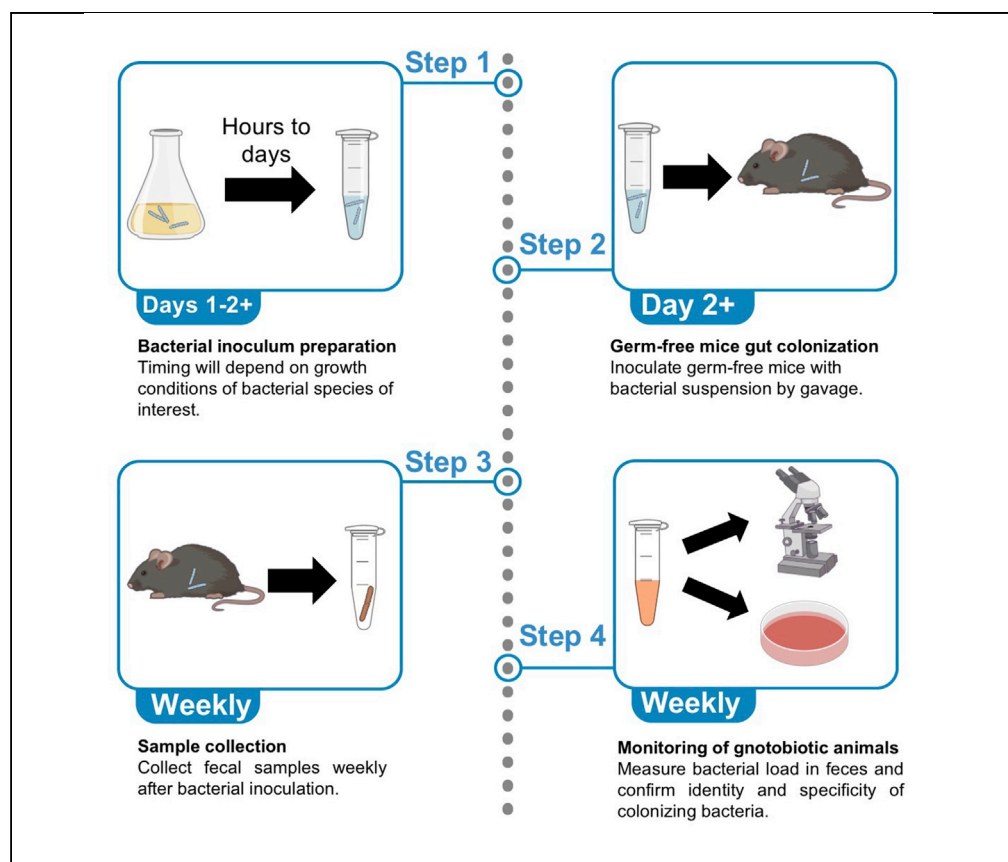


Protocol

Generation, maintenance, and monitoring of gnotobiotic mice



Gnotobiology has revolutionized the study of microbiota-host interactions. This protocol explains how to generate, maintain, and monitor gnotobiotic mice. Three monitoring methods are presented and compared: bacterial culture, microscopy to visualize the presence (or absence) of bacteria using Gram staining or DNA staining, and 16S rRNA gene amplification and sequencing. The generation and maintenance of gnotobiotic animals should be performed in a germ-free and gnotobiotic facility to guarantee sterility and precision of gnotobiotic conditions.

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Highlights

Gnotobiotic (GB)
mice are powerful
tools for the study of
microbiota-host
interactions

GB mice are
generated by
introducing specific
bacteria into the gut
of germ-free mice

Gut colonization must
be monitored to
ensure precise
gnotobiotic
conditions

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Protocol

Generation, maintenance, and monitoring of gnotobiotic mice

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SUMMARY

Gnotobiology has revolutionized the study of microbiota-host interactions. This protocol explains how to generate, maintain, and monitor gnotobiotic mice. Three monitoring methods are presented and compared: bacterial culture, microscopy to visualize the presence (or absence) of bacteria using Gram staining or DNA staining, and 16S rRNA gene amplification and sequencing. The generation and maintenance of gnotobiotic animals should be performed in a germ-free and gnotobiotic facility to guarantee sterility and precision of gnotobiotic conditions.

For complete details on the use and execution of this protocol, please refer to McDonald et al., 2020.

BEFORE YOU BEGIN

Sterilize materials for generation of gnotobiotic animals

⌚ Timing: 5 h

This section describes the preparation of materials necessary for the generation of gnotobiotic mice (i.e., mice with precisely defined microbiota composition) from germ-free (GF) mice using a single gavage of bacteria.

