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Authors

Tong, Maomeng
Jacobs, Jonathan P
McHardy, Ian H
et al.

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Sampling of intestinal microbiota and targeted amplification of bacterial 16S rRNA genes for microbial ecologic analysis

Maomeng Tong[1], **Jonathan P. Jacobs**[1], **Ian H. McHardy**[1], and **Jonathan Braun**[1]

[1]University of California Los Angeles David Geffen School of Medicine, Los Angeles, California

Abstract

Dysbiosis of host-associated commensal microbiota is emerging as an important factor in risk and phenotype of immunologic, metabolic, and behavioral diseases. Appropriate collection and pre-processing of biospecimens from humans or mice is necessary for accurate analysis of microbial composition and functional state. Methods to sample intestinal luminal and mucosal microbiota from humans and mice, and to profile microbial phylogenetic composition using 16S rRNA sequencing are presented here. Data generated using this protocol can be used for downstream quantitative analysis of microbial ecology.

Keywords

intestinal microbiota; mucosal microbiota; lavage; Illumina sequencing; 16S rRNA

INTRODUCTION

Accurate analysis of microbial composition necessitates appropriate collection and pre-processing of biospecimens from humans or mice. We describe an efficient procedure to I) sample intestinal luminal and mucosal microbiota from humans and mice, and II) profile microbial phylogenetic composition using 16S rRNA sequencing. Basic Protocols 1 – 3 cover different human sampling strategies, and Basic Protocol 4 describes the sampling of intestinal microbiota in mice. Products from protocols 1–4 are suitable for genomic DNA extraction and subsequent targeted amplification of hyper-variable region 4 (V4) of 16S rRNA gene as described in Basic Protocol 5.

Basic Protocol 1 – Human stool sample collection and processing

Stool collection is performed routinely for clinical microbiologic analysis. As such, few modifications are necessary for research purposes. Stool sample collection is usually performed by research subjects themselves at home or at a clinical facility, following instruction provided by their physicians.

Materials

Medium Ambient IATA Transport Box (Therapak Corporation No.:23650)

8 oz. Refrigerant Pack (Therapak Corporation No.:56400)

Para-Pak Clean Vials

Powder-free nitrile exam gloves

Class II, Type A2 biological safety cabinet

Liquid nitrogen

Cryovials

Metal spatulas

Mortar & pestle (100mm Fisher #S337621)

Bench diapers

Aluminum foil (Super Strength VWR#89093-584)

Bleach

Dry ice

70% Ethanol

Biohazard container

1. Before the patient visit, prepare a collection kit containing the following items, and label it accordingly:
   - 1 Transport Box
   - 1 8 oz. Refrigerant Pack
   - 2 Biohazard Specimen Transport Bags
   - 1 copy of specimen collection instruction
   - 1 pair of nitrile exam gloves
2. Consent participating subjects as per the Institutional Review Board protocol. Collect clinical metadata (including potentially demographics, clinical questionnaire, and medication history) according to the study design.
3. Provide subjects with labeled collection kits and detailed instructions as shown below. Review the instructions with the patients to ensure that they understand it completely.
   1. Urinate before you collect your stool specimen (urine cannot be mixed with stool).
   2. Label the plastic container with the date and time of collection.
   3. Lift the toilet seat and place plastic wrap over the toilet bowl.
   4. Lower the toilet seat and sit on the toilet seat.
   5. Pass stool onto the plastic wrap.
   6. Take the plastic spoon and scoop stool into the plastic container with your label.
   7. Close the lid of the plastic container tightly.

8. Place the plastic container into the plastic bag with ice pack.
9. Place the plastic bag with the ice pack and container into the freezer. Keep the sample frozen until you can bring it to the clinic.

