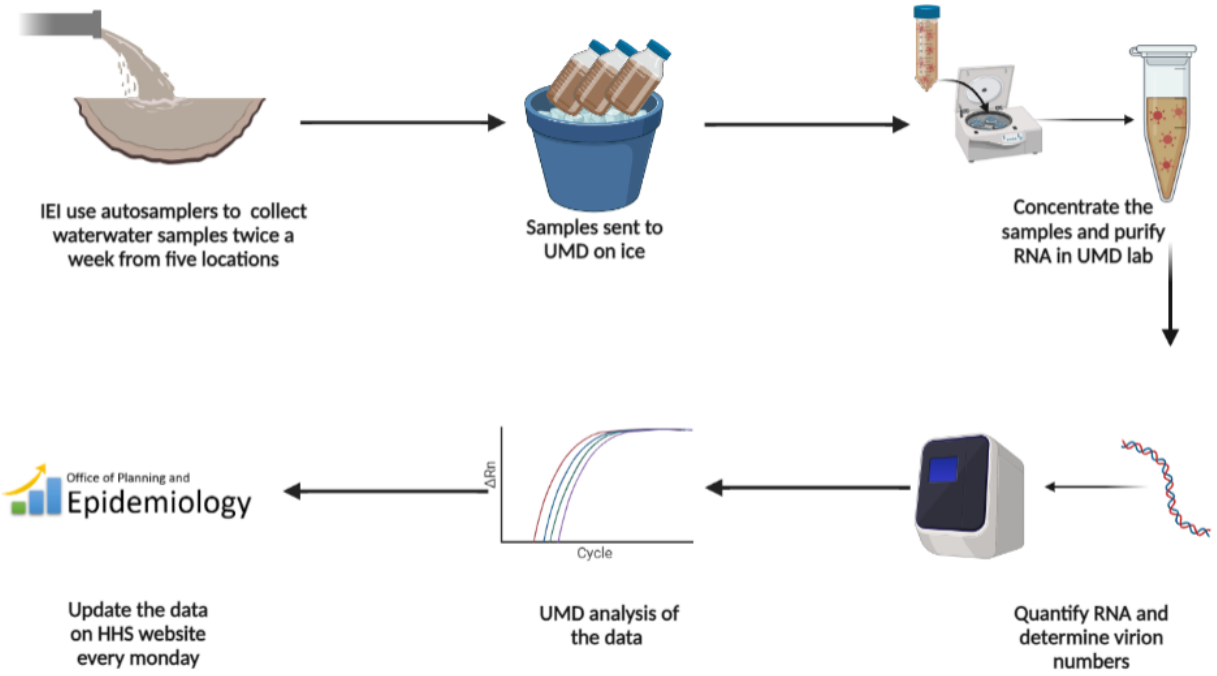
Flow chart of water sample analysis



Scientific protocol used in lab

Samples:
Samples are collected from five locations by IEI and will be collected twice a week by using autosamplers.

Sample Pick up:

1. Gloves and masks are ALWAYS required to pick up samples. Lab coats are not necessary, but recommended.
2. A cooler or other container with ice is required to place the samples in prior to drop-off.
    a. Ice can be obtained in the Chemistry building, among other places. Ice packs can be located within multiple freezers.
        i. In warmer weather, ice blankets will be needed to be placed at each site to keep accumulating samples cool
    b. Drop-off location is Room 1146 (BLS 2 lab) in Martin Hall.
    c. Samples will be arriving in 500L HDPE bottles and should immediately be processed. All bottles should be shaken and mixed thoroughly and transferred to two 50 mL Falcon centrifuge tubes at a fill volume of 45 mL each. The remaining samples should be frozen at –20C until it either needed or disposed of. In each of the two 45 mL aliquots, the samples should be spiked with BRSV surrogate (see below). If any liquid is to be discarded, it can be poured down the BLS lab 1sink, accompanied by running water and a small amount of bleach solution (10% or pre-made mixture).

Analysis Protocol for SARS-COV-2 Enrichment and Detection from Wastewater Samples

The wastewater samples should be processed immediately after they are delivered to the Lab at UMD. All transport will take place on ice to keep samples at 4°C to avoid viral decay.

