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Fecal (micro) RNA Isolation

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TITLE:

Fecal (micro) RNA Isolation

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KEYWORDS:

microRNA, fecal samples, stool specimens, RNA isolation, total RNA, miRNA

SUMMARY:

This protocol isolates high quality total RNA from fecal samples of animal and human subjects. A commercial miRNA isolation kit is used with significant adaption to isolate pure RNA with optimized quantity and quality. The RNA isolates are good for most downstream RNA assays such as sequencing, micro-array, and RT-PCR.

ABSTRACT:

It is becoming clear that RNA exists in the gut lumen and feces in animals and humans. The protocol described below isolates total RNA including microRNAs from fecal samples of animal and human subjects. The aim is to isolate total RNA with high purity and quantity for downstream analyses such as RT-PCR and sequencing. The advantages of this optimized protocol in the miRNA isolation are the purified RNA products with additional washing steps described, increased quantity of RNA obtained with an improved method in the resuspension of sample, and important tips of decontamination. One limitation is the inability to process and purify larger sample of more than 200 mg as these sample sizes would cause a difficulty in the clear formation of the interphase. Consequently, the large sample size may contaminate the aqueous phase to be extracted as described in the protocol with organic matters that affect the quality of RNA isolated in the end. However, the RNA isolates from up to 200 mg samples are sufficient for most of downstream analyses such as RNA sequencing, RT-PCR, micro-array.

INTRODUCTION:

Extracellular RNA is getting recognized as a significant factor that mediates many biological

processes[1]. Extracellular RNA in feces was first reported in 2008 as a marker for colon cancer and active ulcerative colitis[2], and it was recently revealed as a normal component of the gut lumen and feces and mediates host-microbe communications[3–5]. The purpose of this RNA isolation protocol is to extract high quality extracellular RNA from fecal samples collected from animal and human subjects. The protocol was adapted from a commercial miRNA Isolation Kit. The RNA acquired is utilized for downstream analyses such as RNA sequencing, RT-PCR, micro-array. The protocol includes several important and useful tips to maximize the quantity and quality of RNA found in the feces of animals and humans. The reason to develop and optimize this method of RNA (including microRNA) isolation is to decrease microbial RNA in the feces, limit the variables in the research studies and analyze the RNA composition in the gut without accounting various confounding factors and sources of contamination. Of note, this RNA isolation minimizes the release of RNA from living cell and living microbes (cellular RNA). It focuses on extracellular RNAs that have been released by gut cells or been acquired via food intake. This method is not for studies where the microbial transcriptome is investigated.

PROTOCOL:

All methods involving research animals described here have been approved by the Institutional Animal Care and Use Committee (IACUC) of Brigham and Women's Hospital, Harvard Medical School.

All methods involving human research subjects described here are in accordance with the guidelines set by the Partners Human Research Committee.

1. Fecal sample collection

1.1. Autoclave or prepare a sterile and nuclease-free 2 mL microcentrifuge tube with a screw cap for each animal subject in an experiment.

1.1.1. For human subjects, provide an appropriate, nuclease-free, and sterile stool specimen collection device for each subject.

1.2. Collect 25-100 mg (approximately 1-4 fecal pellets for mouse fecal samples) of fecal samples from each animal subject in a sterile environment.

NOTE: Two or more fecal pellets (~50 mg or heavier) are preferred for obtaining RNA of the highest purity.

1.2.1. Utilize all necessary Personal Protective Equipment (PPE) and materials, for example: a pair of laboratory gloves, a disinfectant spray and a sterile paper towel, to sterilize the working area, where the animal research subject is placed on, to avoid fecal sample contamination.

1.2.1.1. For human subjects, instruct each research subject/collector to collect 100-200 mg of stool samples in an environment as sterile as possible. Use standard sterile operation and avoid stool specimen contamination.

1.3. Collect fecal samples from each animal subject directly into a 2 mL microcentrifuge tube with a screw cap without touching any other surfaces to avoid contamination.

1.3.1. For human subjects, instruct each subject to defecate directly into an applicable collection device provided (e.g., a sterile stool specimen collection kit) to avoid contamination.