4. Upon receipt of samples, aliquot each into smaller volumes (around 1g per cryovial) for DNA extraction. Place diapers in the biological safety cabinet along with one mortar and pestle for each sample and KimWipes.
5. Add dry ice to two sample collection boxes. One will be used inside the hood for storing pulverized samples, the other will hold unprocessed samples outside of the hood. Pre-chill sample collection tubes and boxes in a freezer.
6. Line each mortar with aluminum foil. Add liquid nitrogen to one mortar. Carefully add sample directly into the mortar with liquid nitrogen. Use a sterile metal spatula to "chip" the entire sample into liquid nitrogen and then pulverize the sample using a pestle. Allow the liquid nitrogen to evaporate and quickly scoop sample into pre-cooled cryovials. Make several aliquots of each sample if it will be used for multiple experiments. Place in dry ice.

   Note: Make sure to use the Super Strength aluminum foil to line the mortars. Regular thin aluminum foil crumbles easily when pulverizing, which can contaminate both the specimen and the mortar. It is important to keep the specimen frozen. Add more liquid nitrogen if it evaporates completely during pulverization.
7. After pulverizing, remove foil and clean residual sample from the mortar. Decontaminate mortar and pestles by soaking in 10% bleach for 10–15mins, rinsing with H_2O, and UV for 10 minutes.

   Note: Having 3–4 sets of mortars and pestles makes this protocol more manageable. Line each mortar prior to processing each sample and discard foil upon completion. Remove and bleach mortars/pestles as they are used.
8. Once finished, store the aliquots of pulverized stool and the remaining samples at −80°C for downstream analysis. Discard disposables in biohazard containers and soak equipment in 10% bleach. Sterilize the hood by cleaning with ethanol or bleach.

Basic Protocol 2 – Human intestinal tissue biopsy collection and processing

The composition of the mucosa-associated microbiome of human intestinal samples can be assessed by 16S sequencing of biopsy specimens collected during colonoscopy. Approximately 2–3mm biopsy at predefined sites will be collected using standard protocols and instruments (e.g., standard size forceps) during colonoscopy.

Materials

Phosphate buffered saline (PBS)

RNAlater TissueProtect Tubes (Qiagen Cat. no. 76154)

Powder-free nitrile exam gloves

1. Before the colonoscopy procedure, consent patients as per the Institutional Review Board protocol. Collect clinical metadata (including potentially demographics, clinical questionnaire, and medication history) according to the study design. Label the collection tubes accordingly.
2. Irrigate the collection site before taking biopsy to minimize contamination by luminal content.
3. Use a standard size biopsy forceps to obtain 2–3 mm biopsies from pre-defined sites in the intestine.
4. After biopsy acquisition, swish closed biopsy forceps containing tissue specimen in PBS.
5. Open biopsy forceps and flick biopsy into a 1.5mL RNAlater TissueProtect tube. Keep the tube upright. TissueProtect tubes with samples can be left at room temperature during the remainder of the procedure. Keep tube upright and at 4°C overnight (no more than 18 hours).

   Note: Make sure that biopsies are completely submerged in the solution. Flick the tube if the biopsies are sticking to the side of the tube to ensure they fall back into solution.
6. The next day, confirm that the biopsies are still submerged in the solution, then transfer to −80°C for downstream analysis. If samples cannot be processed within 18 hours, they must be placed into the −80°C freezer on the day of collection.

Basic Protocol 3 – Human intestinal mucosal lavage collection and processing

Biopsy specimens cannot be used to assess microbial metabolites and proteins as much of the material would derive from human cells. Routine screening colonoscopy can be slightly modified to collect mucosal lavage samples that can be used for combined phylogenetic, proteomic, and metabolomic analysis. Prior to the procedure, patients scheduled for colonoscopy are consented per IRB requirements. Samples may then be collected any time during endoscopic examination, though physicians have historically preferred to collect samples upon completion and with retraction of the endoscope. During a typical colonoscopy, mucosal washes are routinely collected and discarded. Therefore, only slight modifications are necessitated for implementation of this protocol. Collected mucosal lavage samples can then be processed further and analyzed using many potential methods, including high-throughput sequencing or mass spectrometry for proteomic or metabolomic analysis.