- Each sample should be 45 mL in volume prior to processing. MAKE SURE ALL TUBES ARE LABELED PROPERLY WITH DATE AND SAMPLE LOCATION.
- Add BRSV: Bovine Respiratory Syncytial Virus (BRSV) prepared aseptically according to manufacturer protocol, 1 mL/900mL sample or 0.4 mL / 400 mL of sample, is added to each sample, as an internal control or surrogate to test extraction efficiency, prior to any processing. And shake them well. If samples cannot be processed in 1-2 days, they will be stored at -20°C (see above).
- Quantify copy numbers of each BRSV lot before adding to wastewater, so you know the copy numbers (add at least 10^7 copies/mL)
- Label sample bottles by writing sampling date, sample name, vol, of BRSV added on bottle lid and side of container with sharpie.

A) Virus concentration (1hr-2 hr)

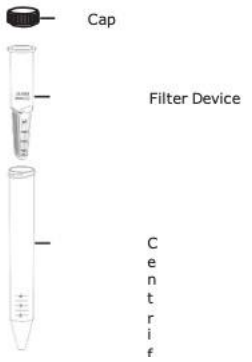
1) Centrifuge:

1. Prepare duplicate samples of each wastewater sample for procedure below
2. Take 45 mL of raw wastewater sample into a sterile 50 mL falcon tube (label tube first)
3. Centrifuge (in room 1148) at 3400 g with swinging-bucket centrifuges (use adapters for falcon tubes) for 15 min at room temperature to remove sediment and large particles
4. Carefully remove supernatant without disturbing the pellet (pellet should be visible)
5. Pour supernatant carefully from the side of the tube opposite to the pellet into a new 50 mL falcon tube.
    a. If any more than minimal (0.1 mL) supernatant cannot be transferred doe to re-suspension of pellet or particles, centrifuge again (5-10 min) as needed.

2) Ultrafiltration method (1 hr-2 hr based on number of samples): The supernatant (from part 1 - aqueous phase containing virus particles) can be further concentrated by using ultrafiltration method through either a) Amicon-15 centrifugal filters with a molecular cutoff of 100kDa (Merck Millipore) following manufacturer's protocol.

Protocol for Amicon® Ultra-15 Centrifugal Filter Devices

Amicon® Ultra 100K device — 100,000 MWCO : (MilliporeSigma, cat no: UFC910024, 100,000 Da)



Required Equipment

- Centrifuge with swinging-bucket or fixed-angle rotor with wells/carriers that can accommodate 50 mL tubes

  CAUTION: To avoid damage to the device during centrifugation, check clearance before spinning.
- Pipettor with 200 microliter (µL) tip for concentrate recovery

Prerinsing (optional and not usually needed): The ultrafiltration membranes in Amicon® Ultra-15 devices contain trace amounts of glycerine. If this material interferes with analysis, rinse the device with buffer or Milli-Q® water before use. (If interference continues, rinse with 0.1 N NaOH followed by a second spin of buffer or Milli-Q® water.).

CAUTION: Do not allow the membrane in Amicon® Ultra filter devices to dry out once wet. If you are not using the device immediately after rinsing, leave fluid on the membrane until the device is used.

Sample Concentration

1. Add up to 15 mL of sample, (total of 6 loads) to the Amicon Ultra filter device.
2. Place capped filter device into centrifuge rotor; counterbalance with a similar device.
3. When using a swinging-bucket rotor, spin the device at 3400 × g (maximum 4000 x g) for 10-15 minutes per load. If ample liquid still remaining in top portion, centrifuge for longer.
4. Discard liquid portion (bottom) of the tube into separate waste receptacle between each load. (Liquid will be disposed of later.)
5. After all sample volume has been processed(90mL)

   • Take the top portion of the Amicon tube carefully and wrap with Parafilm tightly. A second piece may be needed. Vortex for a couple seconds, holding the pink cap on tightly, and tilting to both sides.

   • Take a 100mL or 200mL pipet tip and rinse the duplicate (2nd) membrane with the sample inside, careful flushing out anything inside the membrane but without rupturing it.

