5d)

### Potential solution

Ensure that adult worm extraction is done using autoclaved forceps and that the antibiotic cocktails are filter-sterilized – these mixtures, while extensive, account for microbes in an SPF mouse gut, but not all possible environmental contaminants. Ensure antibiotic concentrations are appropriate and prepare fresh antibiotic stock solutions as freeze-thaw cycles may degrade these reagents. Finally, even in the absence of a visible bacterial or fungal bloom, consider shortening the incubation period of adult worms to lay eggs and ensure the eggs are incubated in antibiotics at 4°C for a full 24 h.

### Problem 4

Contamination of L3 *Hpb* larvae (as assessed by plating on YPD and LB agar – step 8e).

### Potential solution

Assess controls to help determine at what point in the protocol contamination took place. See previous potential solutions if contamination occurred during the growth of the pure *E. coli* HA107 monoculture, or if antibiotic-treatment incompletely sterilized the *Hpb* eggs. If these controls were clear, ensure the handling of the eggs and bacteria when preparing the NGM plates is done carefully. Perform all steps in a sterilized biological safety cabinet. Wipe down all pipettes with 70% ethanol and use filtered tips to combat contaminating aerosols from the pipette interior. Ensure the plastic bags being used to store the NGM plates are completely sterile and that the interior remains clean during handling.

### Problem 5

Low L3 *Hpb* viability or infectivity (step 8d and beyond).

### Potential solution

Ensure that HA107-grown *Hpb* larvae are used within a week of harvest to maximize infectivity. When using larvae for infection, it is essential to keep them on ice. Similarly, harvest the larvae with cold (4°C) sterile water and keep them on ice as you harvest them from the NGM plates. If performing additional washes of the HA107-grown *Hpb* upon harvest, ensure additional centrifugation steps do not impact larval viability (as judged by motility observed under a brightfield microscope). Similarly, ensure all centrifugation steps throughout the protocol are performed at the appropriate speed, as excessive *g*'s may affect egg or larval viability. Make sure that the adult *Hpb* worms extracted to lay eggs are not too old – this is essential as using eggs from older worms (>4 weeks) has been found to lower the infectivity and fitness of HA107-grown larvae.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Irah King (irah.king@mcgill.ca).

### Materials availability

This study did not generate new reagents.

### Data and code availability

This study did not generate computational datasets or code.

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## AUTHOR CONTRIBUTIONS

G.A.R. developed and optimized the methodology, performed the validatory experiments, and wrote the protocol. C.F. performed the gnotobiotic helminth infections. E.F.V. provided the germ-free mice and performed bacteriological assays. G.P. helped write the protocol. S.H. engineered the HA107 strain of *E. coli*. I.L.K. conceived the project.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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