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Sewage sample extraction of total nucleic acid from secondary concentration pellet using a modified QIAamp Fast DNA Stool Mini Kit procedure

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Coronavirus Method Development Community SARS Cov2 ES

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ABSTRACT

This procedure describes a modified extraction procedure to isolate both DNA and RNA from pellet obtained from secondary concentration from BMFS samples using the QIAmp Fast DNA Stool MiniKit. The sample undergoes a lysate preparation process and includes mechanical disruption (bead beating), removal of inhibitors, purification and elution of DNA and RNA using spin columns. Extrinsic controls PhHV (Phocine Herpesvirus) and MS2 are added to each sample during the lysate preparation to evaluate extraction and amplification efficiency. The extracted total nucleic acid (TNA) is then stored at -80°C for testing. To rule out contamination during the extraction process, a blank is also processed through the complete protocol each day extractions are performed.

ATTACHMENTS

Skim milk pellet TNA extraction.docx

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GUIDELINES

Important Notes to Maximize Downstream Assay Performance

1.PhHV and MS2: Careful preparation, storage and use of the MS2 and PhHV are essential because detection of these extrinsic controls is necessary to accurately evaluate the pathogen target PCR results. Detection of MS2 in the sample confirms the extraction and amplification of RNA and DNA were successful. When MS2 is not detected in the sample, it is not possible to accept the results of other RNA targets that were not detected, thus negative RNA target results in that sample would be invalid. MS2 and PhHV detection rates of <90% or Cts consistently above 32 for either PhHV or MS2 may indicate a procedural or reagent problem and should be investigated before continuing with extractions.

Always prepare the lysate buffer/MS2/PhHV mix fresh each day.

2.Extraction Blank: Processing a blank each day/batch of extractions helps to validate sample results in downstream assays. The extraction blank should be positive only for MS2 and PhHV. A pathogen target detected in an extraction blank may indicate a wider contamination problem. Positive results for that target in any sample that was extracted on that same day or within that batch would be invalid. The lab should address possible sources of the contamination before continuing with extractions. The sensitivity of PCR assays requires adherence to good laboratory practices to avoid cross-contamination between samples and laboratory contamination.

MATERIALS TEXT

A. Materials:

QIAamp® Fast DNA Stool Mini Kit

1. **Qiagen Catalog #51604** plus

Collection

Tubes **Qiagen Catalog #19201**

2. PhHV (*store working solution at* **4 °C** *)*

Note: The concentrated Stock PhHV is stored at **-80 °C** (see Appendix 2 for instruction)

3. MS2 working concentration (see Appendix 1 for instruction)
4. Bead beater
5. Vortexer
6. Microcentrifuge
7. Dry bath **70 °C** and **95 °C**
8. **-70 °C** to **-80 °C** freezer
9. Pipettes and Pipet tips
10. 96%-100% Ethanol
11. **2 mL** screw cap tubes, compatible with bead beater
12. **2.0 mL** microcentrifuge tubes

Glass beads acid-washed 212-300 µm (50-70 U.S. sieve) **Sigma**

13. **Aldrich Catalog #G1277**
14. **0.5 mL** screw cap tubes for TNA storage

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B. QIAamp Fast DNA Stool Mini Kit Reagent Preparation and Kit Components:

Each kit can do 50 sample extractions

Store all kit components at Room Temp (15 °C - 25 °C) for up to 1 year after opening.

- **InhibitEX Buffer**: Mix Buffer thoroughly by shaking before use. If a precipitate has formed, incubate at 35 °C to 70 °C for ~ 00:15:00 to ensure any precipitate has fully dissolved. A color change to orange during storage can be expected. It does not effect the buffer performance.
- **Buffer AL**: Mix Buffer AL thoroughly by shaking before use. If a precipitate has formed, incubate at 35 °C to 70 °C for ~ 00:15:00 prior to use to ensure all precipitate has fully dissolved.
- **ProteniaseK**: If ambient temperature often exceeds 25 °C , store at 4 °C - 8 °C for up to one year. Never add proteinase K directly to Buffer AL.
- **Buffer AW**1: Buffer AW1 is supplied as a concentrate. Before using for the first time, add 25 mL ethanol (96–100%) as indicated on the bottle. Thoroughly mix before use.
- **Buffer AW2:** Buffer AW2 is supplied as a concentrate. Before using for the first time, add 30 mL ethanol (96–100%) to Buffer AW2 concentrate as indicated on the bottle. Thoroughly mix before use.
- Buffer ATE

C. Bead Beating Time: instrument adjustments:

1. Bead disruption for too long will overheat the sample and cause nucleic acid degradation, too little time is ineffective and will not enhance the extraction of DNA. Care should be taken in determining bead beating time for the instrument used. If immediately after bead beating the tube is hot, decrease the time.
2. Fastprep FP120 from Bio101 Thermo Electronic Corporation (Fisher NC9646109)- Bead disruption at maximum speed for 3 minutes is sufficient without over heating the preparation.
3. BioSpec Mini-Beadbeater (BioSpec693, Mini-Beadbeater-8) -Bead disruption at maximum speed for 2 minutes is sufficient.

