bio-protocol

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Cost Effective Method for gDNA Isolation from the Cecal Content and High Yield Procedure for RNA Isolation from the Colonic Tissue of Mice

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Abstract

Microbiome studies are quickly gaining momentum. Since most of the resident microbes (consisting of bacteria, fungi, and viruses) are difficult to culture, sequencing the microbial genome is the method of choice to characterize them. It is therefore important to have efficient methodology for gDNA isolation of gut microbes. Mouse models are widely used to understand human disease etiology while avoiding human ethics-related complications. However, the widely used kit-based methods are costly, and sometimes yields (in terms of quality and quantity) are sub-optimal. To overcome this problem, we developed a straightforward, standardized DNA isolation procedure from mouse cecal content for further microbiome-related studies. The reagents we used to standardize the procedure are readily available even in a not-so-well-equipped laboratory, and the reagents are not expensive. The yield and quality of the DNA are also better than those obtained by the readily available kit-based methods.

Additionally, we modified the kit-based method of RNA isolation from the colon tissue sample of the mouse for better yield. Churning the tissue with liquid nitrogen at the beginning of the procedure improves RNA quality and quantity.

Keywords: DNA isolation, RNA isolation, Cecal DNA, Colonic tissue, DNA isolation by lysis, Cost-effective, High yield

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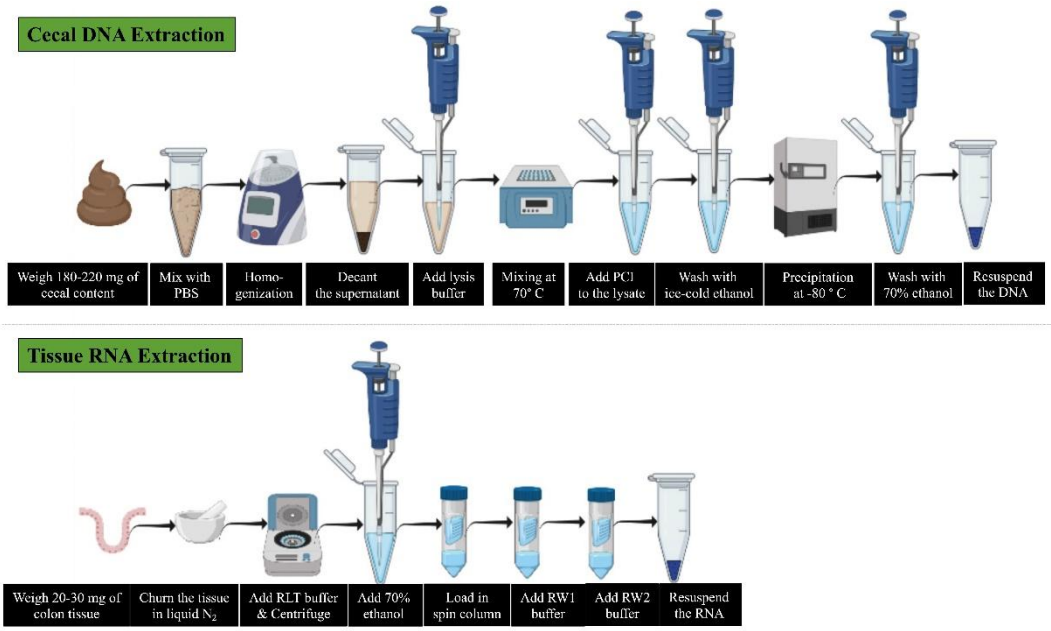
Graphical abstract:



Background

Efficient extraction of high-quality (concentration: 800–2,200 ng/μL) and high molecular weight (>50 kb) genomic DNA and tissue RNA from the limited amount of available samples is the key challenge for downstream applications like next-generation DNA sequencing (NGS) or transcriptomics studies, among others. The expanding research on human diseases related to gut microbiota and associated gut health resulted in a plethora of methodologies used to profile the gut microbial composition and associated inflammatory status of the gut tissue.

Each step in the downstream data analysis process is subject to technical variation depending on the protocol or materials used for DNA or RNA isolation. Researchers have found striking differences in the results obtained from different kit-based methods within the same laboratory (Ferrand *et al.*, 2014; Videnska *et al.*, 2019; Gryp *et al.*, 2020). Therefore, standardizing the protocols is of utmost priority for researchers to obtain consistent, good quality, and robust representative data.