NOTE: Instruct subject to avoid contamination by toilet surfaces, water, urine, or any other non-sterile surfaces/objects.

1.4. Freeze fecal samples collected in 2 mL microcentrifuge tubes immediately at -80 °C or place in a bucket of dry ice for the better quantity and quality of RNA before feces resuspension as described in the steps below.

1.4.1. For human stool specimens, aliquot each fresh specimen of 200 mg into 2 mL microcentrifuge tubes with screw caps and freeze them at -80 °C before feces resuspension as described below.

1.4.1.1. For the storage of stool specimens not in 2 mL microcentrifuge tubes with screw caps, weigh and transfer 100-200 mg each of frozen specimens into separate 2 mL microcentrifuge tubes with screw caps before feces resuspension as described below.

NOTE: For human stool specimens, avoid taking more than 200 mg of stool specimens for RNA isolation as it may cause difficulties in the following steps. With an overloaded sample in a 2 mL microcentrifuge tube, the aqueous phase, organic phase and interphase may not be clearly formed and separated. The procedure can be paused here.

2. Preparations of wash solutions

2.1. Add 21 mL of American Chemical Society (ACS) grade 100% ethanol to the Wash Solution 1 provided in the miRNA Isolation Kit, (see **Table of Materials**), to reach the final volume of 30 mL, as shown on the bottle. Vortex till everything dissolves in the bottle.

2.2. Add 40 mL of ACS grade 100% ethanol to the Wash Solution 2/3 provided to reach the final volume of 50 mL, as shown on the bottle. Vortex for 5 s or until the final mixture is well blended.

3. Preparations of equipment and materials

3.1. Spray the working area and equipment, for example: the laboratory bench, the working area in the chemical fume hood and the micro-centrifuge tube racks, with a Ribonuclease (RNase) decontamination solution (e.g., commercially available RNase decontamination solution). To avoid contamination, apply with the RNase decontamination solution on the surfaces wherever and whenever deemed necessary.

3.2. Don a clean laboratory coat, put on a facial mask, and wear appropriate laboratory gloves to protect the RNA in the fecal samples from nucleases present on human skin. Spray gloves with a RNase decontamination solution and change gloves frequently to avoid contamination.

3.3. Prepare a bucket of dry ice for fecal samples stored at -80 °C to prevent thawing before feces resuspension and a bucket of ice for materials, for example: Acid-Phenol: Chloroform, to prolong the shelf life.

NOTE: Ensure materials including the media used in the protocol are sterile without contamination of nucleases.

4. Feces resuspension

4.1. Resuspend 25-100 mg of fecal samples in 600 µL of sterile 1x Dulbecco's Phosphate-Buffered Saline (DPBS).

CAUTION: Fecal samples should be processed immediately when thawed from -80 °C without even partial thawing to ensure all RNases released and captured as ice crystals rupture both interior and exterior cellular compartments when cells thaw.

4.1.1. Add 600 µL of 1x DPBS to the 2 mL microcentrifuge tube with a screw cap containing fecal samples at room temperature (RT).

4.1.2. Incubate the mixture of fecal samples submerged in 600 µL of 1x DPBS in the 2 mL microcentrifuge tube capped for 30 min at RT.

4.1.3. Resuspend the mixture by mashing with 1 mL pipette tip and vortex well with the 2 mL microcentrifuge tube capped. To optimize and increase the quantity and quality of RNA, resuspend the mixture with a homogenizer with the setting for one cycle at S4000 (or 4000 rpm) and 45 s.

5. Organic extraction

CAUTION: Use the hazardous chemical fume hood for the following steps until Step 6 with the use of acid-phenol: chloroform and ACS grade 100% ethanol due to their toxicity and inflammability. Change PPE as needed and follow proper standard precautions when dealing with hazardous material.

5.1. Extract RNA with 600 µL of acid-phenol: chloroform (the volume of acid-phenol: chloroform required equals to the initial volume of added 1x DPBS in Step 4.1).

5.1.1. Add 600 µL of acid-phenol: chloroform to the suspension from Step 4.1.

NOTE: Withdraw acid-phenol: chloroform from the lower phase in the bottle as the upper phase

is mixed with an aqueous buffer. If the interphase between these two phases is disturbed, then wait and withdraw acid-phenol: chloroform only when the interphase re-establishes itself to avoid contamination.