1. Sterilize gavage kit

- a. Sterilize a stainless-steel gavage needle by baking at 180°C for 3 h.
- b. Add the following to an autoclavable container (Figure 1A):
 - i. Sterilized gavage needle
 - ii. 1 × 1 mL syringe (pull apart the barrel and plunger)
 - iii. 1 × 50-mL conical tube, uncapped
 - iv. 1 × 2 mL microcentrifuge tubes, uncapped (1 tube per 5 mice)
 - v. 1 × 1.5 mL microcentrifuge tube, cap open (1 tube per cage of mice) for collection of fecal samples (to confirm germ-free status prior to inoculation).
- c. Double-wrap the container in autoclavable drapes by wrapping in an inner drape (rectangular fold) followed by an outer drape (diamond fold). Seal the package with autoclave tape (Figure 2).



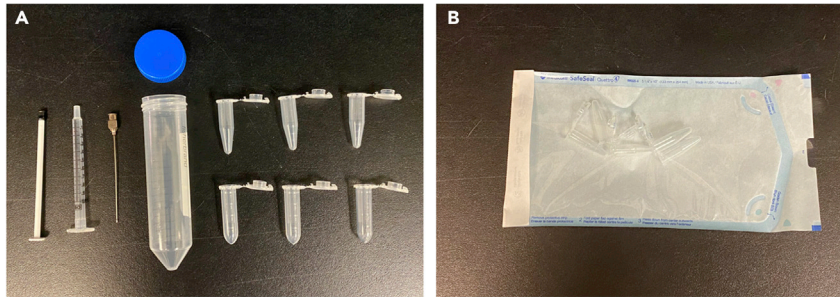


Figure 1. Materials for the generation and monitoring of gnotobiotic mice

(A) Materials for the generation of gnotobiotic mice, including syringe, gavage needle, conical tube to wash the bacterial culture, 2 mL microcentrifuge tubes to store the bacterial inoculum and pre-weighted 1.5 mL microcentrifuge tubes for fecal sampling of germ-free mice prior to bacterial inoculation. The materials must be placed in an autoclavable container and wrapped in autoclavable drapes.

(B) Materials for the monitoring of germ-free or gnotobiotic mice by fecal sampling. 1.5 mL microcentrifuge tubes are placed in an autoclavable envelope and must be wrapped in autoclavable drapes.

- d. Autoclave the wrapped gavage kit (121°C, 20 min, 120.7–122.8 kPa).
2. Sterilize surgical drapes and gowns
 - a. For each experimental group (i.e., gnotobiotic condition) you will need two surgical drapes to create a sterile work surface, and two surgical gowns for the researcher.
 - b. Obtain surgical drapes that are sufficiently large to cover the bottom of a biosafety cabinet (BSC). Accordion-fold the surgical drapes and double-wrap the drape within an inner drape (rectangular fold) followed by an outer drape (diamond fold) (as shown in [Figure 2](#)). Seal the package with autoclave tape.
 - c. Fold the surgical gown inside-out, and double-wrap the gown within an inner drape (rectangular fold) followed by an outer drape (diamond fold) (As shown in [Figure 2](#)). Seal the package with autoclave tape.
 - d. Autoclave the wrapped drapes and gowns (121°C, 20 min, 120.7–122.8 kPa).

Prepare bacterial culture

⌚ Timing: hours to days

3. Prepare a bacterial broth culture sufficient for 1×10^8 CFU per animal. Prepare at least 30% extra volume of culture to account for losses during pipetting, as well as dead-space volume of gavage syringe. As an example from the associated reference article ([McDonald et al., 2020](#)), *Lactobacillus intestinalis* ASF360 is cultured in 10 mL of De Man, Rogosa and Sharpe (MRS) broth for 48 h under anaerobic condition (10% CO₂, 10% H₂ and balanced N₂). In our hands, 1 mL of culture contains approximately 1×10^8 CFU.

⚠ **CRITICAL:** Please note the time required for this step will depend on the growth kinetics and culture conditions of the bacteria being studied. Of note, we have suggested 1×10^8 CFU per animal as an example based on the reference article for this protocol ([McDonald et al., 2020](#)). However, for any new gnotobiotic study, we recommend pilot experiments to determine both the bacterial growth kinetics in culture, as well as the optimal CFU for inoculation of GF mice.