The diversity of the gut microbes in gut ecosystems can be categorized based on their morphological, structural, biochemical, and genetic properties. Gut microbes also have notable differences regarding cell wall structure and cell membranes, which enclose their cytoplasm and genomic contents. Harsh sample treatments can affect DNA quality, while a mild process may cause partial lysis of the microbial community (Bag *et al.*, 2016). Moreover, the unknown composition of the lysis buffer of kit-based methods presents different biases, drastically changing the results obtained from the same sample (Brooks *et al.*, 2015). Therefore, it is important to optimize cell lysis methods to obtain genomic DNA from all groups of microbes present in the gut.

We developed a very efficient, cost-effective cell lysis method. Reagents used to standardize the process are readily available, even in a comparatively less equipped laboratory, and the reagents are not too expensive. The yield and quality of the DNA are also better than those obtained by the readily available kit-based methods.

In an RNA isolation procedure from gut tissue, it is essential to standardize the amount of tissue required to obtain good quality RNA. Proper tissue homogenization is another crucial step for a high yield of RNA. To get high quality and quantity of RNA, we standardized the needed amount of colonic tissue and the liquid nitrogen-based homogenization process.

These methodologies were published in a recent article (Pradhan *et al.*, 2022).

Materials and Reagents

A. For gDNA isolation

1. Filter MAXIPENSE™, Low retention, clear, sterile tips 1,000 µL (Tarsons, catalog number: 527106), 200 µL (Tarsons, catalog number: 527104), and 10 µL (Tarsons, catalog number: 527100)
2. Spinwin™ Micro Centrifuge clear, sterile tubes 1.5 mL (Tarsons, catalog number: 500010) and 2 mL (Tarsons, catalog number: 500020)
3. Dissection box
4. Spatula
5. Tissue paper
6. Kimwipes
7. Nuclease-free water (Promega, catalog number: P1193)
8. 1× sterile PBS (HiMedia, catalog number: TL1022)
9. Phenol-chloroform-isoamyl alcohol (24:24:1) (Sigma-Aldrich, catalog number: 516276)
10. Ice-cold absolute ethanol (Sigma-Aldrich, catalog number: 459836)
11. RNase A (Sigma-Aldrich, catalog number: R6513)
12. Tris-base
13. HCl
14. EDTA
15. NaCl
16. SDS
17. Milli-Q® ultra-pure water
18. 70% ethanol (1,000 mL) (see Recipes)
19. Lysis buffer (100 mL), pH 7.2 (see Recipes)

B. For tissue RNA isolation

1. Filter MAXIPENSE™, Low retention, clear, sterile tips 1,000 µL (Tarsons, catalog number: 527106), 200 µL (Tarsons, catalog number: 527104), and 10 µL (Tarsons, catalog number: 527100)
2. Spinwin™ Micro Centrifuge clear, sterile tubes 1.5 mL (Tarsons, catalog number: 500010) and 2 mL (Tarsons, catalog number: 500020)
3. Dissection box
4. 1 mL syringe
5. Weighing paper (Fisher Brand, size 4" × 4")
6. Tissue paper
7. Kimwipes
8. Mortar and pestle
9. RNA*later*® (Sigma-Aldrich, catalog number: R0901)
10. RNaseZAP™ (Sigma-Aldrich, catalog number: R2020)
11. Nuclease-free water (Promega, catalog number: P1193)
12. Liquid nitrogen
13. RNeasy Mini Kit (Qiagen, catalog number: 74104)
14. DNase I (Qiagen, catalog number: 74106)
15. RDD buffer (Qiagen, catalog number: 74106)
16. 70% ethanol (1,000 mL) (see Recipes)

Equipment

1. Pipette (1,000 µL, 200 µL, 20 µL, 2 µL) (Thermo Fisher, Massachusetts, U.S)

2. -80 °C freezer (Thermo Fisher, Massachusetts, U.S)
3. CO_2 chamber for mice (Thermo Fisher, Massachusetts, U.S)
4. Vortex (Fisher Biotec, Wembley WA 6904, Australia)
5. Microcentrifuge (Eppendorf, Hamburg, Germany)
6. Laminar airflow (Thermo Fisher Scientific, Columbus, OH, USA)
7. Thermoshaker (Thermo Fisher, Massachusetts, U.S)
8. Rotospin (Tarsons, Kolkata, India) or any test tube rotator
9. Nanodrop 2000 machine (Thermo Fisher Scientific, Columbus, OH, USA) or any UV-VIS spectrophotometer (minimum sample volume 0–5 µL)
10. Qubit4 fluorometer (Invitrogen, California, USA) or any normal fluorometer
11. Horizontal gel electrophoresis apparatus (California, U.S)

Procedure

Part I: Microbial gDNA isolation from mouse cecal content

A. Collection of cecal content

1. Dissect the mouse aseptically and carefully remove the cecum.
2. Collect the cecal content in a sterile 2 mL microcentrifuge inside the laminar airflow to avoid any kind of environmental contamination. Label the sample properly. Immediately snap freeze the cecal content and store it at -80 °C for further use (**Graphical abstract step 1**).

