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Extraction of DNA from Murine Fecal Pellets for Downstream Phylogenetic Microbiota Analysis by Next-generation Sequencing

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[Abstract] Mouse models are widely used to evaluate the potential impact of the gut microbial composition on health and disease. Standardized protocols for sampling and storing murine feces, as well as for extracting DNA from these fecal pellets are needed to limit experimental variation between different studies. Both efficient lysis of the microbiota and the quality of the obtained fecal DNA are important for allowing the downstream next-generation sequencing to cover the phylogenetic diversity of both Gram-negative and Gram-positive bacteria living in the mouse gut. Here we present a detailed protocol for fecal sample collection and DNA extraction that we validated in a study on the impact of inflammasomes on the murine gut microbiota. This protocol for DNA extraction from murine fecal pellets utilizes a combination of mechanical and chemical lysis, which aligns with the procedure that was recently recommended as a benchmark protocol for DNA extraction from human feces.

Keywords: Gut microbiota, Fecal DNA, Mouse feces, DNA extraction, 16S phylogenetic analysis, Next-generation sequencing

[Background] Limiting technical variation within as well as between laboratories is imperative for reproducibility and hence for scientific progress from experimental research. Within the expanding gut microbiota research community, a plethora of methodologies are used to profile the phylogenetic composition of the intestinal ecosystem. Each step in this microbiota analysis process is subject to technical variation depending on the protocol or the materials used. For instance, comparing several protocols to extract DNA from murine feces showed striking differences in the obtained results even within the same laboratory (Ferrand *et al*., 2014). Therefore, it is clear that standardized protocols are needed to enable meta-analyses of multiple different studies.

For analyzing the human fecal microbiota, a large international consortium of researchers recently compared the effects of numerous technical approaches in every single step of the gut microbiota analysis pipeline in several independent laboratories (Costea *et al*., 2017). This study identified differences in the DNA extraction method as the biggest influence on the downstream gut microbiota analysis results. Based on the obtained DNA quality as well as on the reproducibility between different laboratories, the so-called ‘Protocol Q’ was identified as the best one and was proposed as a benchmark for extracting DNA from human feces (Costea *et al*., 2017).

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Although such multi-centered comparative studies have not been performed for murine fecal DNA extraction protocols, we recently reported a gut microbiota profiling study in mice using a protocol similar to the Protocol Q recommended for human fecal DNA extraction (Mamantopoulos *et al*., 2017). Like the latter, our protocol uses a combination of mechanical bead-beating and chemical lysis with the QIAGEN QiaAmp® Stool Kit. Indeed, it has been reported that mere chemical lysis of feces results in an underrepresentation of DNA from Gram-positive bacteria that have a thicker cell wall (Salonen *et al*., 2010). In contrast, both Protocol Q and our protocol detailed below are expected to result in efficient lysis of both Gram-positive and Gram-negative bacteria.

Materials and Reagents

1. Filter tip, clear, sterile F. Gilson P1000, 60 PCS/Box (Greiner Bio One International, catalog number: 740288)
2. Filter tip, clear, sterile F. Gilson P-200, 96 PCS/Box (Greiner Bio One International, catalog number: 739288)
3. Standard filter tip, 20 µl, clear, universal, sterile, 96 pieces per rack (Greiner Bio One International, catalog number: 774288)
4. Soil grinding SK38 2 ml tubes (Bertin Technologies, catalog number: KT03961-1-006.2)
5. Eppendorf® Tubes 3810X, 1.5 ml, g-safe® centrifugation stability, Eppendorf Quality™, colorless, 1,000 pcs. (Eppendorf, catalog number: 0030125150)
6. Ethanol absolute, EMSURE® ACS, ISO, Reag. Ph. Eur. analytical reagent (Merck, MilliporeSigma, catalog number: 1.00983.1000)
7. UltraPure™ DNase/RNase-Free Distilled Water (Thermo Fisher Scientific, Gibco™, catalog number: 10977035)
8. QIAamp® Fast DNA Stool Mini Kit (QIAGEN, catalog number: 51604), containing the following:
    a. QIAamp Mini Spin Columns
    b. Collection Tubes (2 ml)
    c. InhibitEX® Buffer
    d. Proteinase K
    e. Buffer AL
    f. Buffer AW1 concentrate
    g. Buffer AW2 concentrate
    h. Buffer ATE

Equipment

1. Finnpipette F1, 100 to 1,000 µl (Thermo Fisher Scientific, catalog number: 4641100N)
2. Finnpipette F1, 20 to 200 µl (Thermo Fisher Scientific, catalog number: 4641080N)
3. Finnpipette F1, 2 to 20 µl (Thermo Fisher Scientific, catalog number: 4641060N)

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4. Beakers
5. -80 °C freezer
6. Precellys®24 (Bertin Technologies, catalog number: EQ03119.200.RD00.0)
7. Thermoshaker with heating block for 24 x1.5 ml microtubes (Grant Instruments, catalog number: PHMT-PSC24N)
8. Microcentrifuge 5417R with rotor for 1.5/2 ml tubes (Eppendorf, model: 5417 R, catalog number: 22 62 180-7)
9. Vortex mixer (Merck Eurolab, catalog number: MELB 1719)
10. NanoDrop spectrophotometer

Procedure

A. Fecal sample collection

1. Collect 1-2 fresh fecal pellet(s) from mice into a soil grinding SK38 2 ml tube. Fresh fecal pellets are collected by holding the mouse in one hand, during which the mouse can defecate directly in an SK38 2 ml tube held in the other hand. Alternatively, individual mice can be put in sterile beakers for a couple of minutes, after which fresh fecal pellets can be collected from the beakers. It is important to collect fecal samples from all animals within the experimental cohort at the same time period of day, since it was reported that the gut microbiota composition displays diurnal oscillations (Thaiss *et al*., 2014).
2. Store the soil grinding SK38 2 ml tubes containing the fecal pellet(s) at -80 °C until further processing. Fecal DNA extraction can also be initiated immediately from fresh fecal pellets, but all samples within an experimental cohort should be either all fresh or all frozen to avoid storage artifacts, as freezing fecal samples was shown to influence the ratio of *Firmicutes* to *Bacteroidetes* (Bahl *et al*., 2012).