D. Procedure Notes:

1. Wear gloves throughout the entire procedure. In case of contact between gloves and sample, change gloves immediately.
2. Change pipet tips between all liquid transfers. The use of aerosol-barrier pipet tips is recommended.
3. After all vortexing steps, to avoid contamination pulse centrifuge the tubes to remove drops from the inside of the lid.
4. Pipet the sample into the QIAamp Mini spin column without moistening the rim of the column.
5. Avoid touching the QIAamp membrane with the pipet tip.
6. Close the QIAamp Mini spin column before placing it in the microcentrifuge.
7. Open only one QIAamp Mini spin column at a time.
8. For efficient parallel processing of multiple samples, fill a rack with collection tubes.

E. Specimens :
Use screw cap tubes compatible with the bead beater, Consider batch pre-filling the tubes with beads

1. Secondary concentration pellet sample: combine all the pellets from the conical tubes from the secondary concentration (combined pellet can be stored in -80 °C until extraction)

Transfer and combine all the pellets into a 2 mL screw cap tube that is compatible with the bead beater.

2. Blank (no specimen) include once each day extractions are performed:
Begin with step 3 below and process through the entire protocol.

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Extraction of total nucleic acid from secondary concentration pellet using a modified QIAamp Fast DNA Stool Mini Kit procedure

21m

1 

15m

Mix InhibitEX Buffer thoroughly by shaking before use. If a precipitate has formed, Incubate at **37 °C** - **70 °C** for ~ **00:15:00** or until all precipitate has fully dissolved.

InhibitEX buffer is a lysis buffer, designed to break open the cells. Important components of the lysis buffer are detergents, enzymes, and salts. Sometimes, the salts form precipitates in the solution and fall to the bottom, so when you pipet some of the solution to use in DNA extraction, it will be missing the salts and might not work properly and might not break open the cells.

2 

Prepare fresh each day: Add PhHV and MS2 working solutions to InhibitEX buffer: prepare only enough for N+1 samples to be processed that day: **1 mL** InhibitEX buffer + **1 µl** PhHV+ **1 µl** MS2 is needed per sample (e.g. For 10 samples **11 mL** InhibitEX+ **11 µl** PhHV+ **11 µl** MS2), mix well.

PhHV (Phocine Herpes Virus) is a double-stranded DNA virus and MS2 is a single stranded RNA virus.They are used as internal controls to ensure the DNA extraction and PCR worked properly. We add them to the DNA extraction buffers in a known quantity and if they are not detected in the final sample at a specific level, we know that either 1) some kind of problem occurred in DNA extraction or 2) there were inhibitors in the PCR. During analysis we might consider removing these samples or adjusting the results in some way to account for the inhibition.

3 

If the beads have not been pre-aliquoted to empty tubes: Add : ~ **370 mg** (one eppendorf tube capful) of Sigma beads to the aliquot of stool (or blank tube).

Some bacterial cells and parasite cells are difficult to break open with just chemical lysis buffers. So, we also use mechanical lysis (using a physical thing – beads – to break open the cell).

4 

Add **1 mL** of the InhibitEX/PhHV/MS2 solution to the first skim milk pellet in **50 mL** conical then resuspend the pellet using a **2 mL** serological pipette tip then resuspend the 2nd pellet with the suspension from the first tube and continue until all 6 pellets are suspended and combined (or blank).

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DNA-degrading substances and PCR inhibitors present in the stool sample are separated from the DNA by the InhibitEX buffer.

5 

Add the combined suspension into a **2 mL** screw cap tube with the glass beads.

6 

1m

Vortex for **00:01:00** .

This step is just to ensure that the lysis buffer is mixed well and all beads are moving (not stuck in the bottom of the tube) so that in the next step the cells will be agitated fully with both the chemical and mechanical lysis.

7 Bead beat at maximum speed for **00:02:00** - **00:03:00** .

5m

This step is mostly for mechanical lysis of cells with the beads.

8 

5m

Incubate the suspension for **00:05:00** at **95 °C** .

Human cells lyse at room temperature, but many bacterial and parasite cells are stable at this temperature, even in the presence of chemical lysis buffers. So, here we heat the solution to assist in the lysis of the cells that might have survived the chemical and mechanical lysis in the previous steps.

9 

1m 15s

Vortex for **00:00:15** then centrifuge sample at full speed (approximately **20000 x g**) for **00:01:00** to pellet the stool particles.

We centrifuge to pull down all large components remaining in the sample matrix (like food particles remaining in the stool, cell wall debris, or other things not suspended in the liquid portion of the buffer).

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10 

Pipet **25 µl** proteinase K into a new **2 mL** microcentrifuge tube, (snap caps acceptable).