• Extract this liquid (volume will vary), up to 200 mL. Check total volume of liquid. If less than 1 mL, add RNAse-free water to reach a total volume of 1 mL. If final volume is greater than 1 mL, make note of the volume amount.

6. Take concentrated sample into RNase free Tubes (2ml or 1.5ml).
7. Perform RNA extraction according to specific procedures (see below)
8. Store extracted sample at -20C/-80C for long-term storage, keep them at 4C if will process them in 24-48 hours.

B) RNA extraction: Zymo Quick RNA mini prep (1.5-2 hr. based on number of the samples)

Clean and decontaminate all work surfaces, pipette, centrifuges and other equipment using RNAZap (RNAse-Away) or 10% freshly prepared bleach prior to use.

- Use RNase-free Tips, Tubes, and H_2O.
- Clean and decontaminate all work surfaces, pipettes, centrifuges, and other equipment prior to use.
- Decontamination agents should be used such as 5% bleach, 70% ethanol, and RNase AWAY to minimize the risk of nucleic acid contamination.

Kit: Zymo Quick RNA mini prep (R1055 or 1054 : 200-50 extraction)

All centrifugation steps should be performed between 10,000-16,000 x g. (default 12,000 g – use mini-centrifuge)

1. Follow manufacturer procedure
2. Take 0.30 mL of concentrated sample (or pellet in RNAse free water) into a clean RNAse-free 2 mL Eppendorf tube
3. Add 600 uL RNA Lysis Buffer to the sample
4. Skip Sample Clearing and gDNA Removal
5. Follow RNA Purification Step of the protocol, which is as follows:
6. Add 1 volume ethanol (95-100%) to the sample in **RNA Lysis Buffer** (1:1). Mix well.
7. Transfer the mixture to a **Zymo-Spin™ IIICG Column**[1] (**green**) in a **Collection Tube** and centrifuge for 30 s-1m. Discard the flow-through.
8. Add 400 µl **RNA Prep Buffer** to the column and centrifuge for 30s-1m. Discard the flow-through.
9. Add 700 µl **RNA Wash Buffer** to the column and centrifuge for 30s-1m. Discard the flow-through.
10. Add 400 µl **RNA Wash Buffer** and centrifuge the column for 2 minutes to ensure complete removal of the wash buffer. Transfer the column (the top green part) carefully into an RNase-free tube (1.5 mL). Liquid bottom and container can be discard.
11. Add 100 µl **RNase-Free Water** directly to the column matrix and let it sit at RT for 1-2 min and centrifuge for 1 min.
    Alternatively, for highly concentrated RNA use ≥50 µl elution.
12. The eluted RNA can be used immediately or stored at -80°C. (can be kept at -20C overnight, but not longer)

C) Measure RNA Concentration

1. Clean the Nanodrop with RNaseZap (or RNAse-away) in advance.
2. Pipette 2 µL Nuclease-free H_2O to start the Nanodrop
3. Select the RNA-40 in the Sample type option, and pipette 2 µL RNase-free/DEPC-treated H_2O (the water resuspends the RNA pellet) as a blank control.

4. Pipette 2 µL RNA sample and start the measurement.
5. (the ideal result is high concentration, with OD260/OD280: ~2.0, OD260/OD230: 2.0-2.2)

D) Reverse-transcription: First Strand cDNA Synthesis (Standard Protocol) (NEB #M0368) (1-1.5hr)

Note: Plate set-up configuration can vary with the number of specimens and workday organization. NTCs (negative controls) and nCoV-PCs (positive controls) must be included in each run.

1) In the reagent set-up room clean hood, place RT-PCR buffer, enzyme, and primer/probes on ice or cold-block. Keep cold during preparation and use.
2) Mix buffer, enzyme, and primer/probes by inversion 5 times.
3) Centrifuge reagents and primers/probes for 5-10 seconds to collect contents at the bottom of the tube, and then place the tube in a cold rack.
4) Label one 1.5 mL microcentrifuge (Eppendorf) tube for each primer/probe set.
5) Determine the number of reactions (N) to set up per assay. It is necessary to make excess reaction mix for the NTC, nCoVPC, HSC (if included in the RT-PCR run), and RP reactions and for pipetting error. Use the following guide to determine N:
- If number of samples (n) including controls equals 1 through 14, then N = n +2
- If number of samples (n) including controls is 15 or greater, then N = n + 2-5

