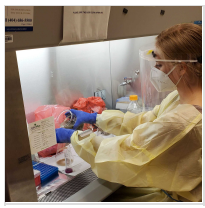

Jan 21, 2022

Skim Milk Flocculation and RNA Extraction for SARS-CoV-2 Viral Capture

Jillian Dunbar[1], Stephen P Hilton[1], Jamie VanTassell[1], Julia Raymond[1], Marlene K Wolfe[1], Pengbo Liu[1], Christine Moe[1]

[1]Center for Global Safe WASH, Rollins School of Public Health, Emory University, Atlanta GA USA

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Stephen Hilton

This protocols describes the use of Skim Milk Flocculation to concentrate and extract SARS-CoV-2 from Moore swab wastewater samples. Included in this protocol are: materials and equipment, steps for processing Moore swabs, and steps for concentrating and extracting the virus using the Qiagen RNeasy Mini Kit.

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Equipment:

- Stainless Steel Potato Ricer or Lab Paddle Stomacher (note: a Stomacher is more expensive but requires less effort for lab technician)
- Erlenmeyer flask
- pH Probe
- Centrifuge Tube
- 50 mL centrifuge tube
- 2 mL microcentrifuge tube
- 4in. by 4in. gauze
- Centrifuge
- Orbital Shaker
- RNeasy spin column
- Eppendorf Research Plus Single Channel Pipette
- LabGard Biological Safety Cabinet - Class 2 A2 Biosafety Cabinet
- Autoclave

Materials:

- Elution solution (contains: NaPP, Tween 80, Antifoam A, 10X PBS)
- Skim milk powder
- 5% HCl
- 5% NaOH
- 10^4 Bovine Respiratory Syncytial Virus (BRSV) per sample
- 70% molecular ethanol
- Qiagen RNeasy Mini Kit (Cat. No. 74106)

Moore Swab Processing 54m 30s

1 Two alternative methods are provided herein for initial swab processing: Lab Paddler Stomacher (Step 2) or a Potato Ricer (Step 3).

2 If using a Lab Paddler Stomacher:

2.1 Set the stomacher settings to paddle at speed 2 for **00:00:30** . 30s

2.2 Place Moore swab sample in a clean stomacher bag with **100 mL** of Elution Solution (see Appendix I for preparation steps).

2.3 Place bag in stomacher so that the top of the bag is above the stomacher and close the handle. The stomacher will begin to paddle the swab for the programed time.

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2.4 Fold the stomacher bag in half length-wise and pour the fluid into a labeled centrifuge bottle.

- Squeeze the swab through the stomacher bag while pouring to release sample fluid contained in the swab.
- Be careful not to let the swab fall out of the bag while pouring the sample fluid into the centrifuge bottle.

2.5 Repeat Steps 2.1 - 2.4 until you have **300 mL** of sample volume.

3 If using the potato ricer method:

Picture of stainless steel potato ricer used for squeezing wastewater fluid out of Moore swab.

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3.1 Place Moore swab sample in a potato ricer and squeeze all the liquid out into a 600 mL beaker.

- The first squeeze typically yields ~100mL of wastewater fluid.
- It is easier to squeeze the fluid into a beaker than a flask due to the wider mouth of the beaker.

3.2 Pour the squeezed liquid into a labeled 500 mL Erlenmeyer flask.

- The 500 mL flask is used to measure the amount of sample volume (final volume will be approximately 300 mL).
- The beaker will be used to mix the swab with the Elution Solution. You will therefore need one flask and one beaker for each swab sample.

3.3 Place the Moore swab back in the beaker. Add **100 mL** of Elution Solution (see Appendix I for preparation steps) into the beaker.

3.4 Gently knead the Moore swab 10 times using the pointed end of a clean 50 mL conical tube. Then, use the top/flat surface of the tube to knead the swab 10 more times.

- Our lab team has found that the 50 mL conical tubes are a good tool for kneading the swabs; however, other instruments could be used to knead the swab.
- It is vital that the instrument should be either sterilized or replaced for each new sample to prevent cross-contamination.

3.5 Turn the swab over and repeat Step 3.4.

3.6 Put the swab back into the potato ricer, and squeeze all the liquid out into the beaker.

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3.7 Pour the liquid into the 500 mL Erlenmeyer flask.

3.8 Repeat Steps 3.3 through 3.7 until there is **300 mL** of sample liquid in the 500 mL Erlenmeyer flask.

3.9 Pour contents into a labeled centrifuge bottle.

4 Ensure all of the centrifuge bottles have similar masses (+/- 0.5g) so that the centrifuge is balanced.

- Using a benchtop scale, weigh each centrifuge bottle (with the lid). Identify the heaviest sample, record its weight, and set it aside.
- Using DI water, adjust the weight of the remaining samples so that each bottle is within 0.5 g of each other.

5 Wipe down the benchtop scale after use using 10% bleach followed by 70% ethanol.

6 Centrifuge for **00:15:00** at **5000 rpm** . 15m

- Utilize the full capacity of the centrifuge. For example, if your centrifuge can hold six 300 mL centrifuge bottles, prepare six samples for the centrifuge. While samples are centrifuging for 15 minutes, you can continue processing the remaining samples.
- If you have a number of samples that cannot be balanced, fill a centrifuge bottle with DI water to be within 0.5 grams of the other bottles and place in the centrifuge.

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Centrifuge loaded at full capacity.

Balanced centrifuge.

7 Pour only the supernatant back into the flask (should be approximately **250 mL**).

- Take care not to let the aggregated solids fall into the flask as you are pouring the supernatant.
- Make sure you are using the same flask as the sample was originally processed in (flask should be labeled to prevent accidental cross-contamination).