5.2. Vortex the mixture for 60 s to thoroughly mix. Alternatively, to optimize and increase quantity of RNA in the yield, mix by using a homogenizer with the setting for one cycle at S4000 and 45 s.

5.3. Centrifuge for 15 min at 10,000 x *g* at RT to separate the aqueous and organic phases with a microcentrifuge. After centrifugation, the interphase should be compact. If not, repeat the centrifugation.

NOTE: If the interphase could not be as compact as desired possibly due to uneven ratio of the initial volume to the volume of added acid-phenol: chloroform after several repeats of centrifugation, proceed to recover the aqueous phase with a greater care to avoid contamination.

5.4. Recover the aqueous phase and transfer it to a new 2 mL microcentrifuge tube with a hinge cap (not provided by the miRNA isolation kit).

5.4.1. Remove the aqueous (or upper) phase carefully without disturbing the lower phase and transfer it to a new 2 mL microcentrifuge tube with a hinge cap. Note the volume transferred (e.g., ~500 µL).

NOTE: When the interphase is compact and the upper phase is clearly separated, there are possibly a few tiny residual particles floating on the top of the aqueous phase. Pipette carefully to avoid these residues and only recover visibly and clearly separated aqueous phase to ensure a quality RNA yield, even if you could only obtain a small volume of the aqueous phase.

6. Final RNA isolation

6.1. Add 1.25 volumes of RT ACS grade 100% ethanol to the aqueous phase in the 2 mL microcentrifuge tube (e.g., add 625 µL of 100% ethanol if 500 µL of aqueous phase is recovered from Step 5.4.). Vortex 3 s.

6.2. Load the aqueous phase/ethanol mixture through the filter cartridge provided in the miRNA isolation kit.

6.2.1. For each sample, place the filter cartridge into one of the collection tubes supplied by the kit.

6.2.1.1. Pipette and load 600 µL of the aqueous phase/ethanol mixture into the filter cartridge.

NOTE: Vortex the mixture briefly to thoroughly mix the ethanol with aqueous phase before

pipetting. No more than 700 µL of the aqueous phase/ethanol mixture can be loaded at a time.

6.2.2. Centrifuge at 10,000 *x g* for 90 s to filter through the mixture. Spinning at a higher speed may damage the filter.

6.2.3. Discard the filtrate and repeat Steps 6.2.1 to 6.2.2 until all the mixture is filtered through the same filter membrane in successive applications. Keep and reuse the same collection tube for washing steps below.

6.2.4. Wash the filter with 700 µL of miRNA Wash Solution 1.

CAUTION: miRNA Wash Solution 1 contains guanidine thiocyanate that can cause skin irritation and serious eye damage. Wear necessary PPE, for examples: gloves, face shield, protective laboratory coat. Change gloves frequently as necessary.

6.2.4.1. Apply 700 µL of miRNA Wash Solution 1, the working solution prepared with the ACS grade 100% ethanol, into the filter cartridge.

6.2.4.2. Centrifuge for 60 s to filter the miRNA Wash Solution 1 through the filter cartridge.

6.2.4.3. Discard the filtrate from the collection tube and place the same filter cartridge into the same collection tube.

6.2.5. Wash the filter with Wash Solution 2/3 one time each with volumes of 700 µL, 500 µL, and 250 µL consecutively.

6.2.5.1. Apply 700 µL of Wash Solution 2/3, the working solution prepared with the ACS grade 100% ethanol, into the filter cartridge.

6.2.5.1.1. Centrifuge at 10,000 *x g* for 1 min.

6.2.5.1.2. Discard the filtrate from the collection tube and place the same filter cartridge into the same collection tube.

6.2.5.2. Apply 500 µL of Wash Solution 2/3 into the filter cartridge.

6.2.5.2.1. Centrifuge at 10,000 *x g* for 1 min.

6.2.5.2.2. Discard the filtrate from the collection tube and place the same filter cartridge into the same collection tube.