6) For each primer/probe set, calculate the amount of each reagent to be added

Prepare Reaction Mixture: M1

Reagent	Volume (x1), uL	Vol. of Reagent Added per Rxn
Random Hexamer (50 uM Invitrogen Cat. no: N8080127	1.5	N*1.5 µL =
dNTP mix (10mM) Invitrogen Cat. no: 18427013	1.25	N*1.25 µL =
H_2O (RNase-free)	2.25	N*2.25 µL =
total	**5**	**N* 5 uL=**

- After addition of the reagents, mix reaction mixtures by pipetting up and down. Do not vortex.
- Centrifuge for 10 seconds to collect contents at the bottom of the tube, and then place the tube in a cold rack.
- Dispense 5 uL of M1 into each well.
- And add 6 uL RNA

Dispense M1	5 µL
Total RNA (upto 1 ug)*	6 µL (adjust if concentration is low)
total	**11** µL

* 1 ng-1 µg total RNA or 50 pg-100 ng poly(A)-RNA

- Mix well, denature mix (sample RNA/primer) 70C denature for 5mins and then cool down at 4C forever (till next step) or immediately on ice for 2 mins, centrifuge briefly. (cycle cDNA1 on ThermoCycler)

Prepare Reaction Mixture: M2

Reagent	Volume (x1), µL	Volume (N Rxns), µL
5X ProtoScript II Buffer Comes with Protoscript II	5	5 *N=
0.1M DTT Comes with Protoscript II	2.5	2.5 *N=
SUPERase (RNAse inhibitor) (20U/ul) Invitrogen Cat no: AM2696	0.5	0.5 *N=
ProtoScript II RT (200U/ul) M-MLV NEB cat no: M0368X	1.25	1.25 *N=
H_2O (RNase-free)	4.75	4.75 *N=
total	**14**	**14 *N=**

- After addition of the reagents, mix reaction mixtures by pipetting up and down. Do not vortex.
- Centrifuge for 10 seconds to collect contents at the bottom of the tube, and then place the tube in a cold rack.
- Dispense M2 into each well containing M1+RNA, so now the total rxn volume is 25 uL.
- Incubate the samples at 42C* for 1hr; Inactivate the enzyme at 70°C for 20 minutes (cycle cDNA2 on ThermoCycler)

  **The cDNA product should be stored at -20°C. In general, the volume of cDNA product should not exceed 1/10 of the PCR reaction volume.

E) Real-time PCR (RT-qPCR) (1-2 hrs)

Preparing the reaction mix as shown in table:

- If frozen, Thaw the TaqMan® Fast Advanced Master Mix on ice, then mix thoroughly but gently.
- If frozen, Thaw samples on ice, then vortex and briefly centrifuge to resuspend.
  - If number of samples (n) including controls equals 1 through 14, then N = n + 2
  - If number of samples (n) including controls is 15 or greater, then N = n + 2-5

Prepare Reaction Mixture (M3) for each Assay: genes N1, N2, or BRSV (surrogate): check table 3 and 4 for details

Reagents	Volume (x1), µL	Volume for N Rxns, µL
2x TaqMan Master Mix Applied Biosystems cat no: 4444557	10	N*10=
Primer/probe *	X	
H_2O (RNase-free)	W=17-10-X:	N*W=
Total	**17**	**N*17 =**

*primer/probe for N1 and N2: they are premixed assays (Table 1). Check Table 3 and 4 for target of interest (e.g., N1, N2, or BRSV)

- Vortex briefly to mix.
- Centrifuge briefly to bring the reaction mix to the bottom of the tube and eliminate air bubbles.