8 Adjust the pH of the sample to **pH 3.5** using 5% HCl and 5% NaOH using a pH probe.

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9 Aliquot **10 µL** of 10^4 Bovine Respiratory Syncytial Virus (BRSV) as a positive control to the sample. Mix well by pipetting the BRSV up and down 20 times in the sample before expelling it.

Use a new tip for each sample.

10 Add **1 % volume** of Skim Milk Solution (see Appendix II for preparation steps) to the sample volume.

For example, if sample volume is 250 mL, add 2.5 mL of Skim Milk Solution.

11 Place aluminum foil over the opening of the flask.

12 Place sample on shaker **120 rpm, Room temperature , 02:00:00** .

If a shaker is not available, place a sterilized magnetic stir bar in the sample and stir at room temperature at low speed for 2 hours.

RNA Extraction 54m 30s

13 Pour sample from flask into sterilized and labeled centrifuge bottle.

14 Centrifuge sample for **00:30:00** at **12000 rpm** . 30m

15 Pour supernatant out (you will not use this liquid for RNA extraction).

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16 Gently scrape off and discard a large portion of the pellet, leaving behind a thin film of pellet in the centrifuge bottle.

17 Buffer

- Aliquot **800 µL** of RLT **Qiagen Catalog #79216** from the Qiagen RNeasy Mini Kit (Cat. No. 74106) into the centrifuge bottle.

18 Buffer

Mix well by pipetting the pellet and RLT **Qiagen Catalog #79216** up and down about 30 times or until the mixture looks homogeneous.

19 Pipette **800 µL** of homogenized mixture into a labeled 2 mL DNA LoBind microcentrifuge tube.

20 Centrifuge tube for **00:03:00** at full speed. 3m

21 Transfer all of the sample into a new labeled tube.

22 Aliquot **800 µL** of 70% molecular ethanol.

- Mix well by pipetting the mixture up and down several times.
- Do not vortex after this step.

23 Transfer **700 µL** of the sample mixture into a labeled RNeasy spin column. Centrifuge for **00:00:30** at full speed. Discard filtrate. Repeat until all of the sample is filtered through the spin column. 30s

24 Buffer 30s

Aliquot **700 µL** of RW1 **Qiagen Catalog #1053394** to the RNeasy spin

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column. Centrifuge for **00:00:30** at full speed. Discard filtrate.

25 30s

Buffer

Aliquot **500 µL** of RPE **Qiagen Catalog #1018013** to the RNeasy spin column. Centrifuge for **00:00:30** at full speed. Discard filtrate.

26 2m

Buffer

Add **500 µL** of RPE **Qiagen Catalog #1018013** to the RNeasy spin column. Centrifuge for **00:02:00** at full speed. Discard filtrate.

27 1m

Transfer spin column to new 2 mL collection tube and centrifuge for **00:01:00** at full speed. Discard filtrate and collection tube.

28 1m

Place the RNeasy spin column into a 1.7 mL labeled microcentrifuge tube. Aliquot **50 µL** of RNAse-free Water **Contributed by users** (from RNeasy kit). Incubate for approximately **00:01:00** at room temperature.

> This labeled microcentrifuge tube will contain the final RNA for PCR. Ensure that the label will be able to withstand storage in a freezer until sample is ready for PCR or for archiving purposes.

29 1m

Centrifuge for **00:01:00** at full speed.

30 1m

Aliquot another **50 µL** of RNAse-free Water **Contributed by users** into the the RNeasy spin column in the 1.7 mL labeled microcentrifuge tube. Incubate for approximately **00:01:00** at room temperature.

31 1m

Centrifuge for **00:01:00** at full speed.

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32 Close the cap of the 1.7 mL tube containing the final RNA (you will have a final volume of **100 µL**).

33 Store RNA at **-20 °C** until it is ready for PCR.

Appendix I - Prepare Elution Solution

34 Prepare the Tween 80 stock by dissolving **1 mL** of Polyoxyethylene-80 (TWEEN 80) **Bio Basic Inc. Catalog #TB0562.SIZE.500ml** in **100 mL** of distilled water.

35 Prepare the Antifoam A stock by dissolving **1 mL** of Antifoam A in **100 mL** of distilled water.

36 Prepare the NaPP stock by dissolving **1 g** of NaPP in **100 mL** of distilled water.

37 Prepare the 10X PBS Solution

37.1 Mix together:

- **80 g** NaCl
- **2 g** KCl
- **14.4 g** Na_2HPO_4
- **2.4 g** KH_2PO_4
- **800 mL** Ddwater

37.2 Adjust the solution to pH **7.4** by adding 5% NaOH and/or 5% HCl.

- Swirl the mixture after adding either NaOH or HCl.
- Use a pH probe in between adding NaOH or HCl to see if the pH has

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reached 7.4 yet.
- Wash off tip of probe with DI water in between uses.

37.3 Add more double distilled water (ddH2O) **Contributed by users** until the volume is **1000 mL** .

38 Create the Elution Solution by mixing the following:
- **880 mL** of distilled water
- **100 mL** of 10 X PBS Solution
- **10 mL** of NaPP stock
- **10 mL** of Tween 80 stock
- **1 mL** of Antifoam A stock.

39 Autoclave Elution Solution before using.

Appendix II - Prepare Skim Milk Solution

40 Dissolve **5 g** of Skim Milk Powder **Contributed by users** in **100 mL** of distilled water using a magnetic stir bar.

41 Autoclave Skim Milk Solution before using.

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