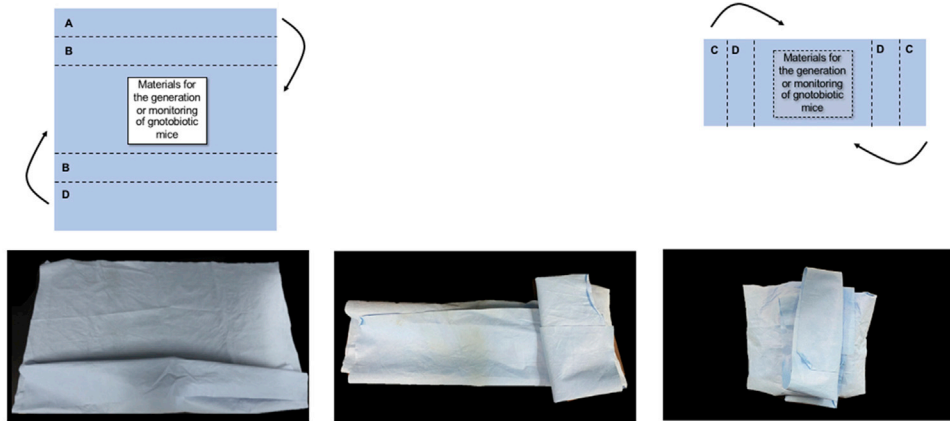
Biosafety cabinet decontamination

⌚ Timing: 30 min

PART 1: INNER DRAPE

Step 1: Place materials for the generation or monitoring of gnotobiotic mice enclosed in an autoclavable container in the center of the inner drape. Accordion-fold sections A and B of the inner drape, covering the materials.

Step 2: Accordion-fold sections C and D of the inner drape, adding another layer on top of the enclosed materials



PART 2: OUTER DRAPE

Step 3: Place the folded inner drape in the center of the outer drape. Fold the drape in half following the instructions below. Secure with autoclavable tape.

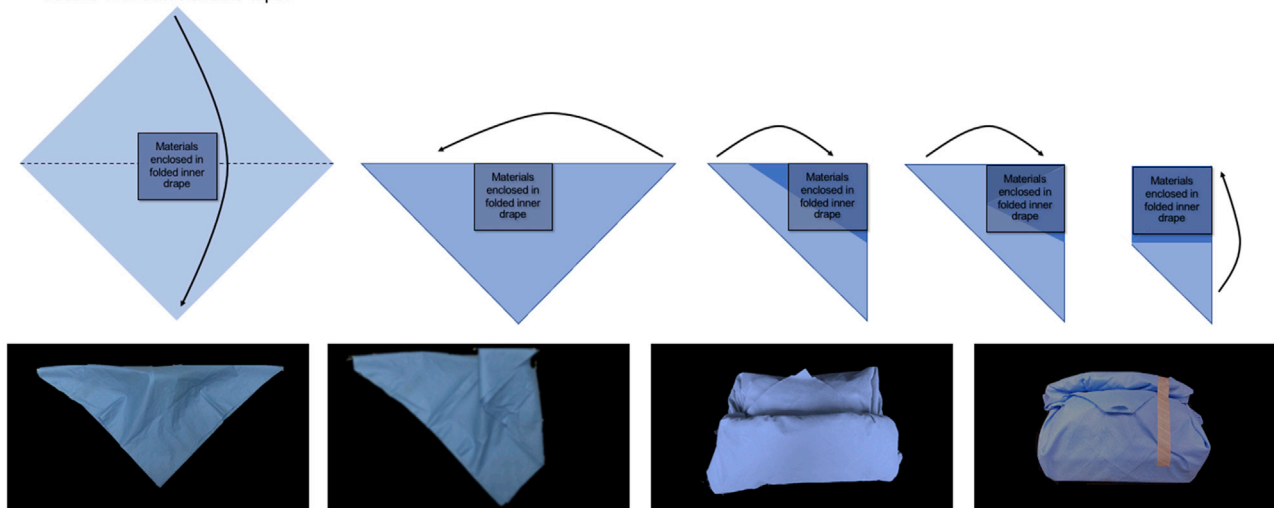


Figure 2. Instructions for wrapping of materials for autoclaving

Any autoclavable material to be used for handling, inoculation, sampling, or monitoring of gnotobiotic mice must be double wrapped with autoclavable drapes. This includes: materials for generation and monitoring of gnotobiotic mice, drapes to cover the bottom of the decontaminated BSC, and surgical gowns.

This section includes procedures necessary for the preparation of a sterile experimental work area within a BSC for animal handling and inoculation. A BSC decontamination is required any time gnotobiotic animals are handled and is therefore critical for all steps in the generation, maintenance, and monitoring of gnotobiotic animals.

4. Turn on BSC laminar air flow.
5. Spray Virkon™ 1% solution thoroughly on surfaces (bottom and walls). Wipe with autoclaved paper towels after 10 min.
6. Repeat step 5.
7. Spray all surfaces with 70% ethanol solution. Wipe with autoclaved paper towels after 10 min.

8. Repeat coating of all surfaces with 70% ethanol solution and allow it to air dry (do not wipe).

△ **CRITICAL:** After use, spray BSC thoroughly with water to remove residual Virkon™ to avoid corrosion of metal surfaces.

Sterilize materials for monitoring of germ-free and gnotobiotic animals

⌚ Timing: 2 h

This section describes the preparation of materials necessary for the monitoring of germ-free or mono-/oligo-colonized gnotobiotic animals using fecal samples.