6.2.5.3. Apply 250 µL of Wash Solution 2/3 into the filter cartridge.

6.2.5.3.1. Centrifuge at 10,000 *x g* for 1 min.

6.2.5.3.2. Discard the filtrate from the collection tube.

6.2.5.4. Transfer the filter cartridge into a new collection tube and spin the assembly for 5 min to remove residual fluid from the filter.

7. Elute RNA with 50 µL nuclease-free water

7.1. Transfer the filter cartridge into a new collection tube. Pipette 50 µL of nuclease-free water to the center of the filter and cap the collection tube.

7.1.1. Incubate at RT for 10 min.

7.1.2. Spin for 5 min at 8,000 x *g* to recover RNA into the new collection tube.

7.1.3. Determine the concentration and purity of recovered fecal RNA using a fluorometer. Recovered fecal RNA can be stored at -80 ˚C.

REPRESENTATIVE RESULTS:

Representative RNAs were isolated from 50 mg mouse fecal samples (2 mouse fecal pellets) and 100 mg human stool specimens respectively and eluted in 50 µL nuclease-free water. Spectrophotometer analysis of the concentration suggests a total amount of 49 µg and 16 µg RNA were isolated respectively (**Table 1**). The RNA purity was high as indicated by an A260/A280 ratio of ~2.0 and an A260/A230 ratio of ~1.8 (**Table 1**). As reported [3], the majority of RNAs in the feces are microRNA and those microRNAs can exist in the exosome. Consistent with this, a chip-based electrophoresis assay of RNA suggests that representative RNA isolates from mouse and human feces are low in or lack of 18S and 28S rRNA compositions, and the size of RNA isolates falls in the small RNA region (**Figure 1A**). A further small RNA electrophoresis with the chip-based electrophoresis reveals that a large portion of the RNAs are of microRNA size (**Figure 1B**), consistent with the observation that the quantification done with small RNA bioanalyzer is comparable to that obtained with previous assays[6].

FIGURES AND TABLES:

Table 1: Representative nanodrop analysis of RNA isolated with this protocol. Representative RNAs were isolated from 2 mouse fecal pellets or 100 mg human stool specimens, eluted in 50 µL nuclease-free water. RNA concentration, ratio of A260/A280, and ratio of A260/A230 were measured with nanodrop.

Figure 1: Representative chip-based electrophoresis analyses of size distribution of fecal RNA isolates. (**A**) Representative RNAs isolated from 2 mouse fecal pellets (left panel) and 100 mg human stool specimens (right panel) using the protocol descripted here were characterized using the chip-based electrophoresis assay, which suggests the majority of RNA isolates were small RNA. (**B**) The isolates were then subjected for the small RNA electrophoresis with the chip-based

electrophoresis system to analyze the size distribution of the isolates.

DISCUSSION:

It is important to use RNase-free technique to prevent RNase contamination during the isolation[7]. After centrifugation and the formation of a compact interphase, it is key to avoid the interphase, lower phase, and the particle contaminant floating on the top of the aqueous phase when recovering the aqueous phase. Additionally, two washing steps with 500 µL and 250 µL Wash Solution 2/3 are added to eliminate contaminants in the filter membrane for optimized quality. Furthermore, a start sample material of more than 200 mg is not recommended. Similarly, a sample material of less than 25 mg is not recommended as it may not be sufficient to extract enough RNA samples for downstream analysis.

The incredible growth in microbiome study has driven the measurements of microbial species, genes to metatranscriptional studies of the microbial profile[8]. MicroRNAs in the stool have been studied as markers for diseases[9–11]. Since the first report of fecal microRNA mediating host-microbe interactions[3], increasing studies start to investigate the contributions of host and diet in the gut ecosystem[12–14]. Noteworthily, due to the richness of microbes in the gut lumen and feces, studies focusing on host arm of the host-microbe interaction demands minimal RNA contamination from microbes. Thus, an RNA extraction protocol that includes steps of cell lysing[15] is not ideal for the study of RNAs released from host and diet. We have adapted the protocol by eliminating the lysing steps to minimize RNA contaminations from living bacteria and living host cells in the feces.

