Prepare bovine coronavirus (BCoV) solution V.3

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ABSTRACT

Prepare bovine coronavirus (BCoV) solution from vaccine

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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

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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 40-200 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 warmed PM1 buffer solution RNeasy PowerMicrobiome (provided in Kit **Qiagen Catalog #26000-50**) + 5 µL of beta-mercaptoethanol..

7 Extract nucleic acids of the duplicates

Nucleic acid extraction - RNeasy PowerMicrobiome Kit (Qiagen)
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7.1 For **HA filter** extraction, add 6.5 µL of beta-Mercaptoethanol to each of the 2-mL ZR BashingBead Lysis tubes. Go to **step 2**.

Add the beta-Mercaptoethanol while samples are still frozen. Let thaw at room temperature.

For **BCoV/BRSV** extraction, add 5 µL of BCoV/BRSV solution to the 2-mL tube containing the warmed PM1 + beta-Mercaptoethanol solution. Vortex for 15 seconds (speed 7 out of 10) and let sit for 10 min. Skip step 2.

For **Direct extraction**, add 150 µL of wastewater to the 2-mL tube containing the warmed PM1 + beta-Mercaptoethanol solution. Vortex for 15 seconds (speed 7 out of 10) and let sit for 10 min. Skip step 2.

7.2

For **HA filter** extraction, place the 2-mL tubes in the bead beater.

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

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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**

7.4 For **HA filter** extraction, transfer 450 µL of supernatant to a Collection Tube (provided in the RNeasy PowerMicrobiome kit).
For **BCoV/BRSV/Direct extraction**, transfer all supernatant.

7.5 For **HA filter/BCoV/BRSV** extraction, add 150 µL of Solution IRS.
For **Direct extraction**, add 100 µL of Solution IRS.

7.6 Vortex briefly to mix (speed 7 out of 10). Incubate at 2–8°C for 5 min. **00:05:00** 5m

Place the tubes in a cold rack (stays in the refrigerator).

7.7 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.

7.8 Add 650 µL each of Solution PM3 and Solution PM4. Vortex by inverting the tubes 15 times.

Visually, check that the 3 solutions are well mixed.

7.9 Load the mixture into an MB Spin Column.

7.9.1 **Using centrifuge:** 1m

- Load 650 µL of the mixture into an MB Spin Column

- 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.

7.9.2 **Using manifold:** 1m

- Load the all mixture into an MB Spin Column (see picture)

- Centrifuge at max speed for 1 min **150000 rpm, Room temperature , 00:01:00** . Discard the flow-through.

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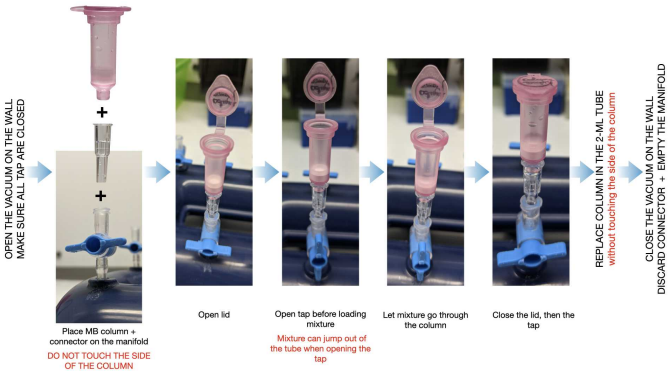

7.10 Shake well to mix Solution PM5 and add 650 µL to the MB Spin Column.

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 5 min **00:05:00** . 5m

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.

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

Quantification by Droplet Digital PCR (ddPCR)
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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 

Plate Set up: see sub-steps below.

This step requires to keep the mixture cold/on ice.

9.2.1 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 other ingredients, such as another assay (e.g., duplex assay) or another RNA template (e.g., inhibition test).

1. Keep all reagents on ice during the process as well as the matrix.

2. Always prepare a total of 8* (N columns) wells for droplet generation, or use ddPCR Buffer Control for Probes (#1863052) to fill empty wells on the last cartridge.

3. Make sure sticky reagents are added the correct volumes and not left in the tips, i.e., Supermix, RT.

9.2.2 Place a 96-well PCR plate onto a low temp PCR rack, or on ice. Disperse 16.5 µL of reaction matrix into each well of a 96-well PCR plate. For runs with multiple columns, calculate (the matrix total volume/8) and evenly distribute the matrix into an 8-well PCR strip. Then use an appropriate multichannel pipette (e.g., 2- 20 µL range) to add 16.5 µL of matrix into each well, column by column.

1. Keep the PCR plate cold/on ice during the process.

2. Lower the pipetting speed to avoid liquid leftover to the inner side of the tips.

9.2.3 Gently vortex at half speed to mix the RNA sample. Make sure no liquid is attached on the lid. Add 5.5 µL of sample RNA into each well containing 16.5 µL of reaction matrix, making the total volume of each reaction 22 µL.