9. Sterilize materials for fecal sampling
 - a. Add microcentrifuge 1.5 mL tubes, cap open, for collection of mouse fecal samples (at least one tube per animal) to an autoclavable container (i.e., plastic bucket or envelope) ([Figure 1B](#)).
 - b. Double-wrap container with tubes within an inner drape (rectangular fold) followed by an outer drape (diamond fold). Seal the package with autoclave tape.
 - c. Autoclave (121°C, 20 min, 120.7–122.8 kPa).
10. Prepare and sterilize one surgical drape and gown per experimental group (i.e., gnotobiotic condition) following step 2 under [Sterilize materials for generation of gnotobiotic animals](#) section above.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
<i>Lactobacillus intestinalis</i> ASF360	(Geuking et al., 2011)	N/A
Chemicals, peptides, and recombinant proteins		
5× GoTaq® Flexi Reaction Buffer	Promega	M8901
Crystal Violet	VWR	10143-182
De Man, Rogosa and Sharpe (MRS) Broth	Sigma-Aldrich	69966
dNTP Mix	Promega	U1511
GoTaq G2 Hot Start Polymerase	Promega	M7401
Paraformaldehyde, 97%	Alfa Aesar	A11313
Safranin	VWR	10143-254
Stabilized Iodine	VWR	10143-256
SYTOX™ Green Nucleic Acid Stain - 5 mM Solution in DMSO	Invitrogen	S7020
Critical commercial assays		
MinElute PCR Purification Kit	QIAGEN	28004
QIAamp PowerFecal Pro DNA Kit	QIAGEN	51804
Experimental models: organisms/strains		
Germ-free C57BL/6 mice (<i>Mus musculus</i>), male, 6- to 12-week-old	International Microbiome Centre (Calgary, Canada)	N/A
Oligonucleotides		
fD1: AGA GTT TGA TCC TGG CTC AG	(Wyss et al., 2020)	N/A
fD2: AGA GTT TGA TCA TGG CTC AG	(Wyss et al., 2020)	N/A
rP1: ACG GTT ACC TTG TTA CGA CTT	(Wyss et al., 2020)	N/A
Software and algorithms		
Nucleotide BLAST	NCBI	blast.ncbi.nlm.nih.gov/

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
Stainless-steel gavage needle	N/A	Gauge size 8 to 12 for C57BL6 mice 6-week-old or older. Gauge size 8 or 10 for C57BL6 mice younger than 6 weeks. Length: 4 to 6 cm
Medical drape (single ply sterilization wrap)	MEDLINE	GEM2140
Nitrile Powder-Free Sterile Surgical Gloves	NitriDerm	Various
Virkon™ S tablets	Virkon	N/A

MATERIALS AND EQUIPMENT

PCR mix for amplification of the 16S rRNA gene (full-length)			
Reagent	Stock concentration	Final concentration	Volume per reaction (μL)
5× Green GoTaq Flexi buffer	5×	1×	5
dNTP Mix	10 mM	200 μM	0.5
Primer fD1	10 μM	200 nM	0.5
Primer fD2	10 μM	200 nM	0.5
Primer rP1	10 μM	200 nM	0.5
DNA	Approximately 100 ng/μL	Approximately 4 ng/μL	1
GoTaq G2 Hot Start Polymerase	5 units/μL	0.05 units/μL	0.25
Molecular grade H ₂ O	N/A	N/A	16.75
Total	N/A	N/A	25

STEP-BY-STEP METHOD DETAILS

The steps described in this section must be performed by two people unless stated otherwise. One person will don a sterile surgical gown and sterile gloves and be responsible for handling the sterile materials and experimental tasks within a biosafety cabinet (BSC). The other will remain “non-sterile” and assist with all tasks that require contact with non-sterile materials.

Prepare materials for inoculation by gavage

⌚ Timing: 60 min

The gavage kit will contain all materials necessary to introduce a bacterial species or consortium into the gastrointestinal tract of a germ-free animal.

- Set up:
 - Decontaminate BSC to create a sterile working surface following the steps above under [biosafety cabinet decontamination](#) (steps 4–8 of [before you begin](#) section).
 - Wash hands with antiseptic soap, and don sterile surgical gown and gloves. Refrain from making contact with the inner surface of the decontaminated biosafety cabinet while performing the next steps.
 - Cover the bottom of a decontaminated biosafety cabinet with a sterile drape, thus creating a sterile work surface.
 - Clearly delineate a working area for the person handling the sterile materials and a working area for the individual handling materials which are not sterile on the outside, e.g., bacterial culture flask, phosphate-buffered saline for washing, micropipette.
 - Open the “gavage kit” (described above in step 1 of the [before you begin](#) section) and place the sterile internal contents on the sterile drape-covered working surface.

