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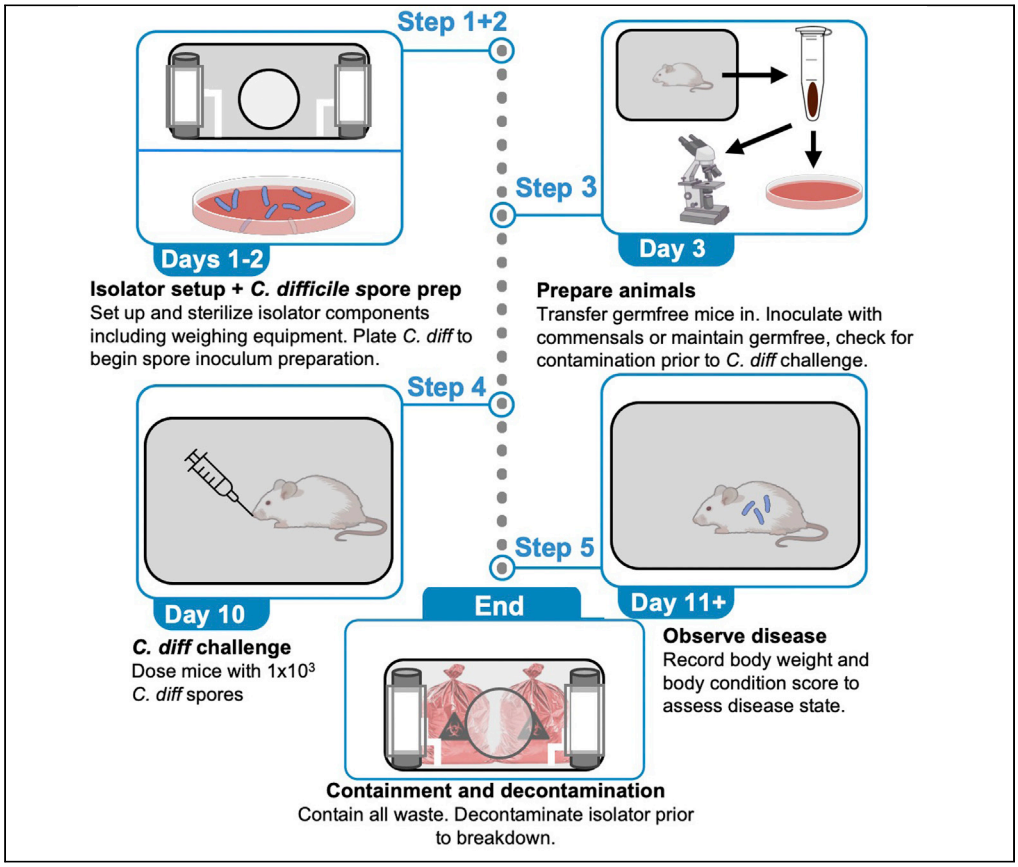
Protocol

Protocol for using negative pressure isolator systems to study BSL-2 organisms in gnotobiotic murine models



Here, we present a protocol for the use of negative pressure isolator systems to maintain defined association and contain BSL-2 pathogens in germ-free and gnotobiotic mouse studies. We describe setup and operation of negative pressure isolators with integrated microbiologic procedures, using the BSL-2 pathogen *Clostridioides difficile* as a working example. This approach supports experimental systems with defined-association mice and enables high-resolution mechanistic studies of pathogen-commensal interactions and their impacts on host phenotypes.

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Highlights

Protocol to set up and operate negative pressure gnotobiotic isolators

Enables defined-association mouse studies with BSL-2 pathogens

Supports longitudinal studies that require frequent mouse procedures

Enables study of complex host-pathogen-commensal interactions

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Protocol for using negative pressure isolator systems to study BSL-2 organisms in gnotobiotic murine models

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SUMMARY

Here, we present a protocol for the use of negative pressure isolator systems to maintain defined association and contain BSL-2 pathogens in germ-free and gnotobiotic mouse studies. We describe setup and operation of negative pressure isolators with integrated microbiologic procedures, using the BSL-2 pathogen *Clostridioides difficile* as a working example. This approach supports experimental systems with defined-association mice and enables high-resolution mechanistic studies of pathogen-commensal interactions and their impacts on host phenotypes.

For complete details on the use and execution of this protocol, please refer to Girinathan et al. (2021).

BEFORE YOU BEGIN

Place all reagents needed for *C. difficile* spore culture preparation, plating, harvest, and quantification in the anaerobic chamber at least 24 h before starting each step so that they can pre-reduce.

Ensure all animal experimental procedures are approved by a local IACUC (Institutional Animal Care and Use Committee). This is the institutional committee that reviews and approves all studies working with vertebrate animals.

All equipment comprising the isolators as well as any equipment that will be in the isolators must be sterilized either by exposure to steam in autoclaving (a pre-vacuum cycle that reaches 129°C at 30 PSI for at least 15 min), ethylene oxide gas sterilization (exposure to 17.6 g of ethylene oxide over 12–24 h with a minimum concentration of 500 mg/L at room temperature and ambient humidity), or by contact with a surface sterilant such as Clidox®-S prepared to manufacturer's instructions (https://www.pharmacal.com/clidox-s.html), using a 1:3:1 ratio of Clidox®-S base, water, and Clidox®-S activator. Contact times with Clidox®-S vary with consideration to the time sensitive nature of materials and animals entering the isolator, with a minimum recommended contact time of 15 min.

Clostridium difficile spore preparation

Timing: 17 days total; 2 overnight incubations, a 14-day incubation, and a 2-day incubation to quantify spores, 7 days active work

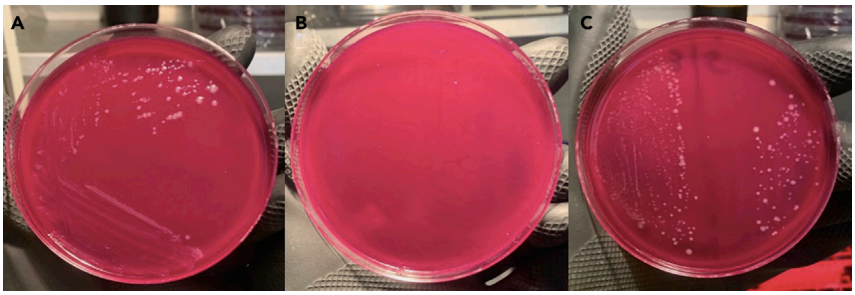

Figure 1. *C. difficile* on Brucella agar

(A) A quadrant streak of *C. difficile* stock to assess purity and isolate single colonies.

(B) A dense lawn of *C. difficile* from plating the overnight BHI culture.

(C) *C. difficile* colonies from serial dilution plating.

This section describes the preparation and isolation of *C. difficile* spores from pure culture and inoculum preparation.

⚠ CRITICAL: All work is carried out in an anaerobic chamber unless otherwise specified. Careful aseptic technique should be employed to limit risk of contamination. Positioning of sterile materials should be considered when preparing the workspace.

Note: Anaerobic conditions may entail different mixtures of gases. We performed experiments using an atmosphere consisting of 10% H_2, 10% CO_2, 80% N_2. The chamber contains palladium catalysts that convert atmospheric oxygen to water and trays of desiccant to control humidity.

1. *C. difficile* ATCC 43255 culture preparation and plating
   a. Streak *C. difficile* ATCC 43255 onto one Brucella agar plate for isolation and incubate plate for 18–24 h at 37°C. Examine plate for purity. *C. difficile* forms 2–5 mm, circular to rhizoid, flat, opaque, grayish colonies on Brucella agar (Figure 1A).
   b. Inoculate PRAS BHI tube with a single colony from the overnight culture and incubate for 18–24 h at 37°C. Examine the tube for turbidity.
   c. Submerge a sterile cotton swab with the overnight broth culture and create a lawn on 20 Brucella agar plates.
   d. Place the plates in a plastic container with the lid slightly ajar to maintain the humidity and prevent the plates from drying out.
   e. Incubate the plates at 37°C for 14 days.

Note: If the plates appear dry, increase the humidity by placing a wet paper towel in the container or wrapping the plates in Parafilm.

2. *C. difficile* spore harvest
   a. Prepare 200 mL of 1× PBS with 0.05% (w/v) cysteine hydrochloride. Sterilize by autoclaving in the conditions described in the "before you begin" section.
   b. Inspect the Brucella agar plates for purity. There should be a dense lawn of *C. difficile* that appears as a uniform, grayish white layer (Figure 1B). There may be small irregularities in the lawn where the swab deposited more cells or shed fibers. Distinct colonies on top of the lawn indicate contamination. Moisten a sterile cotton applicator by submerging the tip in the 10 mL of sterile PBS with 0.05% cysteine hydrochloride.
   c. Gently scrape up the lawn from one plate with the moistened swab and dip it into 20 mL of sterile PBS with 0.05% cysteine hydrochloride in a 50 mL conical tube. Gently shake and twist

the cotton applicator to suspend the *C. difficile*. Press the applicator along the inside wall of the tube to remove as much of the suspension as possible. Remove the swab and discard.

d. Repeat steps for all plates using a new cotton applicator for each plate. It is helpful to have both tubes in a rack for easy access. The workspace should be free of clutter and arranged to minimize reaching over open tubes or uncovered plates.

e. Remove tube with suspension from the chamber and proceed with spore preparation.