1. Keep the PCR plate and RNA samples cold/on ice throughout the process.

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2. Do not over vortex the RNA samples.

3. Pipetting robot may be used to add RNA samples to the matrix.

9.2.4 Seal the PCR plate. Centrifuge down gently at 1000 rpm for 30s. Take out the plate and vortex on a 96-well plate mixer at 1600 rpm for 30s. Centrifuge again at 1000 rpm for 30s to settle down the plate.

9.3 

Droplet generation handling.

It is NOT required to keep the plate cold/on ice during this process.

9.3.1 Prepare materials/reagents on the working bench top, e.g., cartridges, gaskets, ddPCR 96-well plate, droplet generation oil, foil cover. Label the cartridges with corresponding column numbers (e.g., 1 to 12 for a full plate).

9.3.2 Remove the sealing on the PCR plate. Place the cartridge in the cartridge holder. Align well the PCR plate and the cartridge on the bench.
Use an appropriate multichannel pipette (e.g., 2- 20 µL) for liquid transfer. Adjust the pipette at 20 µL and make sure the tips are well positioned. Gently mix the liquid by aspirating up to 2/3 of the tip height and then releasing to a lower level of the tip height, i.e., not to the end of the tip, to avoid creating bubbles. Repeat this mixing step 10 times. In the last movement, slowly aspirate to the full volume of 20 µL.

9.3.3 Transfer the 20 µL reaction matrix to the middle column of the cartridge. Position the tip end to the ridge in well (where the well wall connects to the bottom) at 15º angle. Avoid creating bubbles when releasing liquid from tips into the cartridge; this can be realized by only pressing the plunger to position 2 and not position 3 before pull the tips out from the cartridge. Make sure the cartridge wells are in the same direction as on the PCR plate.

9.3.4 Fill in 70 µl of Droplet Generation Oil into bottom wells of the cartridge and cover the cartridge with a red gasket. Loop outer holes of red gasket around hooks on left and right sides of cartridge holder. Place the gasket equipped cartridge into the droplet generator.

You can start preparing the next column of droplet generation while waiting for the previous column to be done.

9.3.5 When droplet generation is done, take out the cartridge from the droplet generator and remove the red gasket. Using a Rainin multichannel with recommended Rainin tips, in a leaning position, count to 5 to aspirate all the liquid (i.e., 40 µl) from the droplets column, and press against side of wells of the corresponding column in the ddPCR 96-well plate (i.e., not the previous PCR plate), count to 5 to expel the droplets into the wells.

1. Avoid multiple times of liquid transfer.

2. Eye ball the ddPCR plate when droplet generation and transferring are done.

9.4 Turn on the PX1 PCR plate sealer and let heat to 180ºC. Correctly place the plate support block, the ddPCR plate, foil cover (i.e., red line up) and the metal holder. Seal the plate at 180ºC for 5vs and remove the plate immediately from the sealer.

Sealed plate should have indentations around wells. Always check the sealing before loading the plate on the PCR thermal cycler.

9.5 Load the plate on to a PCR thermocycler. Our lab's assay conditions are shown as below. We use Eppendorf Mastercycler Pro and the ramp speed is set to 50% for RT-ddPCR.

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Assay	Step 1	Step 2	Step 3 (40 cycles)	Step 4 (40 cycles)	Step 5	Step 6
N1/N2, BCoV, HepG	50°C 60 min	95°C 10 min	94°C 30s	55°C 1 min	98°C 10 min	4°C 30 mins and hold
PMMoV	50°C 60 min	95°C 10 min	94°C 30s	60°C 1 min	98°C 10 min	4°C 30 mins and hold

9.6 List of assay primers and probes.

A	B	C	D
CDC N1	GACCCCAAAATCAGCGAAAT	FAM-ACCCCGCATTACGTTTGGTGGACC- BHQ1	TCTGGTTACTGCCAGTTGAATCTG
CDC N2	TTACAAACATTGGCCGCAAA	HEX-ACAATTTGCCCCCAGCGCTTCAG-IowaBHQ	GCGCGACATTCCGAAGAA
BCoV	CTGGAAGTTGGTGGAGTT	FAM - CCTTCATATCTATACACATCAAGTTGTT-BHQ1	ATTATCGGCCTAACATACATC
HepG	CGGCCAAAAGGTGGTGGATG	HEX-AGGTCCCTCTGGCGCTTGTGGCGAG- BHQ1	CGACGAGCCTGACGTCGGG
PMMoV	GAGTGGTTTGACCTTAACGTTGA	FAM-CCTACCGAAGCAAATG- BHQ1	TTGTCGGTTGCAATG CAA GT

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 near 200,000 copies per microliter, the solution will have to be diluted before use.

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