2. Prepare the bacterial inoculum for gavage:
 - a. Transfer the cultured bacteria (see step 3 of section [before you begin](#) - prepare bacterial culture) into a conical tube and centrifuge at 4°C, 3000 g, for 10 min.
 - b. Discard supernatant and resuspend the pellet of bacteria in phosphate-buffered saline (PBS) to the desired concentration/volume for gavage, which we now refer to as the inoculum. For adult mice, a gavage volume of 0.2–0.4 mL is recommended.
 - c. Transfer inoculum into sterile 2 mL tubes using a 2-person technique within the sterile work surface in the BSC to ensure that the outside of the tubes remains sterile for later use in the germ-free and gnotobiotic facility. This is accomplished by having the gowned and gloved person hold the 2 mL tube while the assistant transfers the inoculum with a sterile micropipette tip without touching the tube.
3. Place the tube(s) containing the prepared inoculum into the sterile “gavage kit”.
 - a. Under sterile conditions, place the bacterial inoculum tube(s) into the “gavage kit” (containing the gavage needle and syringe) and seal properly. The internal contents of this container are now sterile and should only be opened within the sterile work area of a decontaminated BSC.

Inoculation of germ-free mice by gavage

⌚ Timing: 1 or more h (depending on the number of mice and experimental groups)

To guarantee gnotobiotic conditions of your experiment, the next steps should be performed in a specialized animal facility with specific infrastructure and engineering for housing germ-free and gnotobiotic mice.

4. Decontaminate BSC to create a sterile working surface as described above in steps 4–8 of the section [before you begin](#), [biosafety cabinet decontamination](#).
5. Import germ-free animals into the BSC. If animals are being kept in an isolator, they must be imported through a sterilized sleeve. If animals are being kept in isocages, the outside of the cage must be decontaminated with Virkon™ before handling.
6. Open the gavage kit within the BSC and remove the sterile internal contents. Attach the gavage needle to the 1 mL syringe and draw up 0.2 mL of bacterial inoculum (volume required for gavage of one mouse).
7. Restrain the mouse, hold upright, insert tip of gavage needle into the stomach of the animal, and administer the bacterial inoculum. Return mouse to the sterile isocage. Gnotobiotic animals must be maintained under otherwise sterile conditions to avoid contamination from environmental sources (ideally in a specialized animal facility that is engineered to house germ-free and gnotobiotic mice). Of note, the cage, water, chow and bedding must be autoclaved and sterile. For a cage of 5 mice, the sterile water and chow should be changed weekly, and the bedding every 2 weeks.

Monitoring of germ-free and gnotobiotic conditions in mice

⌚ Timing: 1 h to 3 days (depending on the method and number of samples)

Steps 10 to 13 may be performed in a standard laboratory by one person, with sample handling performed inside a biosafety cabinet to avoid contamination. Please note that there are multiple methods to monitor the colonization status of gnotobiotic animals. This section describes and compares three common methods that are optimized for analysis of monocolonized gnotobiotic mice, and how these methods can be used to confirm successful colonization as well as rule-out contamination: bacterial culture, microscopy to visualize the presence (or absence) of bacteria using Gram staining or nuclear DNA staining, and 16S rRNA gene amplification and sequencing.

8. Following colonization by gavage, fecal samples should be collected at least once per week to monitor gnotobiotic status. For all fecal sampling:
 - a. Decontaminate a BCS and create a sterile work surface as described above in steps 4–8 of the section [before you begin, biosafety cabinet decontamination](#).
 - b. Open autoclaved 1.5 mL microcentrifuge tubes onto your sterile work surface (see steps 9 and 10 of section [before you begin](#) above). The number of tubes and number of fecal pellets required varies depending on the monitoring method to be used: For microbiologic culture and Gram staining, collect 1 fecal pellet in 1 tube per mouse; for SYTOXTM Green staining, collect 3–5 fecal pellets in 1 tube per mouse, for 16S rRNA gene amplification and sequencing collect 1 fecal pellet in 1 tube per mouse. Thus, if collecting for all four monitoring methods, three 1.5 mL microcentrifuge tubes are needed per animal.
 - c. Don a sterile surgical gown and sterile gloves and import isocages containing gnotobiotic mice into decontaminated BSC (as described above in step 5) and collect under otherwise sterile conditions.
9. Microcentrifuge tubes containing fecal samples can now be transported to the laboratory for further analysis.
10. Microbiological culture from a fresh fecal sample (10 – 20 min per sample)
 - a. Weigh fecal pellets and add sterile PBS at a concentration of 100 mg/mL. Mix using a vortex mixer for 1 min until a homogeneous suspension is achieved.
 - b. Prepare 10-fold serial dilutions in sterile PBS, and plate 10 μ L of each dilution in triplicate on agar plates. For example, *L. intestinalis* ASF360 is cultured on MRS agar plates at 37°C under anaerobic conditions.
 - c. Incubate in conditions appropriate for the bacterial species or consortium of choice.
 - d. Count CFU, and express results as CFU/g of feces.
11. Sytox Green Staining (2–3 h)
 - a. Homogenize feces (3–5 fresh fecal pellets) in 0.3 mL of sterile 1 \times PBS by vortex mixing.
 - b. Add 0.15 mL of the homogenate to 0.15 mL of 4% paraformaldehyde stock solution (m/v, in PBS, pH = 7.2). Mix using a vortex mixer.
 - c. Incubate for 45 min at room temperature (21°C–25°C).
 - d. In a new tube, prepare a SYTOXTM green working solution by adding 1.5 μ L of 5mM SYTOXTM green nucleic acid stain into 0.9 mL of 1 \times PBS.
 - e. Add 0.1 mL of the fixed fecal homogenate into the SYTOXTM green working solution.
 - f. Protect from light and incubate at room temperature for 1 h.
 - g. Spread 25 μ L on a glass slide and air dry.
 - h. Visualize using a fluorescence microscope at 400 \times to 1000 \times magnification. SYTOXTM green excitation/emission: 504/523 nm.
12. Gram Staining (10 min per sample)
 - a. Homogenize feces as described in step 10a.
 - b. Add 10 μ L of fecal homogenate onto a glass slide. Air dry.
 - c. Fix by quickly exposing the back of the slide over a flame three times.
 - d. Immerse the fixed slide in Crystal Violet solution for 1 min.
 - e. Immerse the fixed slide in Stabilized Iodine for 1 min.
 - f. Wash the slide thoroughly with a mixture of acetone and isopropanol (1:3) using a plastic pipette until excess Crystal-Violet and Stabilized Iodine are removed and the run-off is clear.
 - g. Rinse with distilled water.
 - h. Immerse in Safranin solution for 2 min.
 - i. Rinse with distilled water and air dry.
 - j. Visualize using a bright-field microscope (400–1000 \times magnification).
13. 16S rRNA gene amplification and sequencing (2–3 days)
 - a. Extract genomic DNA from fecal sample using the components of QIAamp PowerFecal Pro DNA Kit following the manufacturer's instructions