3. *C. difficile* spore preparation and aliquoting

Note: *C. difficile* spores survive in ambient air. This work can be performed outside of the anaerobic chamber.

a. Add 20 mL 95% ethanol to the 20 mL of *C. difficile* in sterile PBS with 0.05% cysteine hydrochloride.

b. Replace the cap and incubate in the 50 mL conical tube at room temperature (20°C–25°C) for 60 min.

c. Centrifuge at 5000×*g* for 10 min to pellet the cells. Decant supernatant. Complete these steps twice to remove the ethanol from the spores.

d. Resuspend the pellet in 20 mL of PBS after the final wash.

e. Make 1 mL aliquots in cryogenic tubes.

f. Snap-freeze the aliquots in liquid nitrogen and store at −80°C.

Pause point: Spore aliquots can be stored at −80°C until needed to inoculate gnotobiotic mice.

4. Quantification of harvested *C. difficile* spores

a. Remove frozen *C. difficile* spore aliquot from −80 and thaw in the anaerobic chamber.

b. Vortex the spore stock for 15 s.

c. Prepare serial 10-fold dilutions in sterile pre-reduced PBS with 0.05% cysteine hydrochloride. Continue the dilution series out to 10^{-6} and plate 100 μL onto Brucella agar plates in triplicate.

d. Plate 100 μL of the spore stock in triplicate on blood agar plates and remove from the anaerobic chamber. Incubate in ambient air at 37°C for 48 h. This is to check for contamination by any aerobic bacteria. After plating, the aliquot can be discarded or re-aliquoted for future use.

Note: Each time a spore stock sample is thawed and refrozen, an aliquot must be quantified as repeated freezing and thawing can reduce the biomass.

e. After 48 h, enumerate the *C. difficile* colonies to determine the concentration of spores (Figure 1C). For the most accurate enumeration, use plates that have between 30-300 colonies.

f. Examine the blood agar plates for contamination. When culturing *C. difficile* or any other obligate anaerobe, any aerobic growth is due to a contaminant. Another aliquot should be re-plated on blood agar and incubated aerobically to determine if the contamination was isolated to that aliquot. If both are found to be contaminated, the stock should be discarded.

g. Calculate the concentration of spores/mL using the following:
CFU/mL = Number of Colonies/Dilution factor × Volume plated (mL)

5. Preparation of spore inoculum for *C. difficile* infection

a. Calculate the stock volume required to achieve 5×10^3 CFU/mL using the following equation:

Original stock concentration (CFU/mL) × stock volume = 5×10^3 CFU/mL × total gavage volume required × 1.25 (overage)

Note: The working concentration of 5 × 10^3 CFU/mL is based on a 200 μL dose containing 1 × 10^3 CFU used to challenge mice in the reference article for this protocol (Girinathan et al., 2021)

6. Transfer inoculum into a 50 mL conical tube.

Prepare commensal bacterial culture for co-colonization studies

Timing: hours to days

Culture the commensal bacteria of interest to an appropriate concentration to colonize germ-free mice.

⚠ CRITICAL: Timing and conditions depend on the characteristics and growth kinetics of the commensal species being studied. We have used 1 × 10^8 CFU per mouse based on the reference article for this protocol (Girinathan et al., 2021). For any new bacterial species, we recommend pilot studies to determine the appropriate concentration to dose GF mice.

7. Prepare broth cultures of the commensal bacteria sufficient for 1 × 10^8 CFU per mouse plus 20% extra volume to account for dead space in the gavage syringe. The present example references the article Girinathan et al. (2021) in which *Paraclostridium bifermentans* was cultured in tryptic soy broth with 5% defibrinated sheep blood in the same anaerobic conditions described in the *C. difficile* spore preparation section. Cultures were pelleted and resuspended in sterile pre-reduced PBS with 0.05% cysteine hydrochloride (40 mM) to achieve a concentration of 5 × 10^8 CFU/mL.
   a. Plate prepared inoculum aerobically on blood agar and anaerobically on Brucella agar to ensure purity.
   b. Aliquot enough prepared inoculum to dose the experimental animals in a Hungate tube.

Ethylene oxide gas sterilization of scales

Timing: 2 days, overnight sterilization cycle

All instruments needed in the isolators that cannot be sterilized by Clidox®-S sterilant or by autoclaving such as electronics should be sterilized by ethylene oxide gas exposure.

Note: Scales and other electronics should be sterilized the day before setting up the isolator so that they can be sprayed in during the setup steps. Proper use of the Andersen Products line of ethylene oxide gas sterilization equipment is described here.

⚠ CRITICAL: Ensure that the indicator has changed color appropriately to indicate adequate exposure to ethylene oxide gas and proper sterilization was achieved. This is essential as any bacteria in the bag could contaminate the isolator.

8. Prepare and sterilize equipment
   a. Place scales and other electronics into a sterilization liner bag from the AN71 Refill Kit. Also place an AN87 Dosimeter in the liner bag. This will indicate whether the inside of the bag was sufficiently exposed to ethylene oxide gas.
   b. Unroll the gas release bag from the AN71 Refill Kit containing the gas ampule, without opening it, and place it on top of the items in the center where it can easily be broken to begin the sterilization process.
   c. Turn on the Anprolene® AN74i Classic Sterilizer and press the button next to START. The sterilizer should begin a self-test.

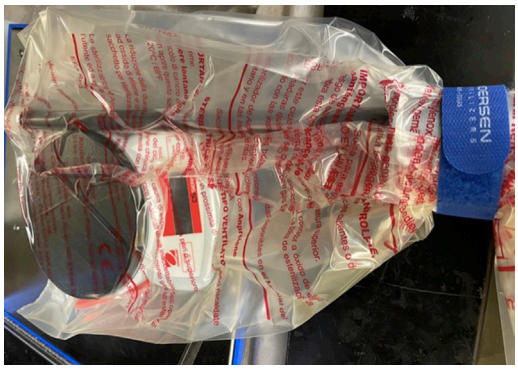

Figure 2. The loaded ethylene oxide sterilizer bag is secured to the purge tube

Once the air is taken out by the purging process, the bag will appear to be vacuumed around the contents. The ethylene oxide ampule can be broken, and sterilization can begin.

d. Take out the purge tube and insert it into the sterilization liner bag outside of the sterilizer cabinet.
e. Position the liner bag around the silver neck of the purge tube and secure it with the Velcro strap so that air cannot enter or escape. If needed, an extra set of twist ties can be used to tighten the seal (Figure 2).
f. When the self-test is complete, the screen should display LOAD STERILIZER BAG/CLOSE BAG OVER TUBE/PURGE BAG. Press the button next to purge. This will create a vacuum inside the bag for about 90 s.
g. When the time is at "00:00:00" the purge is complete. Break the ampule in the bag, place the sterilization liner bag in the cabinet, and close the door.
h. Select the sterilization cycle time (12 or 24 h).
i. There is an additional 2 h ventilation cycle after sterilization is complete. This is important to consider when planning a workflow.
j. When the screen displays UNLOAD STERILIZER, the sterilization process has completed. Press the button next to EXIT.
k. Remove the sterilization bag and purge tube from the cabinet.
l. Carefully remove the purge tube by releasing the Velcro strap and any twist ties and holding the bag tightly as you remove it to prevent air from entering. Immediately close the bag tightly with twist ties. Ensure the dosimeter indicates adequate ethylene oxide exposure before proceeding.
m. Power off the sterilizer.
n. The items are sterilized and ready to be transferred into the isolator as it is being set up.

