Prepare bovine coronavirus (BCoV) solution V.2

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Version 2
Jan 05, 2021
In Development dx.doi.org/10.17504/protocols.io.bpg8mjzw
mclellan lab
McLellan Lab

DOI
dx.doi.org/10.17504/protocols.io.bpg8mjzw

PROTOCOL CITATION
Adélaïde Roguet, Shuchen Feng 2021. Prepare bovine coronavirus (BCoV) solution. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bpg8mjzw Version created by McLellan Lab

CREATED
Nov 07, 2020

LAST MODIFIED
Jan 05, 2021

PROTOCOL INTEGER ID
44288

GUIDELINES
When work is completed, remove equipment and supplies from the cabinet. Wipe the work area with 10% bleach, let stand for 10 min, rinse with water, then with 70% ethanol, and finally with RNAase AWAY.

MATERIALS TEXT
0.6 mL low-bing tubes
☒10X TE pH 8.0 **ROCKLAND antibodes & assays Catalog #MB-007** Step 1
☒Bovine coronavirus **Zoetis Catalog #CALF-GUARD** Step 1
☒RNeasy PowerMicrobiome Kit **Qiagen Catalog #26000-50** Step 6

BEFORE STARTING
1. Clean the working area and all equipment: wipe down with 70% ethanol and let dry. Then, wipe down using RNase AWAY and let dry.

Prepare the BCoV solution

1 ☒Bovine Resuspend one vial of coronavirus **Zoetis Catalog #CALF-GUARD** using 1 to 3 mL of 1xTE buffer. ☒10X TE pH 8.0 **ROCKLAND antibodes & assays Catalog #MB-007**

2 Gently vortex, avoiding foaming.

3 Aliquot 100-200 µL of the BCoV solution into 600 µL low-binding tubes.

The volume aliquoted depends on the number of samples that have to be processed at once, to avoid

freeze/thaw aliquots more than twice.

4 Store the aliquots at -80ºC.

Nucleic acid extraction 1m

5 Thaw on ice a BCoV aliquot

6 In duplicate, transfer 5 µL of the BCoV solution into a 2-mL tube containing 500 µL of PM1 buffer solution (provided in RNeasy PowerMicrobiome Kit **Qiagen Catalog #26000-50**).

7 Extract nucleic acids of the duplicates

Nucleic acid extraction - RNeasy PowerMicrobiome Kit (Qiagen)
by McLellan Lab
PREVIEW RUN

7.1 For **HA filter** extractions, Add 6.5 µL of beta-Mercaptoethanol to each of the 2-mL tubes.

For **direct extraction** of raw sewage, homogenize the sewage sample (avoid foaming) and take 150 µL into a 2-mL tube (provided in the RNeasy PowerMicrobiome kit). Add 4 µL of beta-Mercaptoethanol and 400 µL of Solution PM1. Also add 5 µL of 1:10 diluted BCoV solution.

Add the beta-Mercaptoethanol while the HA sample still frozen. Let thaw at room temperature.

7.2

If you extract from **HA filters**, place the 2-mL tubes in the bead beater.
Skip step 2 if you do **direct extraction**.

Mini-Beadbeater-16
high-energy cell disrupter
BioSpec **607**
1 speed

7.2.1 Bead beat for **00:02:30** 2m

Start the bead beating when the beads start to be loose in the tubes.

7.2.2 Cooldown the samples on ice for **00:05:00** . 5m

7.2.3 Repeat Steps 9.1 and 9.2 once .

7.3 Centrifuge at maximum speed for 1 min at room temperature. **150000 rpm, Room temperature , 00:01:00**

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7.4 To extract from **HA filters**, transfer 450 µL of supernatant to a Collection Tube (provided in the RNeasy PowerMicrobiome kit). Transfer all supernatant for **direct extraction**.

7.5 To extract from **HA filters**, add 150 µL of Solution IRS and vortex briefly to mix. For **direct extraction**, add 100 uL of Solution IRS. Incubate at 2–8°C for 5 min.

7.6 Centrifuge at maximum speed for 1 min **150000 rpm, Room temperature , 00:01:00** . Avoiding the pellet and transfer the supernatant to a new Collection Tube.

7.7 Add 650 µL each of Solution PM3 and Solution PM4. Vortex briefly to mix.

7.8 Load 650 µL of the mixture into an MB Spin Column.

7.9 Centrifuge at max speed for 1 min **150000 rpm, Room temperature , 00:01:00** . Discard the flow-through and repeat until all the mixture has been loaded onto the MB Spin Column.

7.10 Shake to mix Solution PM5 and add 650 µL to the MB Spin Column.

7.11 Centrifuge at max speed for 1 min **150000 rpm, Room temperature , 00:01:00** .

7.12 Discard flow-through. Add 600 µL of Solution PM4.

7.13 Centrifuge at max speed for 1 min **150000 rpm, Room temperature , 00:01:00** .

7.14 Discard flow-through and centrifuge filter at max speed for an additional 2 min **150000 rpm, Room temperature , 00:02:00** .

7.15 Place the MB Spin Column in a clean 2-ml Collection Tube (provided in the RNeasy PowerMicrobiome kit).

7.16 Add 60 µL of RNase-Free Water (warmed to 55ºC) to the center of the MB Spin Column membrane. Incubate at room temperature for at least 1 min **00:01:00** .

7.17 Centrifuge at max speed for 1 min **150000 rpm, Room temperature , 00:01:00** . Discard the MB Spin Column.

The DNA/RNA is now ready for downstream applications. RNA extract may be stored in RNase-free water at -80ºC for 1 year.

Titration 1m

8 Prepare a serial dilution for each duplicate to obtain the following dilution ratio:
1:1, 1:2, 1:8, 1:32, 1:128, 1:512, 1:2048

Dilutions are performed using nuclease-free water in low-binding tubes.

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9 Perform absolute quantification of PCR targets with the Droplet Digital PCR.

Quantification by Droplet Digital PCR (ddPCR)
by McLellan Lab
PREVIEW RUN

9.1 When all reagents are thawed on ice, vortex Supermix, Reverse transcriptase and DTT throughly for 30 seconds. Vortex to mix primers and probes stocks.

9.2 Prepare the reaction matrix (for one well, beside sample RNA) according to the table below. Prepare Use a low-binding tube of appropriate volume to mix all the components according to the reaction numbers. Always include extra wells when setting up reaction to avoid potential volume shortage caused by pipetting.

Component	Volume per reaction, uL	Final concentration
Supermix	5.5	1x
Reverse transcriptase	2.2	20 U/uL
300 mM DTT	1.1	15 mM
Primer mix (forwad + reverse)	1.1	900 nM
Probe	1.1	250 nM
RNase-free water*	5.5	/
Total	16.5	/

* Note: Water volume can be replaced accordingly by another assay (e.g., duplex assay), or another RNA template (e.g., inhibition test).

9.3

10 Formula to back-calculate the BCoV concentration in the initial solution:

$$CopyPerMicroliterReaction * ReactionVolume/VolumeSample * DilutionFactor * ElutionVolume$$

11 The BCoV solution is ready to use.

The titer should be close to ~100,000 copies per microliter. If > 200,000 copies per microliter, the solution will have to be diluted before use.

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