Materials

Sterile water for irrigation (Baxter Cat. No. 2F7114)

60 mL slip tip syringe (BD Cat. No. 309654)

Specimen trap (Bard Mucous Specimen Trap 40cc Cat. No. 0035860)

Powder-free nitrile exam gloves

50 mL high-clarity polypropylene conical centrifuge tube (BD Cat No. 352098)

1. Before the colonoscopy procedure, consent patients as per the Institutional Review Board protocol. Collect clinical metadata (including potentially demographics, clinical questionnaire, and medication history) according to the study design. Label the collection tubes accordingly.
2. During colonoscopy, when the sample is ready to be collected, attach the specimen trap to the colonoscope (female connector to suction instrument or catheter and male connector to suction source).

   Note: Samples may be collected at any time during endoscopy, though physicians have historically preferred to collect samples during withdrawal. Biopsy and mucosal lavage collection can be performed within a single colonoscopy procedure. However, mucosal lavage specimens should be collected first to minimize blood contamination.
3. Inject 50 mL sterile water through the colonoscope port onto unperturbed mucosa. This dissociates a thin mucous film from the mucosal surface which can be collected by vacuum suction. Typically, 20–25 mL of solution can be recovered. When the suction is complete, disconnect the trap and fully secure the female and male connectors.

   Note: Depending on the study design, multiple samples can be collected from different intestinal regions.
4. Place lavage sample (trap) on ice immediately after collection. Keep the sample chilled during transportation.
5. After transporting to a research lab, centrifuge the sample for 15 minutes at 3500g, 4°C. Store the supernatant and pellet separately at −80°C for downstream analysis.

Basic Protocol 4 – Sampling of mouse intestinal luminal and mucosal microbiota

This protocol describes the isolation of luminal and mucosal microbial samples from mouse intestines. The mucosa-associated microbiome is sampled by first rinsing out luminal contents then releasing epithelial cells (with mucosal microbes) from the intestine in a reducing environment with mechanical agitation.

Materials

Distilled water

D10F medium (DMEM+10% FCS)

D10F + 1mM DTT

Extraction buffer (200 mM Tris (pH 8.0), 200 mM NaCl, 20 mM EDTA)

70 micron nylon mesh filter

37°C orbital shaker

Centrifuge (Beckman J-6M with JS-4.2 rotor)

Razor blades

1.5 mL Eppendorf tubes

P1000 pipette and tips

1. Label 1.5 mL Eppendorf tubes. Dissect out the large and small intestines. Cut them into segments corresponding to the regions of interest (e.g. ileum, cecum, rectum, etc.) and place them into separate petri dishes.

2. Cut the segments into 1–2 cm pieces (if the segments are larger than 2 cm). Fill a P1000 pipette with ddH_2O. Grip the intestinal segment of interest with a forceps then intubate one of the cut ends with the pipette. Hold the segment over a clean 1.5 mL Eppendorf. Press the pipetter to lavage the segment with ddH_2O. If the segment appears to still have visible retained feces, pipette up some of the flow through and repeat (or use more ddH_2O). Alternatively, you can press on the segment with the side of the pipette tip to squeeze out the luminal contents into the Eppendorf. Return the tissue to the petri dish and add D10F until the tissue is submerged.

   Note: Longer segments can be used but the lavage is less effective. Steps 2–4 can be skipped if only the mucosal microbiota are of interest.

3. Close the Eppendorf containing the luminal lavage specimen and vortex for 30 seconds. Spin down on a tabletop centrifuge at maximum speed (approximately 16000g) for 2 minutes.

4. Pipette the supernatant into a new Eppendorf and spin again at maximum speed for 2 minutes down again to pellet residual fecal matter. Store the supernatant and frozen for metabolite or protein analysis if desired. The pelleted material can then be frozen at −80°C for future metabolomic or proteomic analysis. Store the fecal pellets at −80°C for future DNA extraction.

5. Cut along the length of the intestinal segments to expose any remaining luminal contents. Shake vigorously in the petri dishes to remove luminal content. Repeat with at least two clean dishes containing D10F.

6. Prepare 50 mL conical tubes containing 15 mL D10F + 1 mM DTT pre-warmed at 37°C. Place the intestinal sections into the conical tubes and close the lids securely.