Proteinase K digests and degrades proteins during incubation at **70 °C** .

11 

Pipet **600 µl** supernatant from step 8 into the **2 mL** microcentrifuge tube containing proteinase K.

Note: Do not transfer any solid material, if necessary centrifuge sample again.

12 

Add **600 µl** Buffer AL.

Note: Do not add proteinase K directly to Buffer AL.
Sodium (Na+) ions in the buffer neutralize the negative charges on the DNA molecules, which makes them more stable and less water soluble. By optimizing these salt and pH conditions with the AL buffer, we ensure that any remaining digested proteins and other impurities (which can inhibit PCR) are not retained.

13 

15s

Vortex for **00:00:15** , Mix thoroughly to form a homogeneous solution.

14 

10m

Incubate at **70 °C** for **00:10:00** . Centrifuge briefly to remove drops from the inside of the tube lid.

Proteinase K is working during this step to degrade proteins.

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15

Add **600 µl** of ethanol (96–100%) to the lysate, and mix by vortexing. Centrifuge briefly to remove drops from the inside of the tube lid.

DNA is not soluble in ethanol so it will precipitate out of the solution and onto the spin column membrane in the next step.

16 1m

Label QIAamp spin column lid and place in a **2 mL** collection tube. Carefully apply **600 µl** of the lysate from step 14 to the QIAamp spin column without moistening the rim. Close the cap and centrifuge at full speed for **00:01:00** .

The special membrane in the bottom of the spin column will bind the DNA and let other dissolved components pass through into the filtrate that comes out the bottom (which we then discard).

17 Retain QIAamp spin column, place the column in a new **2 mL** collection tube, and discard the collection tube containing the filtrate.

18 1m

Repeat 2 more times to use all of the lysate: carefully apply an additional **600 µl** of the lysate from step 14 to the QIAamp spin column without moistening the rim. Close the cap and centrifuge at full speed for **00:01:00** . Retain the column. Discard the collection tube and filtrate. Repeat until all of the lysate (~ **1800 µl** in total) has been through the spin column.

19 Place the spin column in a new collection tube.

20 1m

Open the QIAamp spin column and add **500 µl** Buffer AW1. Close the cap and centrifuge at full speed for **00:01:00** . Place the QIAamp spin column in a new **2 mL** collection tube, and discard the collection tube containing the filtrate.

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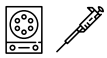
We wash the DNA that is bound to the membrane with Buffer AW1 (Wash Buffer1). This ensures we remove any residual impurities.

21  3m

Open the QIAamp spin column and add **500 µl** Buffer AW2. Close the cap and centrifuge at full speed for **00:03:00** . Retain QIAamp spin column, discard the collection tube containing the filtrate.

Buffer AW2 removes another type of impurity (I don't know the specific things that each wash buffer targets!).

22  3m

To eliminate the chance of possible Buffer AW2 carryover place the QIAamp spin column in a new **2 mL** collection tube and discard the old collection tube with filtrate. Centrifuge at full speed for **00:03:00** .

Note: Residual Buffer AW2 in the eluate may cause problems in downstream applications. An extra centrifugation step ensures that we remove all excess Buffer AW2.

23  5m

Elute the total nucleic acid: Transfer the QIAamp spin column into a labeled microcentrifuge tube. Open the QIAamp spin column and pipet **200 µl** Buffer ATE directly onto the QIAamp membrane. Close the cap and incubate for 1 **00:01:00** - **00:03:00** at **Room temperature** , then centrifuge at full speed for **00:01:00** to elute the TNA.

The ATE buffer re-dissolves the DNA from the membrane back into solution. The incubation for **00:01:00** - **00:03:00** is important to allow enough time for the DNA to unbind from the membrane/ dissolve into the solution. During centrifugation, the DNA passes thru the filter into the final collection tube with the buffer.

24 **Save the filtrate**: this is the TNA. Discard the column.

25 

Prepare two tubes for aliquot storage. Pipet **100 µl** of the extracted TNA into each tube. Store at **-70 °C** to **-80 °C** .

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Appendix 1 Preparation and Storage of MS2/PhHV mix

26 

MS2 and PhHV are incorporated during extraction as extrinsic controls in order to evaluate the extraction and amplification efficiency of RNA and DNA. This protocol describes the preparation and storage of MS2/PhHV mix.

Material

1. Lyophilized MS2/PhHV mix, store at **-80 °C** until use. Reconstitute one vial at a time.
2. STD Diluent, store at **-80 °C** .

Quickly spin the tube containing lyophilized MS2/PhHV mix to bring the pellet to the bottom.

27 

Add **50 µl** STD diluent to each vial, mix well.

28 Use **1 µl** per sample extraction follow the extraction SOP. Store the leftover at **-80 °C** .

Don’t use the reconstituted mix that has been frozen-thawed more than two times.

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