This protocol works for studies where extracellular RNA in the fecal or gut lumen content is an aim of interest. RNA isolated using this protocol is total RNA, including microRNA as major component. This protocol does not distinguish whether the RNA is in exosome, microvesicles, or in a vesicle-free form.

ACKNOWLEDGMENTS:

We received technical assistance from the Biopolymers Facility at Harvard Medical School for bioanalyzer. This work was supported by National Multiple Sclerosis Society research grant RG-1707-28516 (H.L.W. and S.L.).

DISCLOSURES:

The authors declare no relevant or material financial interests that are related to the research method described in this protocol paper.

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Figure 1

Click here to access/download;Figure;Figure 1 Representative V3.tif

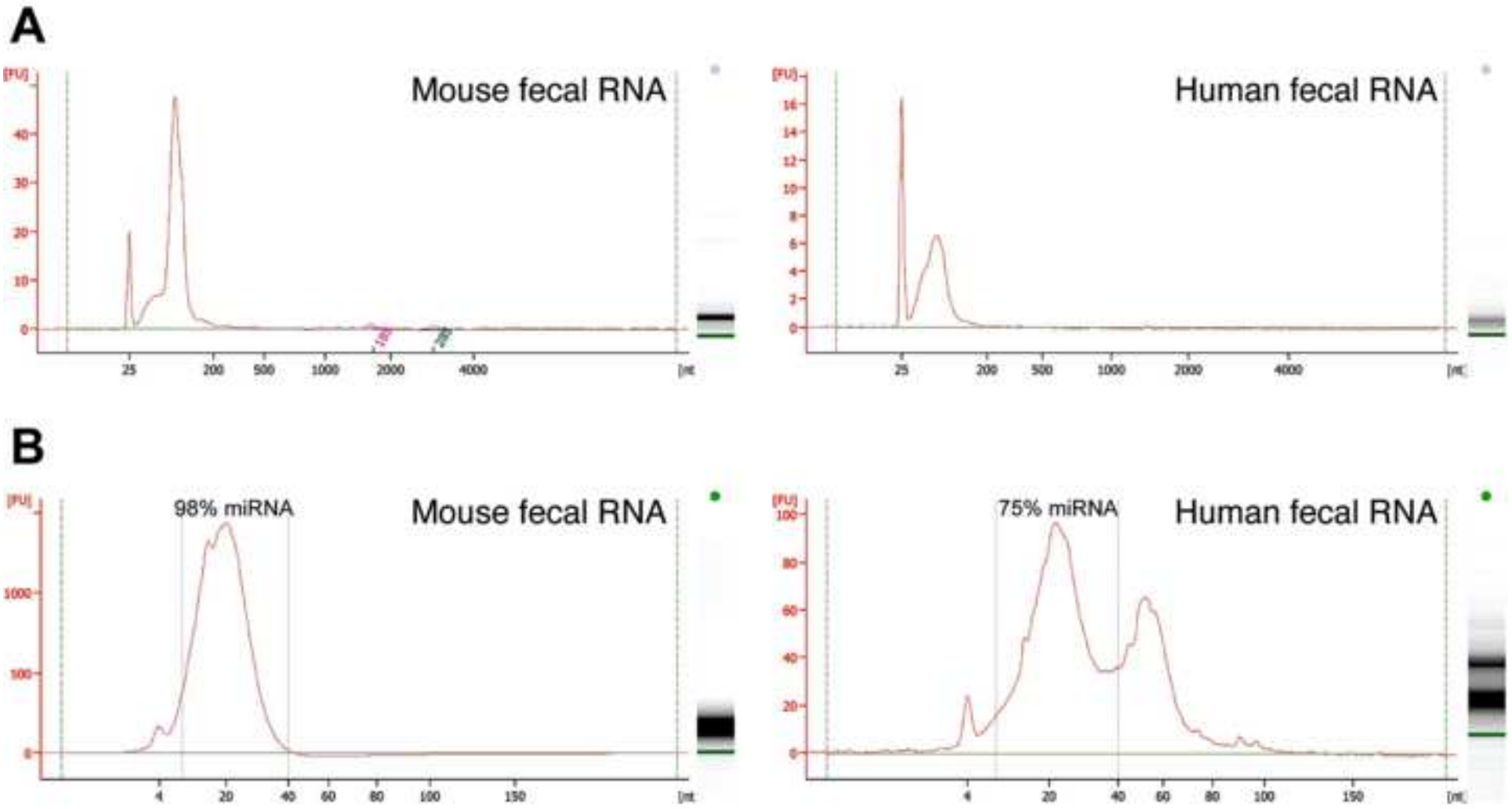

Table 1 Representative Nanodrop results

Click here to access/download;Table;Table 2 Representative Nanodrop results.xlsx

Sample ID	Elution volume (µL)	RNA concentration (ng/µL)
Mouse	50	978.333
Human	50	330.759

A260/A280	A260/A230	Yield (ng)
2.036	1.897	48916.65
1.981	1.849	16537.95

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Acid-Phenol: Chloroform, pH 4.5 (with IAA, 125:24:1)	Thermo Fischer Scientific	AM9720	
DPBS, no calcium, no magnesium	Thermo Fischer Scientific	14190-144	
Gloves			
Microcentrifuge			
mirVana miRNA Isolation Kit	Thermo Fischer Scientific	AM1561	
Nuclease-Free microcentrifuge tubes (1.5 mL, 2 mL)			
Nuclease-Free Water (Not DEPC-treated)	Thermo Fischer Scientific	AM9937	
Pipettor and Nuclease-Free Pipette tips (with filter)			
PowerLyzer 24 Homogenizer	QIAGEN	13155	
RNaseZap RNase Decontamination Solution	Thermo Fischer Scientific	AM9780	
Vortex Shaker			

September 28, 2020

Nam Nguyen, Ph.D.
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Dear Dr. Nam Nguyen,

Thank you for your comments and those of the reviewers regarding our manuscript, JoVE61908 "Fecal (micro) RNA Isolation" by Dhang *et al*. We have revised the manuscript in which we address the editorial and peer review comments below. We have uploaded the files for our manuscript according to the journal guidelines. Thank you for all your help and please let us know if anything else is required.

Sincerely,

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Editorial comments:

Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues (Line 329: impropriate?). Response: The manuscript has been thoroughly proofread to ensure no spelling or grammatical errors.

2. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Response: The table of materials has been removed, updated, and uploaded separately in the form of an .xlsx file.

3. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader. Response: Less than three pages of the Protocol have been highlighted as essential steps for the video production.

4. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Response: The highlighted steps are in accordance with your comments

Reviewers' comments:

Reviewer #1:

Manuscript Summary: Specify in the summary that isolated RNA is mainly from the host (and not the microbiota) and why your protocol allows that discrimination. Response: Due to format limit (50 words maximum for Summary), we did not specify this in the summary. We now discuss in the discussion section (Line 369-374) why RNA isolates by our protocol minimize RNAs from living microbes. We did not state that our protocol allows discrimination of host vs microbial RNAs.

Major Concerns:
no major concern

Minor Concerns:

1. **Line 59-65, in the generality section at the beginning of the protocol, a mention to the kit (with reference) that will be used (with modifications) could be done. Response:** The kit used has been referenced as commented (now Line 52-53).

2. **Line 116. The reference of the kit should be put here (despite referencing the material table) as it is the first time mentioning the kit. Alternatively, the use of this kit could be referenced as commented above. Response:** addressed, see response above to concerns #1

3. **Line 126. Suggestion of reference for the decontamination solution. Response:** addressed, see response above to concerns #1, 2

4. **Line 142. Re-specify the optimal weight of fecal matter to be used or refer to section 1 that explains those aspects. Response:** The optimal weight of fecal matter has been clearly specified again in this section to make it conspicuous that only 25-100 mg of fecal samples should be used.

5. **Line 151. Indicate if incubation should be done at RT. Response:** Indeed, the incubation in this step is and should be done at RT. This has been added.

6. **Line 161: Specify whether working under a hazardous chemical fume hood is necessary until the end of the protocol**. **Response:** It is necessary and recommended to work under a hazardous chemical fume hood until Step 6 of the protocol. The section has been revised stating this.

7. **Line 184: If the tubes are supplied by the kit, please specify. Please do so whenever applicable throughout the protocol. Response:** These tubes noted here are not provided by the kit, as edited for clarification.