7. Place the conical tubes in the 37°C orbital shaker in a horizontal orientation. Shake at 180 rpm for 40 minutes.

8. Vortex for approximately 20 seconds, then pass the digestion solution through a 70 micron nylon mesh filter over a clean 50 mL conical tube. Return the intestinal segments to the original conical tube, add 10 mL D10F, vortex, then pass through the filter. Repeat one more time (total volume around 35 mL).

9. Spin down the filtered supernatant from the DTT extraction at 4200 rpm (approximately 5000g) for 15 minutes at 4°C. Discard the supernatant, then resuspend the pellet in 200 μL buffer A and transfer to a labeled Eppendorf tube. Store the sample at −80°C for downstream analysis.

Note: Consider resuspending in a larger volume and aliquoting into multiple Eppendorf tubes if multiple analyses are to be performed with each sample.

Basic Protocol 5: Targeted PCR amplification using barcoded primers

Specimens from Basic Protocols 1–4 are suitable for genomic DNA extraction and PCR amplification of 16S rRNA. In this step, genomic DNA is first extracted from the bacterial pellet using the PowerSoil DNA Isolation Kit, and primers containing Illumina flowcell adapter sequences are then used to amplify the V4 region of the 16S rRNA gene for downstream semi-quantitative sequence analysis. Given that DNA is amplified, the calculated relative abundance of organisms do not perfectly reflect their relative abundance within the original sample. To help compensate for this variation, PCR reactions are performed in triplicate for each sample and then pooled upon completion. The primer sequences containing Illumina flowcell adapter sequences used here have been published previously (Caporaso et al., 2012). Once complete, the PCR products can then be sequenced using Illumina HiSeq platforms (2000 or 2500).

Materials

PowerSoil DNA Isolation Kit (MO BIO Laboratories)

10× PCR buffer (Sigma-Aldrich, St. Louis, MO, USA)

dNTPs (10 mM)

JumpStart Taq DNA polymerase (Sigma-Aldrich, St. Louis, MO, USA)

DEPC-treated H_2O

Qiagen PCR cleanup kit

Nanodrop spectrophotometer

Thermal cycler

1. Extract genomic DNA using the PowerSoil DNA isolation kit following manufacturer’s instruction.

   Note: Whenever possible, samples being extracted should be kept on ice as target DNA degradation is more likely at higher temperatures. Therefore, batched extractions should be limited to ~24 samples at a time.

2. Take a 96 well plate of barcoded IL_806r unique primers and add ILHS_515f conserved forward primer to each well. Add water so that the 100 μM primer stocks are diluted 1:20 (i.e now 5 μM primer stock)

   Note: The number of multiplexed samples for each sequencing run should be decided according to the desired sequencing depth per sample. For a single read run on Illumina HiSeq-2000, each lane can generate up to 3 million reads per sample (after quality filtering) if 100 samples are pooled and analyzed.

3. Each sample will be analyzed in triplicate. Each reaction contains the following:

   3 μL 10× PCR buffer

0.6 μL dNTPs (10 mM)

0.3 μL JumpStart Taq DNA polymerase

1 μL primer mix (5 μM conserved forward and barcoded reverse primers)

2 μL DNA

23.1 μL H_2O

We recommend first creating the following master mix:

10× PCR buffer	3 μL * 3.3 * #samples
dNTPs (10 mM)	0.6 μL * 3.3 * #samples
JumpStart Taq DNA polymerase	0.3 μL * 3.3 * #samples
H_2O	23.1 μL * 3.3 * #samples

Add 81 μL master mix to every 3rd well in the 96 well plate being used for the PCR reaction. Then add 6 μL sample DNA and 3 μL primer mix to each of these wells. Use a P200 pipette to mix the wells, then pipette 30 μL into two neighboring wells for each triplicate sample.

Note: Jumpstart Taq or another highly specific DNA polymerase enzyme is recommended to reduce non-specific amplification and primer dimer formation.

