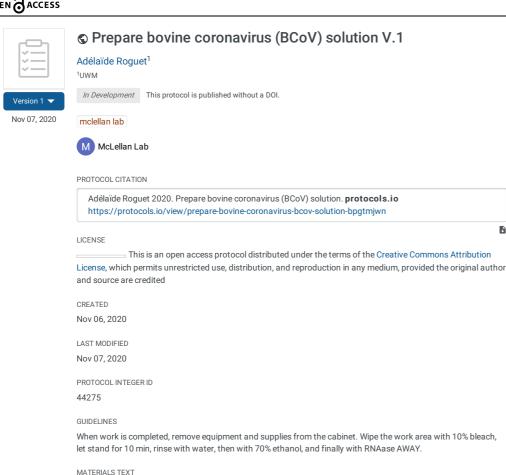


B



Prepare the BCoV solution Resuspend one vial of coronavirus Zoetis Catalog #CALF-GUARD using 1 to 3 mL of 1xTE buffer Gently vortex, avoiding foaming. Aliquot 100-200  $\mu L$  of the BCoV solution into 600  $\mu L$  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

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

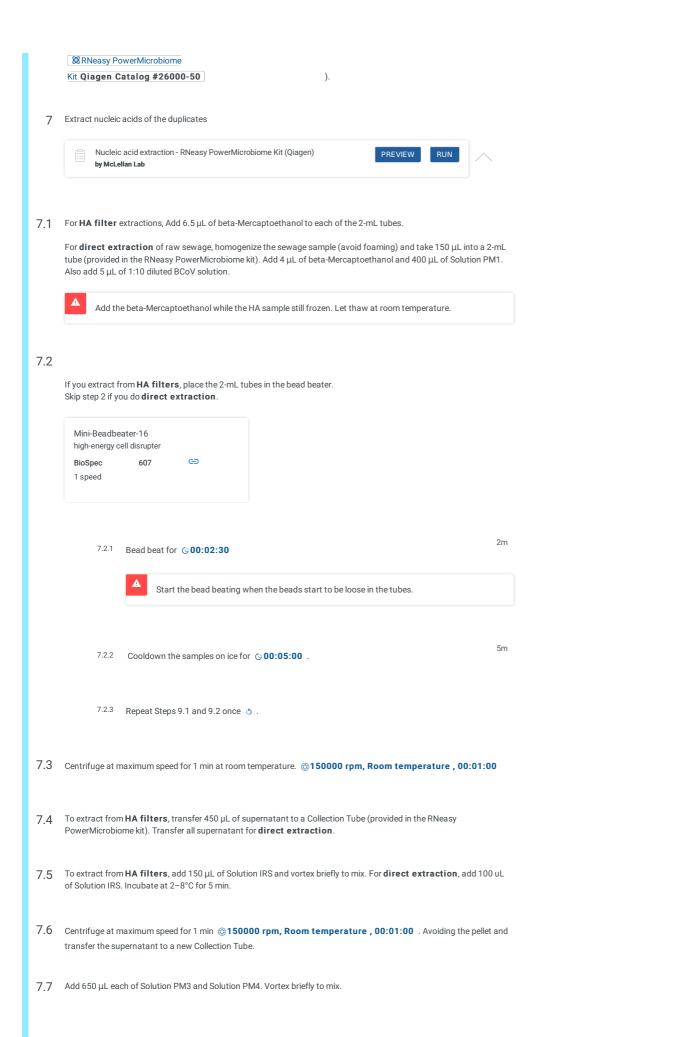
Store the aliquots at -80°C

- 0.6 mL low-bing tubes - TE buffer

RNase AWAY and let dry

Nucleic acid extraction 1m

- Thaw on ice a BCoV aliquot
- In duplicate, transfer 5  $\mu L$  of the BCoV solution into a 2-mL tube containing 500  $\mu L$  of PM1 buffer solution (provided in



7.8	Load 650 μL of the mixture into an MB Spin Column.
7.9	Centrifuge at max speed for 1 min <b>3150000 rpm, Room temperature</b> , <b>00:01:00</b> . 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 $\mu L$ to the MB Spin Column.
7.11	Centrifuge at max speed for 1 min <b>3150000 rpm, Room temperature , 00:01:00</b> .
7.12	Discard flow-through. Add 600 $\mu L$ of Solution PM4.
7.13	Centrifuge at max speed for 1 min <b>®150000 rpm, Room temperature , 00:01:00</b> .
7.14	Discard flow-through and centrifuge filter at max speed for an additional 2 min <b>© 150000 rpm, Room temperature , 00:02:00</b> .
7.15	Place the MB Spin Column in a clean 2-ml Collection Tube (provided in the RNeasy PowerMicrobiome kit).
7.16	Add 60 $\mu$ L of RNase-Free Water (warmed to 55°C) to the center of the MB Spin Column membrane. Incubate at room temperature for at least 1 min $\odot$ <b>00:01:00</b> .
7.17	Centrifuge at max speed for 1 min <b>150000 rpm, Room temperature</b> , <b>00:01:00</b> . Discard the MB Spin Column.
	The DNA/RNA is now ready for downstream applications. RNA extract may be stored in RNase-free water at -80°C for 1 year.
Titratio	n 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) 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 concentra tion
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	/

<sup>\*</sup> 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\*\* ElutionVolumeSample\*\* DilutionFactor\*\* DilutionFactor\*\* DilutionSample\*\* DilutionFactor\*\* Diluti

11 The BCoV solution is ready to use.



The titer should be close to  $\sim$ 100,000 copies per microliter. If > 500,000 copies per microliter, the solution will have to be diluted before use.