- b. Amplify the 16S rRNA gene via PCR using the following thermocycling conditions (refer to the *Materials and Equipment* section for the PCR mix recipe):

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	94°C	5 min	1
Denaturation	94°C	1 min	35
Annealing	43°C	1 min	
Extension	72°C	2 min	
Final extension	72°C	7 min	1
Hold	10°C	Forever	

- c. Run the PCR products on a 1% agarose gel at 120 V. The expected amplicon size is 1,600 base pairs.
- d. Prepare the PCR reaction product for sequencing by cleaning up with QIAGEN's PCR MinElute Purification kit following the manufacturer's instructions.
- e. Elute with 10 μ L of molecular grade water and measure the final DNA concentration.
- f. Sequence the PCR reaction product (Sanger method) and analyze by alignment using a sequence database (e.g., Nucleotide BLAST).

EXPECTED OUTCOMES

SYTOXTM green staining

When analyzing SYTOXTM green-stained fecal samples, the finding of SYTOXTM green-positive cells with the expected size and morphology of the inoculated bacteria indicates colonization (Figures 3A and 3B, arrows). However, this method has limited ability to distinguish the presence of contaminant bacteria. Importantly, SYTOXTM green is a nucleic acid stain, and therefore host epithelial cells shed in the feces may stain positive for SYTOXTM green. They can be differentiated from bacterial cells by size and morphology. Non-digested food may occasionally show weakly positive, amorphous fluorescence that can be easily distinguished from bacterial cells (Figures 3A and 3C, arrowheads). Positive and negative controls using feces from SPF and GF mice, respectively, are helpful to define the thresholds for positive bacterial staining.

Gram staining

When analyzing Gram-stained fecal samples, the presence of bacterial cells with expected morphology and Gram staining indicates successful colonization. The presence of bacteria displaying unexpected morphology or Gram staining suggests possible contamination. Of note, on Gram stains of fecal samples bacterial cells must be distinguished from fecal debris (Figures 4A and 4B,



Figure 3. SYTOXTM green nucleic acid staining

(A) Bacteria stain positive for SYTOXTM green, which binds to nucleic acids (arrows). Non-specific staining is shown by arrowheads. 400 \times magnification; scale bar, 60 μ m.

(B) 1000 \times magnification, scale bar, 15 μ m.

(C) SYTOXTM green staining of germ-free fecal sample showing background staining of fecal debris (arrowheads). 400 \times magnification; scale bar, 60 μ m. Excitation: 488 nm. Emission: 520 nm.