4. Seal the plate with 8-cap strings (make sure they are matched to the plate). Use the following thermal cycler settings for the PCR reaction:

Step	Temp	Time	Cycle
Initial denaturation	94°C	3 min	1
Denaturation	94°C	45 s	35
Annealing	50°C	1 min	
Extension	72°C	1.5 min	
Final extension	72°C	10 min	1
	94°C	5 min	1
	4°C	Hold	

5. Use the Qiagen PCR cleanup kit to purify PCR products from each sample. Elute DNA with 30 μL PCR grade (DEPC-treated) H_2O. Quantify the amplified DNA using a Nanodrop spectrophotometer, then combine 250 ng of each amplified PCR product in one tube to make the library. Library construction can be performed using less concentrated DNA as long as equal concentrations of each sample are combined. This library can then be given, along with proper sequencing primers (Caporaso et al., 2012), to a sequencing facility for highly sensitive DNA quantitation using a Qubit fluorometer (Illumina), cluster generation, and sequencing.

Supporting Protocol: Illumina sequencing and computational analysis

Given the significant capital investment required for any major next-generation sequencing platform, few research laboratories are equipped with their own sequencing machine. The sequencing step is therefore usually done by a core facility or commercial service provider following manufacturer's instruction.

Once the sequencing data has been produced, it must be processed and analyzed before any derived microbial ecology and quantitative analysis. A typical workflow for analyzing 16S rRNA sequencing data generally includes three steps: demultiplexing of raw sequencing data to assign sequences to each barcoded sample, quality filtering to reduce sequencing errors, and assignment of amplicon sequences to operational taxonomic units (OTUs). The output from the workflow is an OTU table, which reports the abundances (number of reads) of each OTU in each sample and also the taxonomy of the OTUs. Several software tools including QIIME (Caporaso et al., 2010), mothur (Schloss et al., 2009) and VAMPS have been developed to perform these analysis. A detailed protocol of 16S rRNA sequencing data processing and analysis using QIIME has been published before (Kuczynski et al., 2012).

In addition to providing phylogenetic information, the OTU table can also be used to infer the metagenomic content of microbial samples using PICRUSt (Langille et al., 2013). In this step, the gene contents and 16S rRNA gene copy number of each detected phylotype are predicted based on its evolutionary similarity with reference genomes from KEGG (Kyoto Encyclopedia of Genes and Genomes). Metagenome imputation enables the comparison of probable metabolic functions across samples.

Commentary

Background Information—The importance of host-associated commensal microbiota in maintaining the health of humans has been well appreciated. Dysbiosis of microbiota is implicated in various chronic diseases including obesity, diabetes, inflammatory bowel disease, HIV, vaginosis, asthma and other immune related chronic disorders (Kinross et al., 2011). Recent advances in sequencing technologies have enabled the profiling of microbial compositions with unprecedented depth and coverage at significantly lower cost, and therefore substantially improved our understanding of host-associated microbiome in different habitats. For any research laboratory interested in establishing microbial ecologic analysis capability, this protocol provides an efficient and feasible guidance to collect biospecimen and perform downstream profiling analysis.

Mucosal immune systems, as the frontiers of host-microbial interactions, consist of a variety of immune cell types including natural killer (NK) cells, dendritic cells, macrophages, B and T lymphocytes. This population of cells actively modulate the composition and activity of microbiome. The mucus covering epithelium consists of two layers: an inner firm layer, and an outer nonattached layer where the normal intestinal microbiota inhabits (Johansson et al., 2011). For discernment of biogeographic relevance in host-microbial interactions, collection of lavage or biopsy samples are warranted for two reasons: first, the phylogenetic composition of mucosa-associated microbiota is distinct from that of the luminal compartment (Eckburg et al., 2005; Tong et al., 2013); second, fecal samples are a mixture

of products from all intestinal regions, which may obscure the unique biogeography of host-bacteria interactions along intestine (Li et al., 2011).