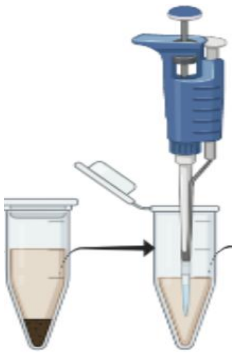
Graphical abstract step 1. Collection of cecal content.

B. Cecal DNA extraction

1. Take the cecal sample from the -80 °C freezer and keep it on ice. Aseptically transfer 180–220 mg of cecal content into a sterile 2 mL microcentrifuge tube.
2. Add 1 mL 1× sterile ice-cold PBS to the sample and homogenize it properly by vigorous vortexing and pipetting.
3. Centrifuge the suspension at 1,500 × *g* for 5 min at room temperature (**Graphical abstract step 2**).

Graphical abstract step 2. Centrifugation of the cecal sample.

4. Carefully remove the supernatant. Add 600 µL of lysis buffer to the pellet and mix it properly by pipetting (**Graphical abstract step 3**).
   Note: Do not use vortex for mixing.

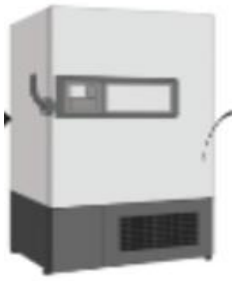


Graphical abstract step 3. Lysis buffer addition.

5. Keep the mixture at 70 °C for 30 min with intermittent mixing by inverting the microcentrifuge tube several times (**Graphical abstract step 4**).

Graphical abstract step 4. Heating at 70 °C.

6. Centrifuge the lysate at 15,700 × *g* for 15 min at 4 °C and collect the supernatant in a sterile 2 mL microcentrifuge tube.
7. Add 1 mL of phenol-chloroform-isoamyl alcohol (24:24:1) to the lysate and mix it properly for 2–3 min by inverting the tube approximately 12–15 times.
8. Centrifuge the mixture at 15,700 × *g* for 15 min at 4 °C.
9. Collect the upper aqueous phase in a new sterile 2 mL microcentrifuge tube. Repeat steps 7–9 at least twice, until the lysate is colorless.
10. Add three volumes of ice-cold absolute ethanol to the colorless lysate and gently mix by simultaneously inverting and rotating in a rotospin instrument for 5–10 min.
11. Freeze the solution at -80 °C for at least 1 h. For better precipitation of DNA, keep it in the freezer for 4–5 h (**Graphical abstract step 5**).



Graphical abstract step 5. Precipitation at -80 °C.

12. Separate the precipitate by centrifugation at 15,700 × *g* for 15 min at 4 °C.
13. Wash the pellet once with 700 µL of 70% ethanol and centrifuge at 15,700 × *g* for 15 min at 4 °C (**Graphical abstract step 6**).

Graphical abstract step 6. Ethanol wash.

14. Dry the pellet at room temperature for 20–30 min.
15. Add 50–100 µL nuclease-free water to the pellet and mix it with gentle tapping.
16. Add 5 µL of RNase A and keep at room temperature for 15 min.
17. Repeat steps 10–15 to remove the RNase A and recover the pure microbial gDNA (**Graphical abstract step 7**).

Graphical abstract step 7. Microbial gDNA.

18. Immediately check the quality and quantity of the RNA, and store it in a -80 °C freezer.

Part II: RNA extraction from mouse colonic tissue

A. Collection of the colon tissue sample

1. Dissect the mouse aseptically and cut the colon portion from the entire gut.
2. Remove the colonic content by flushing with cold 1× sterile PBS.
3. Cut the tissue (80–120 mg) into small pieces and store it in RNALater (1,800 µL) at a -20 °C freezer for further use.

B. RNA extraction

For RNA isolation of the colonic tissue, we use the Qiagen RNeasy Mini Kit with an additional step of churning the tissue with liquid nitrogen for better yield.

1. Weight 20–30 mg (not more than 30 mg) of colonic tissue. Remove the remaining RNALater from the tissue sample using Kimwipes.
2. Place the tissue sample in a mortar. Add ample liquid nitrogen and immediately churn the tissue with the pestle. Ensure that all liquid nitrogen evaporates before grinding (**Graphical abstract step 8**).
   Note: After step 2, follow the Qiagen RNeasy Mini Kit protocol.

Graphical abstract step 8. Tissue grinding.