Prepare isolator filter units and sterilizing cylinders

Timing: 5 h

9. Load the prepared DW4 filter media-wrapped sterilizing cylinder with necessary supplies and equipment.
   a. Line each sterilizing cylinder with a mesh laundry bag.
   b. Divide the following resources among sterilizing cylinders (Figure 3):
      i. Plastic mouse cages with metal lids/food hoppers (minimum one cage per 5 mice)
      ii. Water bottles with stoppers (1/cage + 1 extra)
      iii. Cage card holders (1/cage)
      iv. Cage cards
      v. Microcentrifuge tubes (in pouch)
      vi. Flexible feeding tubes or feeding needles (in pouch)
      vii. Cotton gloves (2)
      viii. Empty brown paper bags (2)

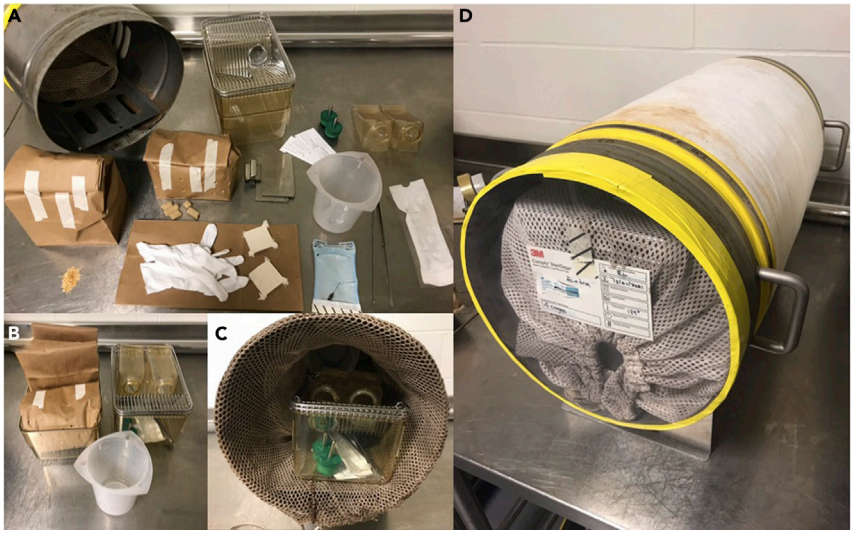

Figure 3. Sterilizing cylinder with supplies

(A) Supply sterilizing cylinder contents including plastic static mouse cages with lids, water bottles and stoppers, cage card holders, paper cage cards, tri-corner plastic beaker, microcentrifuge tubes in a sterilization pouch, feeding needles, cotton gloves, empty brown paper bags, packed diet, bedding, and nestlets.

(B and C) Consolidated items to fit in the sterilizing cylinder and for ease of pulling supplies through the transfer sleeve into the isolator (C) loaded into the cylinder, lined with a mesh laundry bag.

(D) A prepared, autoclaved sterilizing cylinder with a steam integrator card indicating proper sterilization.

ix. Bedding, diet, and nestlets

Note: These are partitioned into brown paper bags, which are folded and taped shut with autoclave tape

x. Large forceps
xi. 1,000 mL plastic beaker

c. When a sterilizing cylinder is filled, pull the string of the mesh bag to close the top.
d. Affix a steam integrator card to the exterior of the mesh bag with a small piece of tape.

10. Seal the filter connector or sterilizing cylinder opening (Figure 4).
    a. Cut out a roughly circular piece of Mylar that extends beyond the edges of the opening by 1 in.
    b. Tape the Mylar sheet over the edge of the opening.
        i. Tape the Mylar sheet in place using small pieces of Mylar tape (Figure 2A).
        ii. Wrap a long piece of Mylar tape over the Mylar sheet around the circumference of the opening, crimping the Mylar sheet as you go along to create a tight seal (Figure 4B).
    c. Next, run the yellow vinyl tape over the Mylar tape seal for at least two full revolutions so that it also is sealed against the metal of the sterilizing cylinder. Pull/stretch the tape as you are running it over the edge to form a tighter seal (Figures 4C and 4D).
    d. Place a piece of autoclave indicator tape on top of the tape seal.
11. Autoclave the filters and sterilizing cylinder (129°C at 30 PSI for at least 15 min). Check the steam integrator card and the run log to ensure successful sterilization (Figure 3D.
12. Wrap the filters with plastic sleeves (Figure 5).
    a. Identify the appropriate plastic sleeves. The outlet sleeve contains a hard plastic shell, whereas the inlet sleeve does not.
    b. Slide the plastic sleeve onto the filter unit so it completely covers the HEPA filter paper.

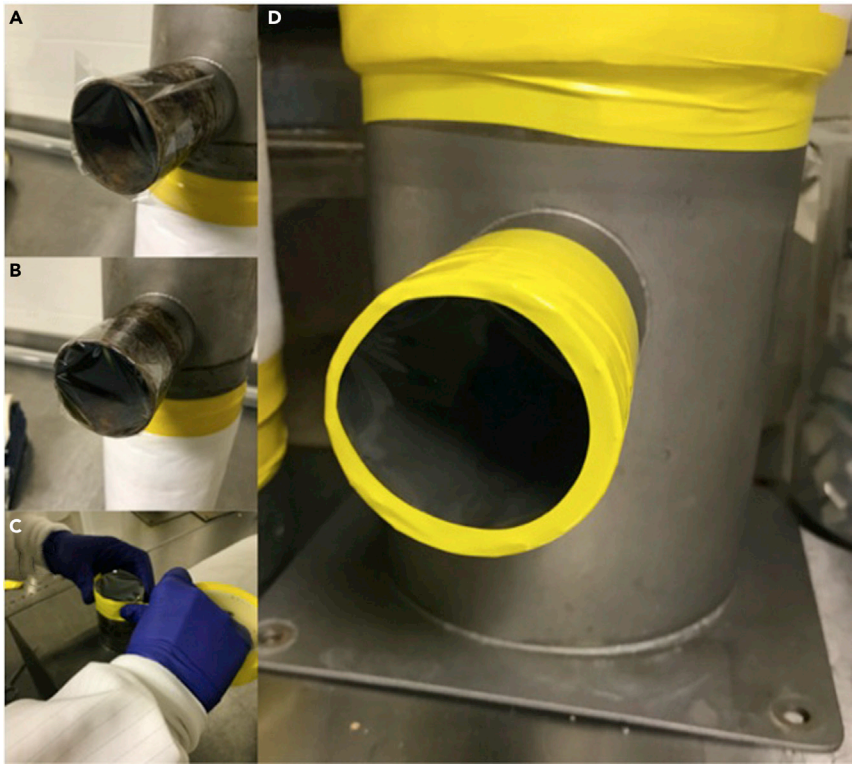

Figure 4. Sealed filters

(A) Hold the Mylar film in place with Mylar tape.

(B) Wrap Mylar tape around the Mylar sheet on the filter opening to create a flat, tight seal.

(C) Wrap yellow vinyl tape around affixed Mylar sheet and opening to reinforce the seal.

(D) Wrapped filter opening.

i. Align the sleeve outlet on the appropriate side of the filter unit so it will connect with the air flow hoses on the isolator frame.

ii. The sleeve seam should be facing the isolator bubble, aligned with the metal connector on the filter unit.

c. Tape over the seams between the plastic sleeve and the filter unit.

i. Use a small piece of tape to affix either end of the sleeve to the filter.

ii. Crimp the seam of the plastic sleeve so the sleeve edge is snug against the filter.

iii. Wrap the seam two to three times using nylon tape.

Set up the negative-pressure containment isolator

Timing: 1–2 days

Negative pressure containment isolators enable the safe execution of gnotobiotic experiments with BSL-2 organisms. Isolator setup includes attachment of the filters, decontamination of the isolator interior, establishment of the negative pressure airflow, and transfer of initial materials for the experiment. The sterilization steps are easier with two people as it requires items to be passed into the port.

13. Collect necessary isolator components

a. Collect the following items and pass them into the isolator interior through the open port:

i. Plastic floor mat

ii. Inner port cover

iii. Rubber band

Figure 5. Filter with cover attached with yellow vinyl tape

iv. Long metal hook wrapped in nylon tape
v. Ethylene oxide gas sterilized equipment in sealed bag

b. Obtain wrapped and sterilized filters
c. Screw the detachable base of the outlet filter into the platform.

14. Attach the isolator filters (Figure 6). For each filter:
    a. Insert the metal connector on the filter into the adapter on the isolator bubble. Lubricate the connector as necessary with a non-corrosive liquid (Figure 6A).
    b. Insert the end of the PVC airflow valve into the outlet of the plastic sleeve (Figure 6B).
    c. Secure the filter units to the platform.
        i. Screw the rectangular base of the inlet filter into the platform.
        ii. Insert the bottom of the outlet filter unit into the metal ring of the detachable base.
    d. Wrap hose clamps around the connections and fasten using a flathead screwdriver or nut driver. Ensure a snug fit, but do not overtighten to avoid damaging the plastic adapters.
    e. The properly attached filter appears in Figure 6C.