B. Fecal DNA extraction

Fecal DNA extraction is performed using the QIAamp® Fast DNA Stool Mini Kit according to the manufacturer's instructions with additional mechanical lysis by bead-beating as detailed below:

1. Add 1 ml InhibitEX® buffer to the soil grinding SK38 2 ml tubes containing fecal pellet(s). Fecal pellets taken from the -80 °C freezer can be used immediately, as equilibrating to room temperature is not required.
2. Homogenize fecal pellets in 1 ml InhibitEX® buffer by bead-beating using the Precellys®24 for 2 x 30 sec at 6,500 rpm.
3. Transfer the fecal homogenate from Step B2 to sterile 1.5 ml microcentrifuge tubes, taking care to avoid transferring the zirconium beads.
4. Heat the fecal homogenate in a thermoshaker for 5 min at 70 °C while shaking at 1,100 rpm in order to promote further lysis of the bacteria.

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5. Centrifuge the fecal homogenate from Step B4 at 20,800 *x g* for 1 min at room temperature in order to pellet the stool particles.
6. In the meantime, pipet 15 µl Proteinase K from the QIAamp® Fast DNA Stool Mini Kit to fresh sterile 1.5 ml microcentrifuge tubes.
7. Pipet 200 µl fecal homogenate supernatant from Step B5 into the Proteinase K containing 1.5 ml microcentrifuge tubes.
8. Add 200 µl buffer AL to the 1.5 ml microcentrifuge tubes containing 200 µl supernatant and Proteinase K (from Step B7) and vortex for 15 sec to form a homogeneous suspension.
9. Heat the suspension in the thermoshaker for 10 min at 70 °C while shaking at 1,100 rpm.
10. Add 200 µl absolute ethanol to the suspension, and vortex to mix.
11. Transfer 600 µl of the resulting lysate carefully onto the QIAamp spin columns provided in the QIAamp® Fast DNA Stool Mini Kit.
12. Close the QIAamp spin column and centrifuge at room temperature for 1 min at 20,800 *x g*.
13. Place the QIAamp spin column in a new 2 ml collection tube and discard the old collection tube containing the filtrate.
14. Add 500 µl buffer AW1 to the QIAamp spin column.
    Note: Buffer AW1 is provided as a concentrate. When using a fresh bottle, first add 25 ml absolute ethanol to the AW1 concentrate and mix thoroughly by resuspending.
15. Close the QIAamp spin column and centrifuge for 1 min at room temperature at 20,800 *x g*.
16. Place the QIAamp spin column in a new 2 ml collection tube and discard the old collection tube containing the filtrate.
17. Add 500 µl buffer AW2 to the QIAamp spin column.
    Note: Buffer AW2 is provided as a concentrate. When using a fresh bottle, first add 30 ml absolute ethanol to the AW2 concentrate and mix thoroughly by resuspending.
18. Close the QIAamp spin column and centrifuge for 3 min at room temperature at 20,800 *x g*.
19. Place the QIAamp spin column in a new 2 ml collection tube and discard the old collection tube containing the filtrate.
20. Centrifuge again for 3 min at room temperature at 20,800 *x g* in order to reduce the chances of buffer AW2 carryover.
21. Place the QIAamp spin column in a fresh 1.5 ml microcentrifuge tube and discard the old collection tube containing the filtrate.
22. Add 200 µl UltraPure™ DNase/RNase-Free Distilled Water to the QIAamp spin column to elute the DNA.
    Note: DNA can also be eluted using 200 µl of the buffer ATE provided by the QIAamp® Fast DNA Stool Mini Kit.
23. Incubate for 1 min at room temperature.
24. Centrifuge at room temperature for 1 min at 20,800 *x g* to collect the DNA.
25. Discard the QIAamp spin columns.

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26. Close the 1.5 ml microcentrifuge tubes containing the eluate and store the DNA at -20 °C until further use. The amount of fecal DNA obtained with this protocol varies per sample, and depends on the amount of stool used to extract DNA. In our hands, the amount of fecal DNA obtained varies between 50 and 500 ng DNA per mg stool, with an average of about 150 ng DNA per mg stool. Since one fecal pellet weighs at least 20 mg, one can expect to obtain at least 1 µg of fecal DNA using this protocol. Thus, as the fecal DNA is dissolved in 200 µl of UltraPure™ DNase/RNase-Free Distilled Water (see Step A22), the obtained concentration of fecal DNA from any given sample will be at least 5 ng/µl.

Data analysis

Concentration and quality of the obtained fecal DNA can be measured by NanoDrop analysis, in which the absorbance ratios at 260 nm/230 nm and 260 nm/280 nm can be determined to evaluate the purity of the extracted DNA, which should be around 2 and 1.8, respectively. Downstream phylogenetic 16S rDNA microbiota analysis starts with a PCR on 25 ng of fecal DNA. Therefore, since this protocol generates at least 1 µg of DNA at a concentration of 5 ng/µl, the quantity of obtained DNA is not a limiting factor. Further procedures for phylogenetic 16S rDNA microbiota analysis are outlined in our previous study (Mamantopoulos *et al*., 2017).

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