3. Add 700 µL of RLT buffer to the mortar and mix the churned tissue with the buffer by pipetting.
4. Transfer the lysate into a 1.5 mL sterile microcentrifuge tube and centrifuge at 14,000 × *g* for 3 min at room temperature.
5. Carefully remove the supernatant by pipetting and add 1 volume of 70% ethanol to the lysate. Mix well by pipetting and centrifuge at 14,000 × *g* for 3 min at room temperature.
6. Carefully remove the supernatant and transfer up to 700 µL of the sample to an RNeasy Mini spin column placed in a 2 mL collection tube.
7. Close the lid and centrifuge at 8,000 × *g* for 15 s at room temperature. After centrifugation, discard the flowthrough.
8. Add 350 µL of Buffer RW1 to the RNeasy column, close lid, and centrifuge at 8,000 × *g* for 15 s at room temperature. After centrifugation, discard the flowthrough.
9. Add 10 µL of DNase I stock solution to 70 µL of Buffer RDD and mix by gently inverting the tube. Give a brief spin.
10. Add 80 µL of DNase I incubation mix directly to the RNeasy column membrane, and keep it at room temperature for 15 min (**Graphical abstract step 9**).

Graphical abstract step 9. Column purification.

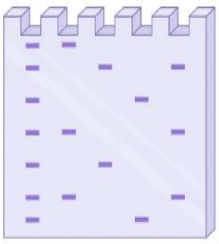
11. Add 350 µL of Buffer RW1 to the RNeasy spin column. Close the lid and centrifuge at 8,000 × *g* for 15 s at room temperature. Discard the flowthrough.
12. Add 500 µL of Buffer RPE to the RNeasy spin column. Close the lid and centrifuge at 8,000 × *g* for 15 s at room temperature. Discard the flowthrough.
13. Add 500 µL of Buffer RPE to the RNeasy spin column. Close the lid and centrifuge at 8,000 × *g* for 2 min at room temperature. Discard the flowthrough.
14. Place the RNeasy spin column in a new 2 mL collection tube. Centrifuge at full speed for 1 min to dry the membrane.
15. Place the RNeasy spin column in a new 1.5 mL collection tube.
16. Add 30–50 µL of nuclease-free water directly to the spin column membrane. Wait for 2 min.
17. Close the lid and centrifuge at 8,000 × *g* for 1 min at room temperature to elute the RNA.
18. Immediately check the quality and quantity, and store the RNA in a -80 °C freezer (**Graphical abstract step 10**).

Graphical abstract step 10. Tissue RNA.

Data analysis

The concentration and quality of the obtained DNA (approximate expected yield/concentration: 800–2,200 ng/µL) and RNA (approximate expected yield/concentration: 700–2,000 ng/µL) can be measured by NanoDrop analysis (**Graphical abstract step 11**). The absorbance ratios at 260 nm/230 nm and 260 nm/280 nm can be determined to evaluate the purity of the extracted DNA and RNA, respectively. DNA and RNA concentration can be validated by the Qubit fluorometer (**Graphical abstract step 12**). DNA and RNA integrity can be assessed by 0.8% and 2% agarose gel electrophoresis, respectively.

Graphical abstract step 11. Quantity check by Nanodrop.



Graphical abstract step 12. Quality check by Agarose gel.

Notes

1. It is important to collect cecal samples from all animals within an experimental cohort at the same time of the day, to avoid diurnal oscillations of the gut microbiota (Thaiss *et al.*, 2014). It is also recommended to isolate the DNA from freshly collected samples of a single cohort at the same time to avoid storage artifacts, as freezing of cecal samples was shown to influence the ratio of *Firmicutes* to *Bacteroidetes* (Bahl *et al.*, 2012).

Recipes

1. Lysis buffer for gDNA isolation (100 mL), pH 7.2

Reagent	Final concentration	Amount
Tris-base	n/a	1.211 g
HCl	0.1M	8.940 mL
EDTA	20mM	0.744 g
NaCl	100mM	0.584 g
SDS	4%	4.000 g
Milli-Q® ultra-pure water	100 mL	Adjust the volume with water

2. 70% ethanol (1,000 mL)

Reagent	Final concentration	Amount
Ethanol (absolute)	70%	700 mL
Milli-Q® ultra-pure water	n/a	300 mL

Acknowledgments

We acknowledge Dr. Biswaranjan Pradhan for helping us to standardize the DNA isolation protocol.
We also acknowledge the two previous reports from our lab by Pradhan *et al.* (2019 and 2022), from which we adopted the mentioned DNA isolation protocol.

Competing interests

The authors declare no competing interests.

Ethics

Committee for Control and Supervision of Experiments on Animals, Govt. of India (CPCSEA) approved this study (IAEC, Reg. No- 1634/GO/ReBi/S/12/CPCSEA, protocol number NISER/SBS/IAEC/AH-186). All experiments were performed as per the approved guidelines.

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