15. Sterilize the isolator interior (Figure 7).

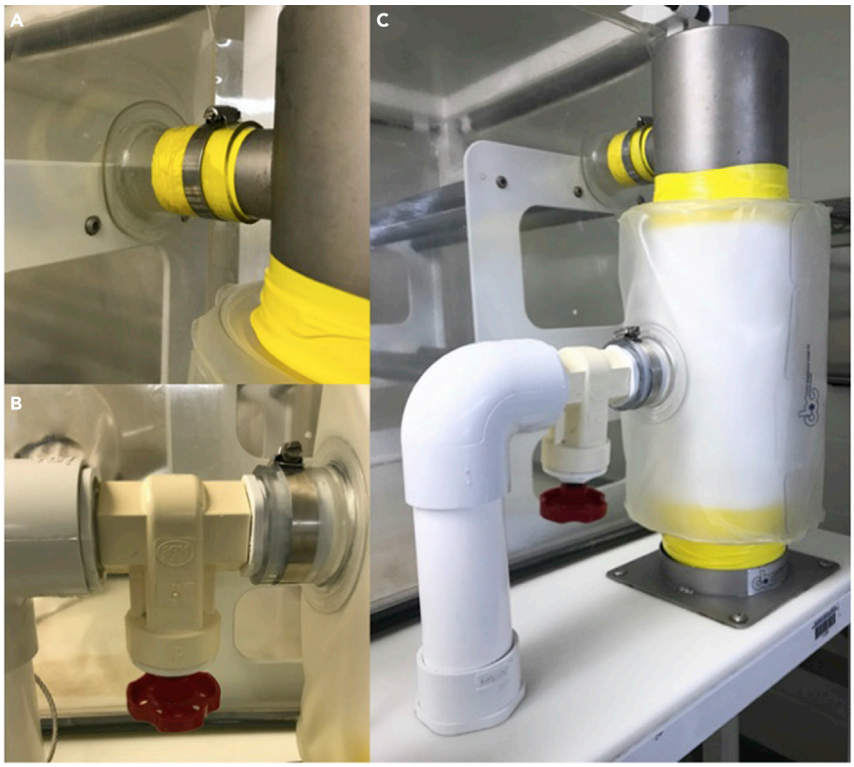

Figure 6. Connecting filters to isolator and air intake/outlet valves
(A) Installed filter connection to isolator adapter.
(B) Installed filter cover connection to PVC airflow valve.
(C) Installed negative pressure outflow filter.

a. Prepare 500 mL of Clidox®-S sterilant in a spray bottle in a 1:3:1 ratio, according to the manufacturer's instructions (https://www.pharmacal.com/clidox-s.html).

⚠ CRITICAL: Clidox®-S base and activator solutions and the prepared solution can cause skin irritation, respiratory irritation, and serious eye irritation. Wear face masks or respirator masks and appropriate eye protection while handling and spraying.

b. Spray to thoroughly cover all surfaces of the isolator interior with Clidox®-S. Be sure to cover any hard-to-reach surfaces, including the interior of the filter adapters, the base of the gloves, and the interior of the port (Figures 7A and 7B).
c. Use the entire length of the gloves to spread the Clidox®-S, covering the walls, floor mat, bagged ethylene oxide sterilized equipment, and any other loose items inside the isolator.
d. Pass the spray bottle with the remaining Clidox®-S out of the isolator.
e. Fog the isolator with Clidox®-S.
    i. Transfer the remaining Clidox®-S from the spray bottle to the atomizer reservoir.
    ii. Spray rubber stoppers with Clidox®-S and plug the outer port cover openings.
    iii. Pass the atomizer into the isolator through the port and attach the outer port cover over the hose. Start at the bottom and slide the outer port cover onto the port.
    iv. From inside the isolator, use the atomizer to fog the isolator (Figure 7C).
    v. Once a fog has developed, pass the atomizer into the port, and attach the inner port cover and band (Figure 7D).
    vi. Remove the outer port cover and atomizer.
f. Replace the outer port cover.
    i. Remove one of the rubber stoppers.

ii. Hold the outer port cover at the top where the seam is and thoroughly spray all interior surfaces with Clidox®-S. Be sure to spray inside of the nipples.
iii. Thoroughly spray the inside of the open port.
iv. Starting from the bottom and without touching the interior, slide the port cover on. It should be a snug fit without any wrinkles or air bubbles.
v. Place the neoprene band and patent clamp on the cover. Press the lever to tighten and lock the patent clamp.
vi. Fog the port by placing the atomizer nozzle in the port cover nipple without the rubber stopper and spraying (Figure 7E). Spray around the inside of the nipple and the tapered side of the rubber stopper. Place the rubber stopper in the opening.
vii. Allow the fogged isolator to sit for 5 h to achieve contact sterilization.

g. Begin negative pressure airflow.
i. Firmly thrust the straight end of the metal hook to puncture the Mylar membrane of the filter inlets.
ii. Turn on the airflow pumps.
iii. Open the valve knobs to initiate airflow through the isolator.

16. Attach a sterilizing cylinder to the isolator port and transfer its contents to the isolator (Figure 8).
a. Prepare 500 mL of Clidox®-S sterilant (1:3:1) in accordance with the manufacturer's instructions (https://www.pharmacal.com/clidox-s.html).
b. Set the sterilizing cylinder on a tiered rack in line with the isolator port and lock the wheels of the rack.
c. Open the outer isolator port cover.
i. Undo the patent clamp and pull off the black neoprene band.
ii. Remove the rubber stopper from one of the port cover openings.
iii. Pry the edges of the port cover off the port to break the seal, then pull the cover off.
d. Using a spray bottle, cover all sterilizing surfaces with Clidox®-S (Figure 8A).
i. The interior of the isolator port, including the outer rim.
ii. The inside of the transfer sleeve, including the plugs.
iii. The sterilizing cylinder membrane, rim, and outer edge.
e. Slide two large rubber bands around the outside of the transfer sleeve.
f. Wrap one sleeve opening around the isolator port, and the other around the end of the sterilizing cylinder (Figure 8B).
g. Slide the rubber bands up to secure the sterilizing cylinder and port to the sleeve (Figure 8C).
h. Use aerosolized Clidox®-S to sterilize the transfer sleeve interior (Figure 8D).
i. Remove the rubber stopper from one plug of the transfer sleeve.
ii. Place the nozzle of the atomizer into the sleeve plug and spray Clidox®-S until a fog develops throughout the sleeve interior.
iii. Spray the stopper surface and replace it in the plug.
iv. Allow the sleeve interior to sterilize for at least 5 h (Figure 8E).
v. Monitor the sleeve for the first 20 min of sterilization to ensure that it remains inflated. Significant deflation during this interval indicates a failed seal in the Mylar membrane, inner port cover, or sleeve connections.
i. Transfer the sterilizing cylinder contents to the isolator.
i. Open the inner port cover.
ii. Thrust the straight end of the metal hook to puncture the Mylar membrane on the sterilizing cylinder. Move the hook in a large circle to create a larger opening (Figure 8F).
iii. Grasp the looped string of the mesh laundry bag with the hook and pull the bag forward into the isolator port (Figure 8G).
iv. Open the bag and transfer the contents to the isolator interior.
j. Replace the inner port cover (Figure 8H).
k. Disconnect the sleeve from the port and sterilizing cylinder (Figure 8I).
l. Replace the outer port cover as in step 15f.

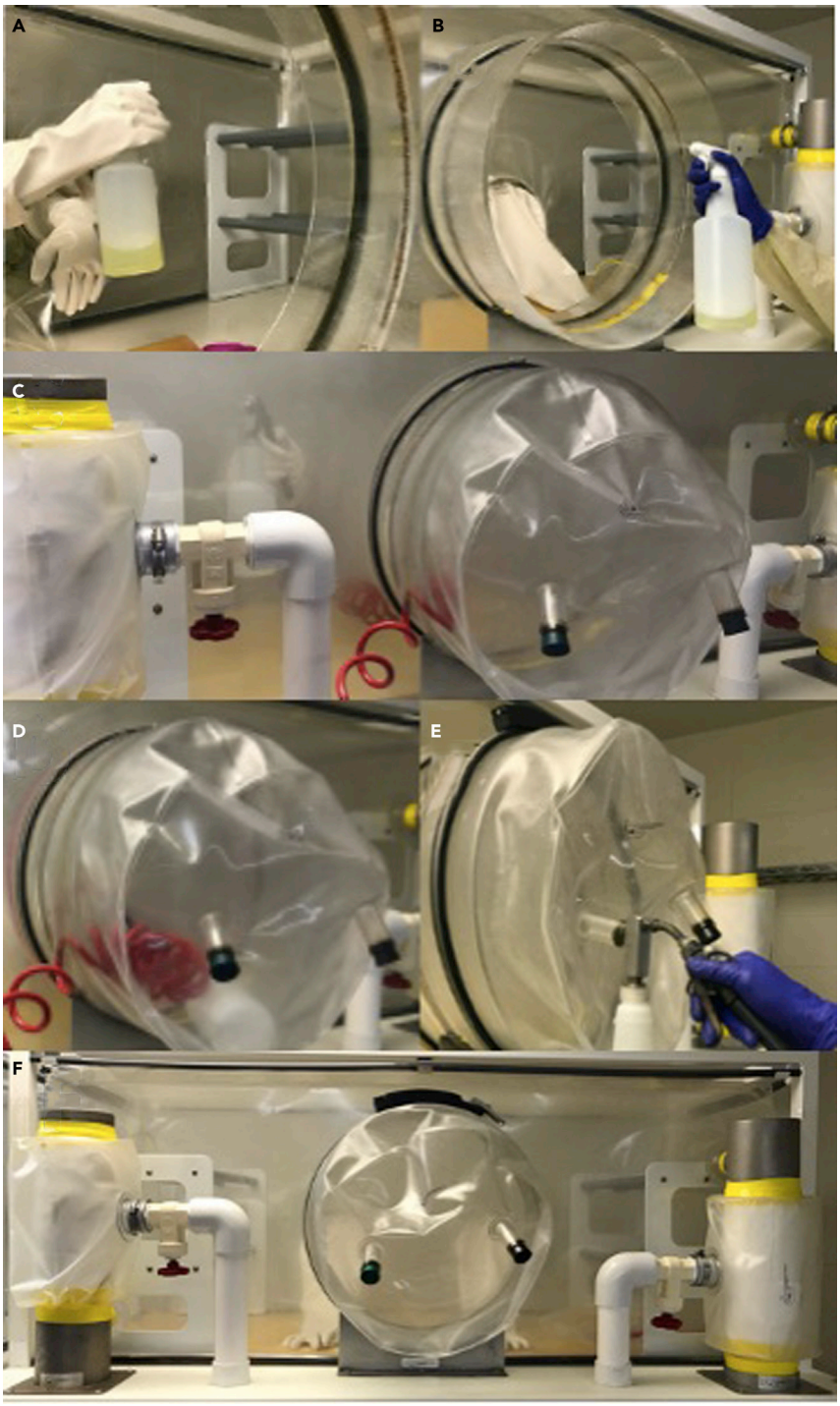

Figure 7. Setup and sterilization of isolator

(A) From the inside of the isolator, spray all surfaces thoroughly with Clidox®-S.