Prepare the PCR reaction plate

- Set up reaction strip tubes or plates in a 96-well cooler rack.
- Dispense 17 µL of each master mix into the appropriate wells (of an optical reaction plate) going across the row as shown below (Table 2):

Nucleic Acid Template Addition

1) Gently vortex nucleic acid sample tubes for approximately 5 seconds.
2) Centrifuge for 5 seconds to collect contents at the bottom of the tube.
3) After centrifugation, place extracted nucleic acid sample tubes in the cold rack.
4) Add cDNA template, 3 µL (1 pg to 100 ng in Nuclease-Free Water), or Nuclease-Free Water for NTC, to each well of the specific assay that is being tested as illustrated in Table 1. Carefully pipette 3 µL of the first sample into all the wells labeled for that sample. Keep other sample wells covered during addition to prevent cross contamination and to ensure sample tracking. Change tips after each addition
   ***Note**: Be sure to adjust the volume of Nuclease-Free Water in the PCR reaction mix for a larger volume of cDNA.*
5) Change gloves often and when necessary to avoid contamination.
6) Repeat steps #4 and #5 for the remaining samples

Master mix: M3	17 µL
cDNA	3 µL for each well
Total	20 µL

Plates can be prepared and stored at 4C to be run later: enzyme is stable at room temperature for up to 72 hours in preassembled reactions

Table 1: Primer/probe info

Target Assay	Sequence (5'>3')	Size (bp)	Cycling conditions
CDC N1	F: GACCCCAAAATCAGCGAAAT R: TCTGGTTACTGCCAGTTGAATCTG P: FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1	72	95°C for 2 min and 45 cycles at 95°C for 10 s, and 55°C for 30 s
BRSV	F: GCAATGCTGCAGGACTAGGTATAAT R: ACACTGTAATTGATGACCCCATTCT P: HEX-ACCAAGACT-ZEN-TGTATGATGCTGCCAAAGCA-3IABkFQ	124	95°C for 2 min and 40 cycles at 95°C for 10 s, and 60°C for 30 s
PMMoV	F: GAGTGGTTTGACCTTAACGTTTGA R: TTGTCGGTTGCAATGCAAGT P: FAM-CCTACCGAAGCAAATG-ZEN/IBFQ	68	95°C for 2 min and 40 cycles at 95°C for 10 s, and 60°C for 30 s

Table 2: Preparation of serial dilutions and qPCR reaction for BRSV

To make dilutions for the RT-qPCR standard curve, use the following table: gblock is used as standard

Dilution #	Source Tube for Dilution	Source Conc (gc/uL)	Vol. of the Source (uL)	Diluent Vol. (uL) (PCR water)	Final Vol of Dilution (uL)	Dilution Conc. (gc/uL)	Dilution Conc. (gc/3 uL)
stock: BRSV :1 ng/uL		7.36E+09	-	-	-	7.36E+09	2.21E+10
1	stock: 1ng/uL	7.36E+09	20	80	100	1.47E+09	4.42E+09
2	1	1.47E+09	10	90	100	1.47E+08	4.42E+08
3	2	1.47E+08	10	90	100	1.47E+07	4.42E+07
4	3	1.47E+07	10	90	100	1.47E+06	4.42E+06
5	4	1.47E+06	10	90	100	1.47E+05	4.42E+05
6	5	1.47E+05	10	90	100	1.47E+04	4.42E+04
7	6	1.47E+04	10	90	100	1.47E+03	4.42E+03
8	7	1.47E+03	10	90	100	1.47E+02	4.42E+02
9	8	1.47E+02	10	90	100	1.47E+01	4.42E+01

qPCR		
Master Mix Preparation		BRSV
Reagents	Final conc. /20 µl rxn	Vol/rxn (µl)
Master Mix (2X)	1X	10
Water		4.5
Fwd Primer, 10 uM	500	1
Rev Primer, 10 uM	500	1
Probe1: hex/zen, 10 uM	250	0.5
DNA		3
rxn mix volume=		20
add 17 uL of Master mix+3 uL of template in well		

Table 3: Preparation of serial dilutions and qPCR reaction for cov-2 N1

To make dilutions for the RT-qPCR standard curve, use the following table: postive controls are used as standard

Dilution #	Source Tube for Dilution	Source Conc (gc/uL)	Vol. of the Source (uL)	Diluent Vol. (uL) (PCR water)	Final Vol of Dilution (uL)	Dilution Conc. (gc/uL)	Dilution Conc. (gc/3 uL)
stock: N, E, RdRP: linearized plasmid	stock: plasmid	2.00E+05	-	-	-	2.00E+05	6.00E+05
1	linearized stock	2.00E+05	10	40	50	4.00E+04	1.20E+05
2	1	4.00E+04	10	90	100	4.00E+03	1.20E+04
3	2	4.00E+03	10	90	100	4.00E+02	1.20E+03
4	3	4.00E+02	10	90	100	4.00E+01	1.20E+02
5	4	4.00E+01	10	90	100	4.00E+00	1.20E+01

qPCR	2 min at 95°C, and then 45 cycles including denaturation at 95°C for 3 s and annealing and elongation at 55°C for 30 s, followed by fluorescence measurement.