The fecal sample from Basic Protocol 1 and the lavage biospecimen from Basic Protocol 3 can also be subjected to other high-throughput analyses in addition to 16S rRNA sequencing. For example, fecal samples can be analyzed for virome, fungome, meta-metabolome, meta-proteome and meta-transcriptome. The supernatant of lavage sample contains soluble components of the mucosal surface, and can generate a rich dataset of host proteome and metabolome (Li et al., 2011; McHardy et al., 2013a). To achieve unprecedented understanding of the ecological structures and biomolecular activities of the gut microbiome, it is necessary to extend the analysis to multiple levels of biological organization – genome content, gene expression, protein expression, and metabolism (Nicholson et al., 2012; Raes and Bork, 2008). Appropriate sampling approaches are needed for multi'omic profiling of the gut ecosystem, to disentangle the complex host-microbial metabolic interplay. Although only a limited number of integrative 'omics profiles of the gut microbiota currently exist (Erickson et al., 2012; McHardy et al., 2013a; Perez-Cobas et al., 2012), multi'omic studies have shown great potential in providing a holistic picture of the metabolic status of the gut microbiota and the host response to functional changes.

Other options for similar analysis—We described here several representative sampling approaches of the intestinal habitat, containing abundant commensal microbiota. Other sampling strategies for luminal and mucosal microbiota have also been widely used. For example, stool samples can also be obtained by swabbing used bathroom tissue, which is adequate for obtaining material for microbial community analysis (Costello et al., 2009). Mucosa-associated microbiota can also be collected from resected tissue or rectal sponges (Frank et al., 2007; McHardy et al., 2013b). The appropriate sampling approach should be chosen on the basis of available resources and the experimental goals.

Critical Parameters—Researchers should be aware of several imperfections inherent to this, and most high-throughput sequencing-based studies. First, complete microbial lysis and genomic release is critical for unbiased analysis. Unfortunately, lysis efficiency for each organism is difficult to control and is thus rarely considered, but may contribute to observed variations of alpha and beta diversity of resulting data. Second, given that the 16S DNA is amplified prior to sequence analysis, random variation in measured abundance is expected and likely not completely accounted for by pooling PCR reactions. Thus, while quantitative data is generated using these methods, the data may only semi-quantitatively reflect the underlying distribution of bacteria. Accordingly, any quantitative analysis (e.g. correlations, linear models) of resulting sequence data should be performed and reported with caution.

Anticipated Results—DNA yield from extraction will depend on input volume. However, if Qiagen PCR cleanup columns are used, a maximum, yet sufficient, concentration of 10μg DNA per sample will be available for downstream analysis. Importantly, since barcoded genomic libraries are combined using overall DNA concentration, sample collection should be performed consistently to equalize contaminating human cells in all samples, which can otherwise introduce variation of microbial DNA concentration and confound downstream data analysis.

Read density per sample is often overlooked during study planning, but is an important parameter to consider for minimizing costs and maximizing resource efficiency and data utility. For example, a study design with 100 samples can result in several million reads per sample using a high-output flow-cell on the Illumina HiSeq 2500 or 2000 platform. Such large read densities are rarely warranted; most microbial survey-based studies are sufficiently robust with read densities of ≤50,000 per sample. Therefore, most researchers choose to pool multiple small-medium sized studies into a single flow cell.

Time Considerations—The protocols described above are self-contained, and do not need to be performed on the same day. Each of the sample collection and pre-processing protocols can be usually done within 1–2 hours, although the total time might be up to several hours if multiple samples are collected and processed at one time. For human sample collection, if the collection site (e.g. clinic or procedure room) is not located near the research lab processing the samples, additional time and personnel are needed to transport samples. The DNA extraction and PCR amplification steps normally take up to one working day in total.

It is crucial to perform human lavage and biopsy sample collection during colonoscopy efficiently to avoid increasing patients' time under sedation (and the risks associated with extended sedation). This requires streamlining the logistics for sample collection with physicians, nurses and endoscopy technicians in advance. Also, the specimen traps and RNAlater TissueProtect tubes should be labeled prior to the procedure.

Mouse sample collections can be time-consuming if many mice are harvested due to the effort required to lavage intestinal segments. If only the mucosa-associated microbiome is of interest, it's prudent to skip the lavage and instead immediately dissect open the intestinal segments then rinse them with media.

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