(B and C) Thoroughly spray the inside of the port and port cover with Clidox®-S before (C) passing in the atomizer and attaching the outer port cover. Use the atomizer to spray Clidox®-S into the isolator until a fog forms.

(D) Once isolator is fogged, pass the atomizer into the port, and replace the inner port cover and band.

(E) Sterilize the outer port cover, replace, and fog the port with Clidox®-S using the atomizer.

(F) Replace the rubber stopper and allow the fogged isolator to sit for 5 h to sterilize.

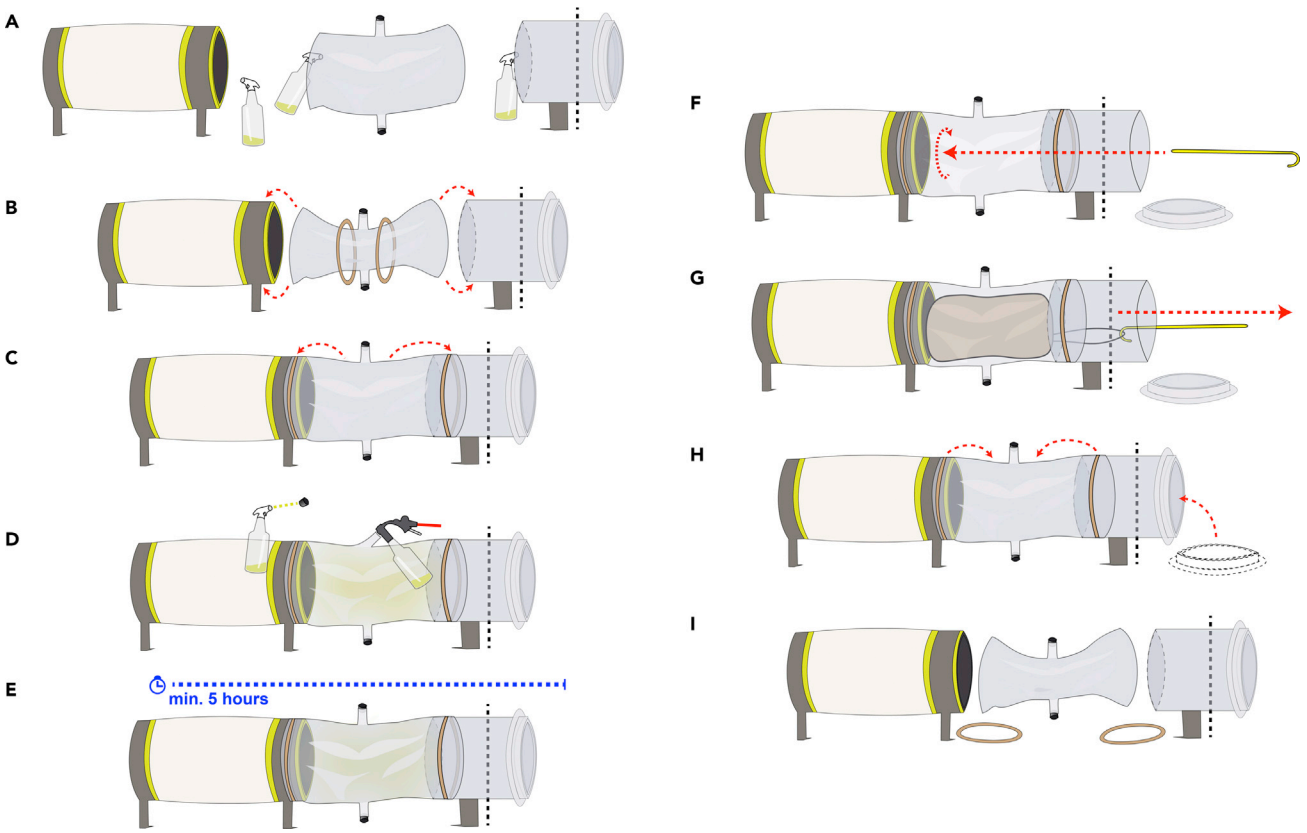

Figure 8. Schematic showing critical steps during the attachment of a sterilizing cylinder supply canister to the isolator port and the transfer of its contents to the isolator interior

In each panel, the vertical black dashed line marks the boundary between the isolator exterior (left) and interior (right).
(A) Sterilizing all surfaces of the transfer tunnel with Clidox®-S,
(B) wrapping the transfer sleeve around the port and sterilizing cylinder ends,
(C) securement of the transfer sleeve with rubber bands,
(D) fogging the transfer tunnel with Clidox®-S,
(E) sterilization of the transfer tunnel over at least 5 h,
(F) opening the sterilizing cylinder by puncturing the mylar,
(G) pulling the payload into the isolator,
(H and I) (H) replacing the inner port cover and pulling back the rubber bands, and (I) removing the transfer sleeve.

Note: Sterile supplies that can be exposed to Clidox®-S, such as pre-packaged irradiated lab markers, sterile 1 mL insulin syringes, and autoclaved bottles of water, may be introduced to the isolator by thoroughly spraying with Clidox®-S and placing in the port and then spraying the port as described in step 15f. Allow the items to sit for 5 hours before pulling into the isolator.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Clostridioides difficile	ATCC	Cat#43255
Paraclostridium bifermentans	ATCC	Cat#638
Chemicals, peptides, and recombinant proteins		
Blood Agar	Thermo Fisher Scientific	Cat#R01202

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Anaerobic Gas Mixture (10% hydrogen, 10% carbon dioxide, 80% nitrogen)	Medical Technical Gases	Cat#3131214H12
PRAS Brain Heart Infusion Broth	Anaerobe Systems	Cat#AS-872
Ethanol, 95%	DeCon Lab Inc	Cat#2801
Phosphate Buffered Saline (PBS)	Thermo Fisher Scientific	Cat#BP-3991
L-Cysteine hydrochloride	Sigma-Aldrich	Cat#C1276
Brucella Agar with sheep blood, hemin, and vitamin K	Thermo Fisher Scientific	Cat#R01255
Clidox®-S Sterilant	Pharmacal Research Laboratories	Activator: Cat#95120F Base: Cat#96120F
Ethylene Oxide Gas (ampules from Anprolene® AN71 Refill Kit)	Andersen Products	Cat#72212663
Compressed CO_2 gas	Medical-Technical Gases, Billerica, MA, USA	N/A
BBL Gram Crystal Violet	Becton Dickinson	Cat#212525
BBL Gram Iodine (Stabilized)	Becton Dickinson	Cat#212542
Acetone	Fisher Scientific	Cat#A949
BBL Gram Safranin	Becton Dickinson	Cat#212531
Experimental models: Organisms/strains		
Germ-free Swiss-Webster Mice (6–7 weeks), male and female	Massachusetts Host-Microbiome Center	Cat#SWGF
Other		
Sterile 50 mL Conical Tubes	Thermo Fisher Scientific	Cat#06-443-18
3M™ Comply™ (SteriGage™) Steam Integrator Load Record Cards	3M	Cat#4171MM
Hungate Tube (16 × 125 mm)	Bellco Glass	Cat#2047-16125
Anprolene® AN74i Classic Sterilizer	Andersen Products	Cat#981912
Anprolene® AN71 Refill Kit	Andersen Products	Cat#72212663
Exposure Indicator stickers	Andersen Products	Cat#AN-85
Ohaus CS-200. Portable Digital Scales.	Ohaus	Cat#72212663
AN-87 EO Gas Dosimeter	Andersen Products	Cat#AN-1087
Double negative pressure isolator	Class Biologically Clean, Madison, WI, USA	Cat#51602424182
DW4 Filter Media 36" × 75 yards	Class Biologically Clean, Madison, WI, USA	Cat#3903675
12" × 24" Sterilizing Cylinder	Class Biologically Clean, Madison, WI, USA	Cat#2812240FC
Stainless steel atomizer	Class Biologically Clean, Madison, WI, USA	Cat#395870A
12" × 12"20mil Frosty Vinyl Transfer Sleeve with 2-1" Nipples and Flanges	Class Biologically Clean, Madison, WI, USA	Cat#231212121
12" × 2" deep transfer port with flange and 2-1" nipples	Class Biologically Clean, Madison, WI, USA	Cat#2212223
12" × 7" ½" deep transfer port cap with 2-1" nipples	Class Biologically Clean, Madison, WI, USA	Cat#2212702
Hypalon 8" cuff, 15 mil thick 32" length, hand size 9	Class Biologically Clean, Madison, WI, USA	Cat#2611532891A
.001 Mylar film roll 54" × 100 feet	Class Biologically Clean, Madison, WI, USA	Cat#39105101
3M Vinyl Yellow Tape#471 1" Wide × 36 Yards long	Class Biologically Clean, Madison, WI, USA	Cat#3900031
North Hypalon Isolator Glove 15 mil, 14" Length, Ambidextrous, Size 9	Class Biologically Clean, Madison, WI, USA	Cat#263Y1549A
18" × 2" deep transfer port cap with flange	Class Biologically Clean, Madison, WI, USA	Cat#2218201
Mesh Laundry Bag, 36 × 24"	Uline, Pleasant Prairie, WI	Cat#S-22977
Alpha Chip Irradiated Bedding, 2.2 cu. ft.	Northeastern Products Corp., Warrensburg, NY, USA	N/A
Autoclavable Mouse Breeder Diet	LabDiet, St. Louis, MO, USA	Cat#5021
30 cm Jar Forceps	Thermo Fisher Scientific	Cat#13-820-077
Sterilized Trans-Tainer Tubes with Filter End Caps	Perotech	Cat#M-10 PCC
Straight Feeding Needle 20G	Kent Scientific	Cat#FNS-20-1.5-2
BD 1 mL Insulin Syringe with Slip Tip	BD	Cat#329654
Nunc Cryogenic Tubes	Thermo Fisher Scientific	Cat#375418
1.5 mL microcentrifuge tubes	Celltreat	Cat#229443
1,000 mL Polypropylene Graduated Tri-Corner Beaker	Thermo Fisher Scientific	Cat#14-955-111F
Superfrost/Plus Microscope slides	Thermo Fisher Scientific	Cat#12-550-15
Pre-packaged Irradiated LabMarker	Micronova	Cat#PEN-40IR
Olympus BX43 Light Microscope	Olympus Life Sciences	Cat#BX43