Master Mix Preparation		N1
Reagents	Final conc. /20 µl rxn	Vol/rxn (µl)
Master Mix (2X)	1X	10
Fwd Primer, 6.7 uM	500	1.5
Rev Primer, 6.7 uM	500	
Probe, 1.7 uM	125	
Water		5.5
DNA		3
rxn mix volume=		20
add 17 uL of Master mix+3 uL of template in well		

qPCR	2 min at 95°C, and then 45 cycles including denaturation at 95°C for 3 s and annealing and elongation at 55°C for 30 s, followed by fluorescence measurement.

Master Mix Preparation		N2
Reagents	Final conc. /20 µl rxn	Vol/rxn (µl)
Master Mix (2X)	1X	10
Fwd Primer, 6.7 uM	500	1.5
Rev Primer, 6.7 uM	500	
Probe, 1.7 uM	125	
Water		5.5
DNA		3
rxn mix volume=		20
add 17 uL of Master mix+3 uL of template in well		

Extra Useful Info:

Primer and Probe Preparation:

- 1) Upon receipt, store dried primers and probes at 2-8°C.
- 2) Precautions: These reagents should only be handled in a clean area and stored at appropriate temperatures (see below) in the dark. Freeze-thaw cycles should be avoided. Maintain cold when thawed.
- 3) Using aseptic technique, suspend dried reagents in 1.5 mL of nuclease-free water (50X working concentration) and allow to rehydrate for 15 min at room temperature in the dark.

- 4) Mix gently and aliquot primers/probe in 300 µL volumes into 5 pre-labeled tubes. Store a single aliquot of primers/probe at 2-8°C in the dark. Do not refreeze (stable for up to 4 months). Store remaining aliquots at ≤ -20°C in a non-frost-free freezer.

CONTROLS

Each real-time RT-PCR assay includes in addition of unknown samples:

• Two negative samples bracketing unknown samples during RNA extraction (negative extraction controls)

• Positive controls (in duplicate); when using in vitro synthesized transcripts as controls include five quantification positive controls (in duplicate) including 10^5, 10^4 and 10^3 copies genome equivalent (ge) of in vitro synthesized RNA transcripts.

• One negative amplification control.

Negative Extraction Control (NEC) preparation

- Prepare at least 1 negative extraction control (NEC) each time RNA is extracted from a sample.
- The NEC is an extraction with no sample added, it is prepared by extracting from RNase/DNase free water. Internal Control is added to extraction system.
- This NEC will serve as the negative control for the entire testing system and to check for contamination during PCR plate set-up.

No Template Control

- DNase/RNase free water is a provided to use as a No Template control (NTC) if required in addition to the NEC
- The NTC is used to check for contamination during PCR plate set-up.

Calculations for viral RNA concentration

Gene copies (GC) per L of sample: to calculate viral RNA concentration (GC/L) in wastewater,

$$\frac{GC}{L} = \frac{copies}{rxn} * \frac{1\ rxn}{Template\ Volume\ (\mu L)} * \frac{Total\ Volume\ of\ RT\ reaction\ (\mu L)}{RNA\ Volume\ used\ in\ RT\ (\mu L)} * RNA\ Elution\ Volume\ (\mu L) * \frac{Total\ Volume\ after\ concentration\ (\mu L)}{Volume\ Extracted\ (\mu L)}$$
$$* \frac{1}{Total\ Volume\ used\ for\ Concentration\ (mL)} * 1000\frac{mL}{L} * Dilution\ Factor$$

Where copies/rxn is the gene copies quantified per qPCR assay for each sample by using the associated target's standard curve.