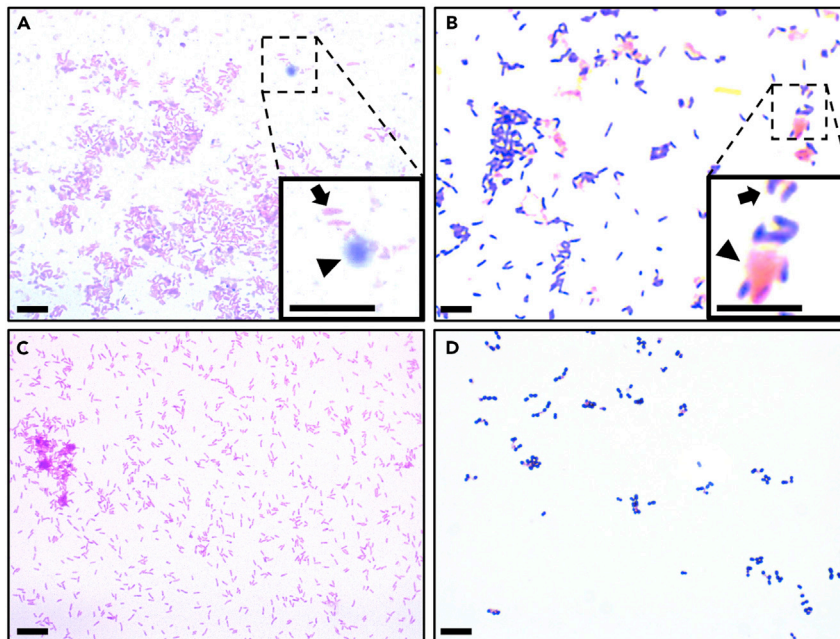


Figure 4. Gram staining of fecal bacteria

Representative images of Gram-stained fecal samples from gnotobiotic colonized by (A) Gram-negative bacteria (arrow) and (B) Gram-positive bacteria (arrow). Bacteria must be distinguished from fecal debris (arrowhead). Representative images of Gram-stained bacteria growing from cultures of gnotobiotic mouse feces showing (C) Gram-negative (D) and Gram-positive bacteria. 1000 \times magnification; scale bar, 15 μ m.

arrowheads), whereas Gram-stains of bacterial colonies cultures from feces should be free of debris and more easily facilitate the identification of bacterial morphology and Gram-staining (Figures 4C and 4D).

16S rRNA full-length gene amplification

The PCR product obtained from 16S rRNA gene amplification should yield a single, 1,600 base pair band on agarose gel in colonized mice, whereas no band is seen if colonization has not been achieved (i.e., germ free) (Figure 5). We recommend the use of positive and negative controls from feces of SPF and GF mice, respectively (Figure 5). Sanger sequencing of the 16S rRNA gene enables confirmation the precise genetic identity of the colonizing bacteria to definitively confirm the presence of expected bacteria, while ruling out a contaminant.

Microbiological culture and Gram staining alone may be sufficient to confirm successful colonization of gnotobiotic mice (for example, finding bacterial growth from fecal samples with appropriate colony morphology and appearance on Gram stain without any evidence of contaminant growth suggest successful monocolonization). However, we recommend also confirming successful colonization (and absence of contamination) by 16S rRNA full-length gene sequencing. Table 1 summarizes how each of these methods may be informative to confirm successful colonization and/or rule-out the presence of contaminating bacteria.

LIMITATIONS

The methods described above for monitoring gnotobiotic mice are optimized for mono-bacterial colonized mice. These methods are not designed to identify non-bacterial microorganisms (e.g., fungi, parasites, viruses), and therefore additional testing may be required if performing gnotobiology using non-bacterial organisms, or if non-bacterial contamination is suspected (for example, by the presence of fungal element seem on microscopy of fecal samples). If fungal contamination is

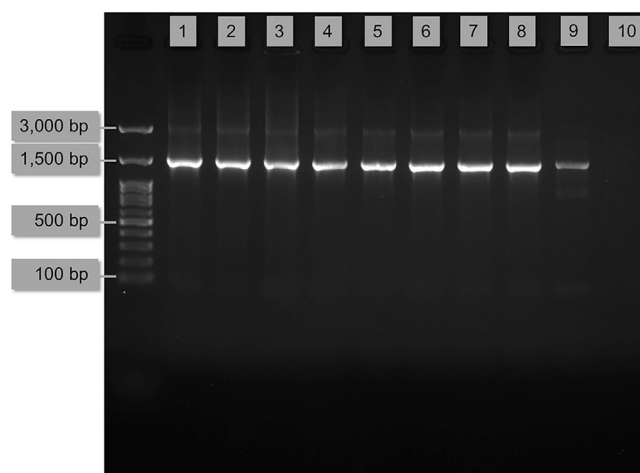


Figure 5. Agarose gel electrophoresis of PCR amplification of 16S rRNA gene

DNA was extracted from bacterial colonies that grew from cultures of fecal samples from gnotobiotic mice. Lanes 1-9, monoclonized gnotobiotic mice. Lane 10, non-template negative control (molecular-grade water only).

suspected, fungal culture and/or internal-transcribed spacer (ITS) gene sequencing should be performed (van Tilburg Bernardes et al., 2020). Viral contamination may be identified by PCR, immunodetection of viral proteins, or transmission electron microscopy (Langdon, 2004).