MATERIALS AND EQUIPMENT

Blood agar and Brucella agar with sheep blood, hemin, and vitamin K are available as prepared plates and should be used according to the manufacturer's instructions (https://tools.thermofisher.com/content/sfs/manuals/IFU1254.pdf). Plates in unopened plastic sleeves may be stored in the dark at 2°C–8°C until the expiration date stamped on the sleeve. Plates from opened sleeves should be stored at 2°C–8°C until just prior to use, and should be used within one week of opening.

STEP-BY-STEP METHOD DETAILS

Transferring germ-free mice

Timing: 30 min

Germ-free mice will need to be transferred from their home isolator into the experimental negative-pressure BSL-2 isolator. Prior to the transfer, Trans-Tainer transfer containers will need to be prepared in a sterilizing cylinder in the isolator the mice are coming from. The receiving negative-pressure containment isolator should be set up with prepared cages prior to the transfer so that the mice can be housed.

We recommend testing the sterility of the isolator and supplies with germ-free mice. Three germ-free mice should be transferred in, allowed to acclimate for one week, and monitored for sterility using the methods described in "Check for sterility or defined association of mice." Once the sterility of the isolator and supplies is confirmed, the experimental mice may be transferred in.

⚠ CRITICAL: The negative pressure should not be turned off if the isolator contains any BSL-2 agents. The protocol below describes the transfer of animals into a newly set up isolator. Negative pressure is turned off when transferring animals in to avoid exerting too much pressure on the inner port cover. If negative pressure is left on and the inner port cover is pulled, unfiltered, unsterilized ambient air and any Clidox®-S vapors in the port can be drawn into the isolator.

1. Loading mice to be transferred (Figures 9A–9D)
   a. Before beginning, prepare fresh Clidox®-S sterilant.
   b. Prepare Trans-Tainer tubes by placing one of the filter caps on and adding a small amount of bedding a few pellets of diet.
   c. Use forceps to hold mice at the base of the tail, gently lift and place in the Trans-Tainer tube(s). One Trans-Tainer tube can hold 3–5 mice depending on size and strain. Do not place mice from different litters in the same Trans-Tainer tube, as they may fight.
   d. Securely fasten the end cap to the open end of the tube.
   e. Set a timer for 20 min. It is important to work quickly to minimize the time the mice are in the tube.
   f. Remove the band and inner port cover and place the Trans-Tainer tube(s) in the port. Quickly replace the inner port cover and band.
   g. Remove the outer port cover and remove the Trans-Tainer tube(s).
      i. The port can be left without disinfecting with Clidox®-S and closing until after the transfer is complete following the procedure described in "before you begin" step 15f to sterilize and close the port.
2. Prepare the experimental negative-pressure BSL-2 isolator to receive mice (Figures 9E–9G).
   a. Turn off negative-pressure airflow by closing the valve.
   b. Remove the outer port cover.
   c. Place the Trans-Tainer tube(s) in the port. Thoroughly spray all surfaces of the tube(s) and the port with Clidox®-S. Spray the outer port cover, replace, and fog the port with Clidox®-S as described in "before you begin" step 15f.

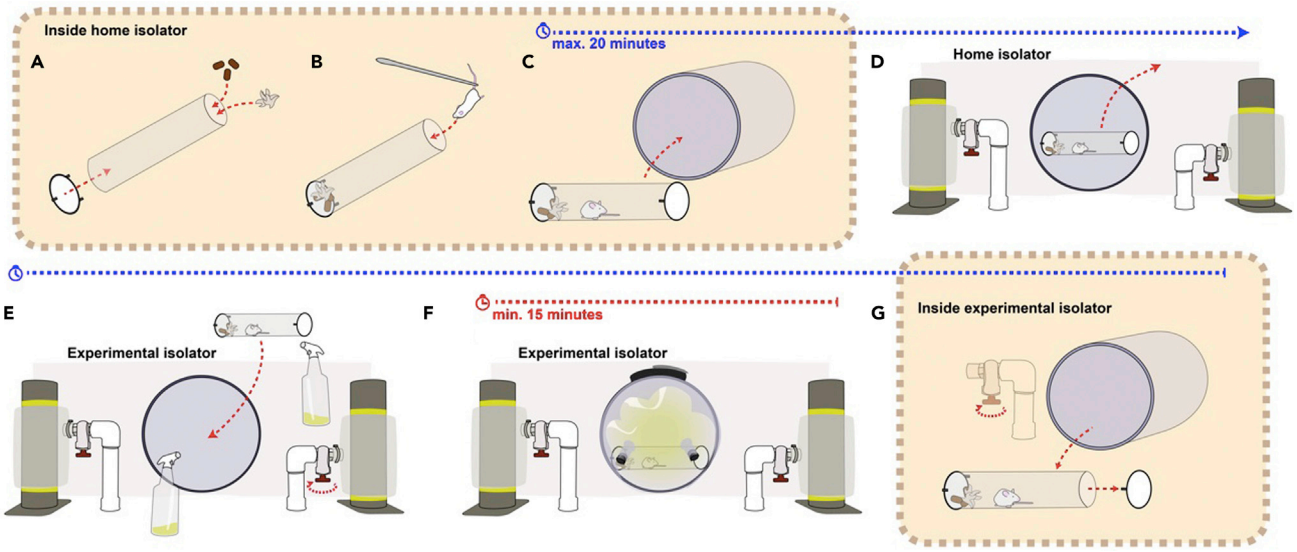

Figure 9. Schematic showing the transfer of germ-free mice using Trans-Tainer tubes

(A) Preparation of Trans-Tainer tube,

(B–D) loading of mice, and (C) transfer out of home isolator, and (D) move to the experimental isolator.

(E–G) Turning off negative pressure and spraying transferred mice in Trans-Tainer into the experimental isolator, and (F) fogging to sterilize port prior to (G) pulling mice into the isolator after 15 min of contact time.

i. Ideally, the physical transfer and closing of the port should take about 5 min.

d. Set another timer for 15 min. This is the time needed for the Clidox®-S to sterilize the port and outside of the Trans-Tainer tube(s).

e. Monitor the mice for signs of distress.

i. If you notice the mice display signs of distress (e.g., difficulty breathing, lethargy), remove the Trans-Tainer tube(s) from the port via the outer port cover and remove a filter cap by breaking one of the tabs. These mice are no longer germ-free and cannot be used for gnotobiotic experiments.

f. After 15 min, remove the inner port cover and pull the mice in. Turn negative pressure airflow back on.

g. Open a prepared cage and one end of the Trans-Tainer tube by breaking a tab. Carefully move the tube to cover the cage and angle the open side down so the mice move into the cage.

h. Close the cage and observe them for 1 or 2 min to ensure they are behaving normally.

i. Distribute mice to other cages as needed by picking them up by the base of the tail using forceps.