8. **Line 208: Specify clearly that no more than 600ul should be applied to the filter cartridge at a time. Response:** No more than 700 µL can be loaded to the filter cartridge at a time. The comment has been noted and addressed.

9. **Line 325-326: Reword the sentence as it is not correct. Second half of sentence missing or first part must be combined with the following sentence. Response:** The sentences have been combined for a clear flow and easily understood.

Reviewer #2:
Manuscript Summary:
This manuscript describes a brief protocol for the extraction of total RNA from mouse and human feces. The authors provide clear and concise methodology to this end.

Major Concerns:
None.

Minor Concerns:

1. **Line 43, could do with grammatical revision. Response:** The grammatical error has been addressed.
2. **Line 56, insert "the" between where and microbial. Response:** The grammatical error has been addressed. The "the" has been inserted between “where” and “microbial”
3. **Line 91 does not sound right. Response:** The clarification has been added that the instruction for human subjects to collect stool specimen.
4. **Line 142, is this sterile PBS. Response:** Indeed, the DPBS used in the protocol is sterile. We have edited accordingly.

5. **Discussion: The 1st paragraph is confusing and should be revised. The authors should also reference the following paper, it was the 2nd publication to describe faecal miRNA:**
   Faecal microRNAs: indicators of imbalance at the host-microbe interface? G M Moloney 1, M F Viola 1, A E Hoban 1 2, T G Dinan 2 3, J F Cryan 1 2 Affiliations expand PMID: 29264965 DOI: 10.3920/BM2017.0013. **Response:** The first paragraph of the discussion section has been revised for clarification and the suggested paper has been referenced.

Reviewer #3:
Manuscript Summary:
I have read with interest the manuscript by Dhang, Weiner and Liu and I think it is valuable protocol and interesting to the scientific community involved in this kind of research.
I have only one Major concern and few Minor concerns that it is important to check and modify in the text.

Major concern:

1. **- Table 1 and Figure 1 should be improved. In Table 1, please report both mouse samples concentration (or specify if you have pooled them), the elution volume (50, 100 µl?) and the amount obtained (as stated in the main text). Please, separate the quantitative evaluation done with nanodrop and that performed by Bioanalyzer so to have two distinct parts. In the bioanalyzer part, please report the concentration of the small RNA part (of the two panels in A), and the concentration of the small RNA part (reported in B in the two panels). If a RIN is available (especially for small RNAs), please report also this data. The quantification done with small RNA kit should be comparable to that obtained with Nano kit, according to the literature (Masotti et al. Journal of Biomedicine and Biotechnology Volume 2009, Article ID 659028, doi:10.1155/2009/659028) that could be cited.**
   Response: We have improved Table 1 (New Table 2) and Figure 1 according to the reviewer's suggestion. Please note that RNA 6000 Nano (Figure 1 A) examines the size distribution of total RNA, which shows the majority of fecal RNAs fall in the small RNA size region (<200 nt). However, it does not quantify the concentration of particular region. Consistently, small RNA kit quantified a majority RNA species to be microRNA. RIN is acquired based on the measurement of 28S rRNA (~5kb) and 18S rRNA (~2kb) components and is not available in small RNA (up to 200 nt) Bioanalyzer measurement. We now have cited the recommended reference.

2. **In the manuscript text, in the Representative Results section, please indicate the approximate amount of the two mouse fecal pellets.**

Response: We have indicated the approximate amount of the two mouse fecal pellets as reviewer suggested.

Minor Concerns:

1. **Abstract line 30: Consider to replace "miRNA" with "miRNAs" (here and thorought the manuscript) where appropriate (lines 324, 326). Response:** The plural form of "miRNA" has been given whenever appropriate

2. **Introduction line 43: please revise "as an approach mediate", something is missing. Response:** The grammatical error has been corrected.

3. **Table of Materials: please, add the Bioanalyzer's kits codes employed (the Nano and the small RNA). Response:** Bioanalyzer was completed per service. We used it to evaluate fecal RNA isolated using our protocol but bioanalyzer itself is not part of the RNA isolation protocol.