In this protocol, we recommend full-length sequencing of the 16S rRNA gene by Sanger sequencing as it offers taxonomic resolution of bacterial colonizers at the species and strain level and is ideal for mono-bacterial colonized gnotobiotic mice. However, for gnotobiotic mice colonized by complex microbial communities/consortia, metagenomic sequencing methodologies (16S rRNA gene hypervariable region amplicon sequencing or shotgun metagenomic sequencing) may be appropriate to simultaneously monitor colonization by multiple bacterial species (Brugiroux et al., 2016).

TROUBLESHOOTING

Problem 1

Generation of gnotobiotic mice: fecal samples from germ-free mice show evidence contamination, e.g., bacteria are detected in the feces of germ-free mice prior to inoculation of the bacteria of interest by gavage (steps 10, 11 or 12).

Table 1. Methods for monitoring gnotobiotic mice

Method	Equipment required*	Time (per sample)	Evidence of colonization	Evidence of contamination
Microbiological culture	Incubator, anaerobic chamber if appropriate	Hours to days	Presence of bacterial growth with expected colony morphology.	Presence of colonies with unexpected morphology.
Gram staining	Bright field microscope	Less than 1 h	Presence of bacterial cells with expected morphology and Gram staining.	Presence of unexpected bacterial morphology or Gram staining.
SYTOX TM green staining	Fluorescence microscope	Less than 2 h	Presence of SYTOX TM green-positive bacterial cells of expected size and morphology	This method is not optimal for the identification of contaminants
16S rRNA full-length gene sequencing	Thermocycler, gel electrophoresis apparatus	Hours to days	Presence of bacterial species inoculated by gavage	Presence of bacterial species other than the one inoculated by gavage

*BSC for sample handling required for all methods

Potential solution

Germ-free mice must be maintained under sterile conditions to prevent contamination by environmental sources. If contaminating microbes are identified at the start of the experiment, these mice should not be used for the generation of gnotobiotic mice.

To prevent contamination of germ-free mice, the cage, bedding, water bottle, water and food must be autoclaved. Sample the water, the food and the surfaces of the cages to identify a suspected contaminant. It is critical to identify the source of contamination to properly eradicate.

Problem 2

Generation of gnotobiotic mice: despite confirming colonization 1 week after inoculation, the bacterial burden in feces decreases dramatically or no bacteria are detected at later time points by microbiological culture (step 10).

Potential solution

Some bacterial species colonize the mouse gastrointestinal tract at very low levels that may be beyond the sensitivity of microscopy and culture-based methods to reliably detect. It is important to note that some bacteria will never stably colonize the mouse intestinal tract of gnotobiotic mice. If it is suspected that mice are colonized at very low (or undetectable) levels based on the results of culture or microscopic analysis of the feces, we suggest employing a more sensitive methodology for bacterial detection (e.g., 16S rRNA gene PCR or qPCR).

Problem 3

Monitoring of gnotobiotic mice: fecal samples from colonized mice show evidence of contamination (e.g., unexpected bacteria present in culture, Gram- or SYTOXTM green staining) (steps 10, 11 or 12).

Potential solution

Contaminating microbes can be the result of true contamination of gnotobiotic animals, or due to contamination of the fecal samples between collection and analysis.

- Re-sample feces from all mice and re-analyze to determine whether contamination is replicated.
- If contamination of the animals is confirmed, perform 16S rRNA full-length gene amplification and sequencing (step 13) to identify the contaminant bacteria.

Problem 4

Monitoring of gnotobiotic mice: failed colonization of germ-free mice (e.g., no bacteria detected in the feces of inoculated mice) (steps 10, 11 or 12).

Potential solution

Always confirm viability of the bacterial inoculum (by culture) that is administered to mice as colonization failure may result from inoculation with non-viable bacteria.

Some microbes selectively colonize proximal segments of the gastrointestinal tract and are not shed in detectable quantities in the feces. Therefore, sampling the intestinal contents from all segments of the small intestine, cecum and colon is advised.

Problem 5

Monitoring of gnotobiotic mice: no detectable PCR product from 16S rRNA gene amplification, despite detection of bacteria with other methods (microbiological culture, Gram staining or SYTOXTM Green staining) (step 13)

Potential solution

Increase the quantity of extracted bacterial DNA in the PCR reaction. It is also advised to include positive and negative controls from SPF and GF mice, respectively, for all monitoring methods noted above.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Braedon McDonald (bamcdona@ucalgary.ca).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze datasets or codes.

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AUTHOR CONTRIBUTIONS

Conceptualization, A.Z.Z. and B.M.; methodology, A.Z.Z., B.M., and K.D.M.; investigation, A.Z.Z. and I.-L.Y.; writing and revision, A.Z.Z. and B.M.; funding acquisition, B.M. and K.D.M.; supervision, B.M. and K.D.M.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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