Alternatives: There are alternative means of transferring germ-free animals into isolators. Trans-Tainer tubes and TransDisks (Class Biologically Clean) allow for the transfer of mice from breeding isolators in a clean room to mice in a negative pressure containment isolator in a room dedicated for BSL-2 work. TransDisks (Class Biologically Clean) have separate compartments and are convenient for transferring both male and female mice or males from different litters. If using germ-free mice from a vendor or other source, mice may be kept in their transport cages and transferred using a Shipper Sleeve (Class Biologically Clean).

⚠ CRITICAL: Once mice are in the isolators, ensure the power supply remains connected. If the power supply is turned off, no air will enter the isolator and the mice will die.

Check for sterility or defined association of mice

Timing: 30 min, 2 days incubation

Mice should be checked for sterility or defined association weekly.

3. Fecal pellet collection from gnotobiotic mice
   a. Use long forceps to gently lift one mouse from the cage by the base of the tail and place into a tri-corner beaker.
   b. Wait ~5 min for mouse to produce two fecal pellets.
   c. While waiting, label one microcentrifuge tube with appropriate isolator number/letter and date.
   d. Upon fecal pellet production, return the mouse to the cage.
   e. Use forceps to gently remove the pellets from the beaker and place into the snap-cap tube.
   f. Open the inner port cover of the isolator and place the sample tube into the transfer port.
   g. Replace the inner port cover and band.
   h. Once the inner port cap is securely fastened, open the outer port cover, and remove the sample tube.
   i. Follow the procedure described in "before you begin" step 15 f to sterilize and close the port.
4. Plating
   a. Immediately homogenize the collected fecal pellet sample in 900μL of pre-reduced 1× PBS with 0.05% cysteine.
   b. Pipette 100 μL of the PBS on a blood agar and a Brucella agar plate and streak evenly across the entire surface of the plates.
   c. For each sample, pipette 100 μL of homogenate on one blood agar and one Brucella plate.
   d. Incubate the sample and PBS blood agar plates aerobically at 37°C and incubate the Brucella plates in the anaerobic chamber at 37°C.
   e. Read the PBS and sample blood agar plates for growth after 24 h and the Brucella plates for growth after 48 h (Figures 10A–10D).
      i. The PBS plates and germ-free fecal pellet plates should have no growth.
      ii. Ensure that plated samples from mono-associated mice appear pure.
5. Gram Staining
   a. After plating, resuspend the fecal homogenate by vortexing briefly.
   b. Pipette 10 μL of the homogenate onto a glass slide and allow to dry.
   c. Heat-fix the sample by quickly passing the slide over a flame a few times.
   d. Flood the slide with Gram Crystal Violet and let sit for 1 min.
   e. Rinse gently with water, being careful to avoid the stream hitting the sample directly.
   f. Flood the slide with Gram Iodine and let sit for 1 min.
   g. Thoroughly rinse the slide with a decolorizing solution consisting of 30% acetone and 70% ethanol until it runs clear.
   h. Rinse with water.
   i. Flood slide with Gram Safranin and let sit for 30 s.
   j. Thoroughly rinse slide with water and let dry.
   k. Observe the slide at 400–1000× magnification with a light microscope. Confirm the absence of bacterial or fungal cells from germ-free samples and that mono-associated mice are free from contamination by other bacteria or fungi (Figures 9E–9H).

Gavage with commensal bacteria

Timing: 1 h

Note: When handling animals in the isolator, wear cotton gloves over the isolator gloves to provide better grip.

⚠ CRITICAL: Separate isolators are needed for different gnotobiotic conditions as microorganisms easily spread throughout the isolator.

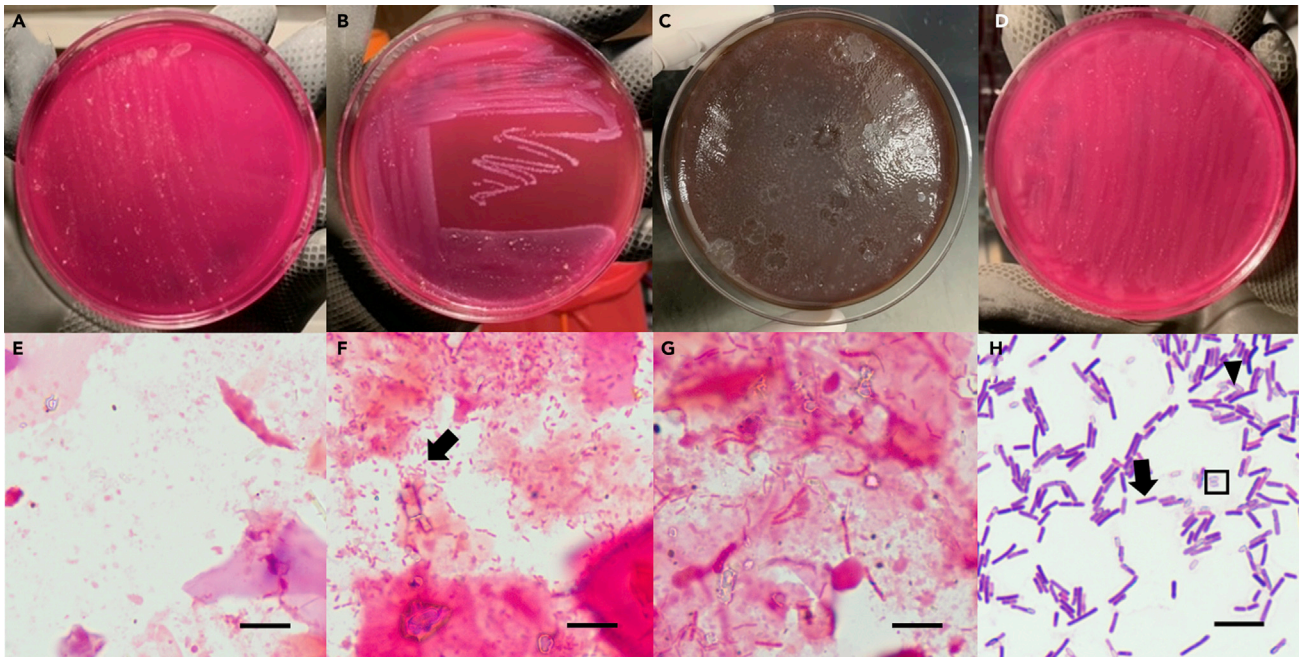

Figure 10. Plated and Gram-stained gnotobiotic fecal material to check sterility

(A–D) Plated feces from (A) germ-free, (B) mono-colonized with a commensal, (C) contaminated, and (D) mono-colonized with *C. difficile* mice.

(E–G) Gram-stained feces from (E) germ-free, (F) mono-colonized with a commensal Gram-negative bacteria (arrow), and (G) mice contaminated with environmental bacteria. Note the multiple morphologies present in the contaminated sample.

(H) Gram-positive *C. difficile* vegetative cells (arrow), sporulating cells (arrowhead), and spores (square) cultured from feces from a mouse mono-associated with *C. difficile*. 1000× magnification, scale bar is 10 μm.

6. Use freshly prepared Clidox®-S to spray the commensal inoculum Hungate tube and 1 mL syringe with safety needle.
   a. Replace outer port cover as described in "before you begin" step 15 f.
   b. Allow 30 min of exposure to Clidox®-S before pulling the items into the isolator.
   c. Open the 1 mL syringe and carefully pierce the septum of the Hungate tube to draw up the inoculum.
      i. Seal needle in the safety cap.
   d. Remove the capped needle and replace it with the gavage tube.
   e. Pick up a mouse by the base of the tail using the forceps and place on the cage lid.
   f. Restrain the mouse by holding by the skin at the nape of the neck, hold upright, and place the tail between the 4^{th} and 5^{th} fingers to hold firmly.
   g. Carefully introduce the gavage tube into the mouth and then into esophagus.
   h. Once the tube is placed, carefully inject 200uL of inoculum.
   i. Carefully remove the tube and return the mouse to its cage.
   j. Observe mouse for up to 5 min for normal behavior.
7. After 48 h, collect fecal pellets from the mice and check for defined association with the commensal bacteria as described in "Check for sterility or defined association of mice."

C. difficile infection

Timing: 1 h, 7 days after commensal inoculation

8. Following the same gavage procedure as described above, administer 200uL of 5×10^3 CFU/mL *C. difficile* spores to achieve a dose of 1×10^3 CFU.

Monitoring *C. difficile* infection

Timing: 1 h, 2× daily after *C. difficile* infection

9. Body weight measurement
   a. Place a tri-corner beaker on the scale weighing tray and tare.
   b. Use forceps to pick up a mouse and place in the beaker.
   c. Record the weight once the mouse is settled and the value stabilizes.
   d. Return the mouse to the cage and repeat.
10. Body condition scoring
   a. Observe the mice in their cages for *C. difficile* infection symptoms and general signs of pain and distress.
   b. Mice must be euthanized if they attain a score of 2-.

Body condition score	Symptoms or behaviors
3	Normal behavior and feeding with no signs of pain or distress
3-	Slight diarrhea (soft stool, less formed than typical pellet)
2+	Slight to moderate diarrhea (soft, wet stool that sticks to bedding and anus), anal inflammation, some signs of pain and distress (hunching, grimace, reduced activity), slight weight loss
2	Moderate diarrhea, anal inflammation, moderate pain and distress, moderate weight loss
2-	Severe diarrhea (runny stool with visible mucus and/or blood), profoundly reduced stool output, anal prolapse, significant weight loss, clear signs of pain and distress, unresponsive, non-ambulatory

⚠ CRITICAL: Evaluate prior recorded weights for a mouse when assigning a body condition score. Loss of 10–15% body weight over 2–3 days or 20% body weight overall contributes to a score of 2- and grounds for euthanasia.

Containment and decontamination of biohazardous material in isolator

Timing: 1 h active time, 8 h including disinfection and autoclaving

⚠ CRITICAL: Before starting, ensure all required equipment for BSL-2 waste management.

BSL-2 wastes and isolator components must be contained and decontaminated at the end of the experiment. Proper decontamination of the isolator, filters, and covers is necessary to prepare them for use in future gnotobiotic experiments.

11. Contain all materials and instruments in the isolator in double autoclavable biohazard bags.
   a. Using freshly prepared Clidox®-S, spray autoclavable biohazard bags and steam integrator strips into the isolator port and replace outer port cover as described in the section "before you begin" step 15f. Allow 15 min of contact time before pulling them into the isolator.
   b. Collect cages, cups, feeding needles, and other instruments in a double biohazard bag with a chemical integrator strip and tie each off. Collect all bedding, diet, and other disposable material in a separate double biohazard bag. Collect scale in a separate double biohazard bag.
   c. Remove the inner port cover. Remove a rubber stopper from the outer port cover and fog the chamber and materials with Clidox®-S sterilant using the atomizer through the opening. Allow to sit for 5 h for contact sterilization to occur.
   d. Pull bagged waste and materials out of the isolator. Dispose of waste in the appropriate biohazard waste containers.

e. Sterilize the bagged scale by ethylene oxide sterilization as described in "before you begin". Ensure the dosimeter indicates the cycle has passed before removing the scale from the bag.

12. Break down, contain, and decontaminate isolator components.
    a. Turn off positive and negative pressure air pumps by unplugging power supply.
    b. Disconnect filters by loosening hose clamps and screws and detach from the isolator. Remove vinyl tape and plastic covers.
    c. Spray all surfaces of plastic covers with Clidox®-S sterilant.
        i. Allow 30 min of contact time before wiping.
    d. Place filter frames in a double autoclavable biohazard bag. Double knot to close.
    e. Decontaminate bagged cages, instruments, and filter frames by autoclaving at 129°C and 30 PSI for at least 20 min. Ensure that the steam integrator strips indicate that the cycle passed. If not, the materials will need to be re-autoclaved until they are properly sterilized.

EXPECTED OUTCOMES

This gnotobiotic model allows for the study of specific commensal bacterial species on *C. difficile* infection. Germ-free mice infected with 1×10^3 CFU *C. difficile* spores exhibit a clear disease phenotype 20 h post-infection, approximately 4 h after bacterial transit through the germ-free intestine, that manifests by weight loss, diarrhea, and worsening symptoms. The dose of spores described for strain ATCC43255 typically leads to a body condition score of 2- in all mice within 48 h post-infection.

Commensal species may vary in their mono-colonization of the gut, so commensal biomass should be checked at least once in fecal pellets before infection. For a gavage of 1×10^8 CFU of *P. bifermentans*, a biomass of 4.8×10^6–6.5×10^7 CFU/g of cecal contents is generally attained 7 days after inoculation. Colonization with *P. bifermentans* at this biomass protected against lethal *C. difficile* disease.

LIMITATIONS

Due to the resource-intensive nature of setting up and maintaining gnotobiotic isolators, it is important to consider the number of isolators needed to conduct an experiment. Separate isolators are needed to house mice with different gnotobiotic conditions, as bacteria readily spread throughout the entire isolator.

The methods described to monitor sterility or specific association of experimental animals are most suitable for the screening and identification of bacteria. Contamination by fungi may be detected by visualization of Gram-stained samples, but contamination by viruses or parasites may evade detection.

TROUBLESHOOTING

Problem 1

Signs of contamination by other microbes in inoculum (steps 4f, 7a in before you begin).

Potential solution

Prepare fresh inoculum and retest for purity. Due to the time and resource-intensive nature of gnotobiotic work, it is crucial to test for contamination at many points throughout the workflow. Be certain to follow strict aseptic technique in preparing and handling inocula to reduce risks for contamination.

Problem 2

Failure to achieve negative pressure in the isolator (step 15 g in before you begin).

Potential solution

Ensure that the negative pressure pump is on, and the valve is open by turning it clockwise. If this is not the source of the problem, check the connections to the outlet filter. The hose clamps should form a tight seal around the connection between the isolator adapter and filter connector as well as the connection between the filter plastic cover and PVC outlet valve. Care should be taken to avoid overtightening the hose clamps, as they can damage or puncture these adapters and compromise airflow and containment. It is crucial to include the rigid plastic insert between the outlet filter and the plastic cover. Without the insert, the cover will become suctioned to the filter due to the negative pressure, restricting airflow.

Problem 3

Growth on germ-free fecal pellet plate and suspected contamination (step-by-step method details 4e).

Potential solution

If few isolated colonies appear on a germ-free fecal pellet plate or if a few colonies of growth other than the defined bacteria appear on a plate, environmental contamination (i.e., fallen dust or debris) has likely occurred during plating. If there is significant unexpected growth (i.e., a "lawn" of growth) across the plate, the sample may have been contaminated during collection or the mice in the isolator have been contaminated.

Contamination of any kind can be confirmed by collecting a fresh set of fecal pellets from one or more mice in the applicable isolator and repeating the plating and staining procedures. Environmental contamination can be avoided by paying greater attention to sterile technique. If significant unexpected growth appears again, the cohort of mice in the isolator should be euthanized and the isolator should be thoroughly disinfected before restarting experimentation.

Sources of contamination and their causes include (Lavin et al., 2018):

Temperature-resistant spore-forming organisms which may produce spores that survive insufficient autoclave cycles. These organisms include thermophilic *Clostridium* or *Bacillus* species and some fungal *Streptomycetes* species. This issue can be addressed by checking autoclave logs to ensure proper temperatures were reached for a sufficient duration and by running biological indicators.

Contamination by common skin flora. These include species of *Staphylococcus* or *Corynebacterium* that can enter through damaged gloves. Both sides of the gloves should be checked regularly for signs of damage. Interior cotton gloves should always be worn when handling mice and materials to avoid punctures and should be examined.

Environmental contaminants, typically fungi or hardy bacterial species, can enter the isolator through tears or through the port if insufficiently sterilized. All surfaces of the isolator, port covers, and sleeves, especially those that are regularly manipulated (e.g., transfer sleeves, port covers, surfaces near the glove openings etc.) should be examined for wear. Also check the rubber band, stoppers, and clamp to ensure the port covers are properly in place as well as the HEPA filter media.

Atypical flora, especially contamination with multiple species could result from contaminated materials or inoculum. Ensure that all cultures entering the isolator are tested for purity. Check the log of when materials were moved into the isolator to determine the source of the contamination.

Problem 4

Failure to colonize germ-free mice with commensal bacteria of interest (step-by-step method details 7).

Potential solution

This issue is relatively rare but can occur with obligate anaerobes and fastidious species. The cultures should be stored appropriately and moved into the isolator as quickly as possible. Cultures of obligate anaerobes should be kept in sealed Hungate tubes prepared in an anaerobic chamber with pre-reduced media containing a reducing agent such as cysteine. Apart from these considerations, try inoculating mice with a higher concentration of cells. If working with a spore-former, it is best to use spores when possible as they are more resistant to changes in ambient conditions.

Problem 5

Sterilization cycle fails (before you begin steps 8 and 11, step-by-step method details steps 11 and 12).

Potential solution

It is imperative that all materials entering the isolator are sterilized. If the steam integrator, chemical dosimeter, or sterilization run log indicate an incomplete cycle, re-run the cycle with a new indicator. Insufficiently sterilized materials should never be placed inside the isolator as they pose a contamination risk. Similarly, all materials from an active BSL-2 isolator must be properly disinfected before reuse.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contacts or communicating author, Aidan Pavao (apavao2@bwh.harvard.edu) and Madeline Graham (mgraham0@bwh.harvard.edu) or Lynn Bry (lbry@bwh.harvard.edu).

Materials availability

This study did not generate any new unique reagents.

Data and code availability

This study did not generate/analyze datasets or code.

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AUTHOR CONTRIBUTIONS

A.P., M.G., O.T., M.D., and V.Y. wrote the manuscript and generated figures and reagent supply tables. L.B. provided oversight and editing.

DECLARATION OF INTERESTS

L.B. is an inventor on patents for defined live bacteriotherapeutic products against *C. difficile* and is the scientific founder, SAB chair, and a stockholder in Pareto Biosciences, Inc.

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