



# ⌚ High-throughput Wastewater SARS-CoV-2 Detection Pipeline

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## ABSTRACT

Large-scale wastewater surveillance offers a great tool for group tracking of infection dynamics especially for large communities where continuous individual testing is not feasible. However, current methods for viral wastewater detection are severely lacking in terms of cost and scaling up for high throughput. In this study, we employed a full streamlined, cost efficient process for sample collection, magnetic-bead based concentration, nucleic acid extraction, and multiplexed PCR for wastewater based detection of SARS-CoV-2 viral particles in up to 96 samples per run. The method compares favorably to existing used methods for viral wastewater detection and can be used to process up to 100 samples per day with a 4.5 hour turnaround time from sampling to results. Currently, the high-throughput protocol is being used in UCSD for processing sewage samples from the 121 campus autosamplers as well as the SD county sewage samples (~120 samples run per day).

## EXTERNAL LINK

<https://msystems.asm.org/content/6/2/e00045-21>

## THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

<https://msystems.asm.org/content/6/2/e00045-21>

## ATTACHMENTS

Multiplex RT-PCR Ladder  
for N1, N2, and E Genes.jpg

## DOI

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MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

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## KEYWORDS

Coronavirus Method Development Community, University of California - San Diego, wastewater epidemiology

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## IMAGE ATTRIBUTION

This protocol's photos were taken by Caroline Homa Sheikhzadeh @ HOMA Photographic Art.

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## LAST MODIFIED

Jun 02, 2021

## PROTOCOL INTEGER ID

47381

## SAFETY WARNINGS

Personal Protective Equipment should be used at all times (i.e. masks, gloves, face shields, lab coats) when collecting wastewater samples. Transfer of wastewater samples should be done under a biosafety hood (BSL2+). CDC Guidelines are as follows: "Concentration of SARS-CoV-2 from wastewater requires bioaerosol-generating processes. CDC recommends conducting these processes in a Biosafety Level 2 (BSL2) facility with unidirectional airflow and BSL-3 precautions, including respiratory protection and a designated area to don and doff personal protective equipment. Laboratory waste from wastewater samples that may contain SARS-CoV-2 should be autoclaved and managed in accordance with BSL2 biosafety guidelines."

## Collection

- 1 Sample collection from the Autosampler can be done by 1-2 people, PPE must be worn at all times. All autosamplers (HACH AS950) were retrofit with 1L Nalgene bottles for ease of sample retrieval. The bottles were placed in a secondary container for spill containment.



PPE: Before collecting samples, make sure to wear two face masks (layered), double gloves, and a face shield. Use a spray bottle of 70% ethanol to sterilize everything, and have clean bottles with caps to replace the full bottle in each sampler. Do not reuse PPE. Wipe down face shield with 70% ethanol before disposal. If disposable lab gowns are used, dispose of them as biohazard. Bag them separately in a small biohazard bag, seal the bag and then discard in the designated biohazard waste bin. Use 70% ethanol to sterilize equipment and gloves when deemed necessary. Gloves to be disposed as biohazard waste as well.

- 2 Remove the top of the autosampler (unclip all three clips attaching the lid to the base/middle section). Press the [Run/Halt Button]. Use the [Down Arrow] to select "Stop Program". Press the button for "Select". The pump should start running to purge any wastewater left in the tube before stopping.



- 3 Unclip the three clips attaching the middle section of the sampler to the base. Grab the black handles on either side of the middle section of the autosampler and lift above the base of the autosampler.



Autosampler retrofit with 1L bottles placed in secondary containment. The bottles are tethered using suspension cables to avoid any leakage

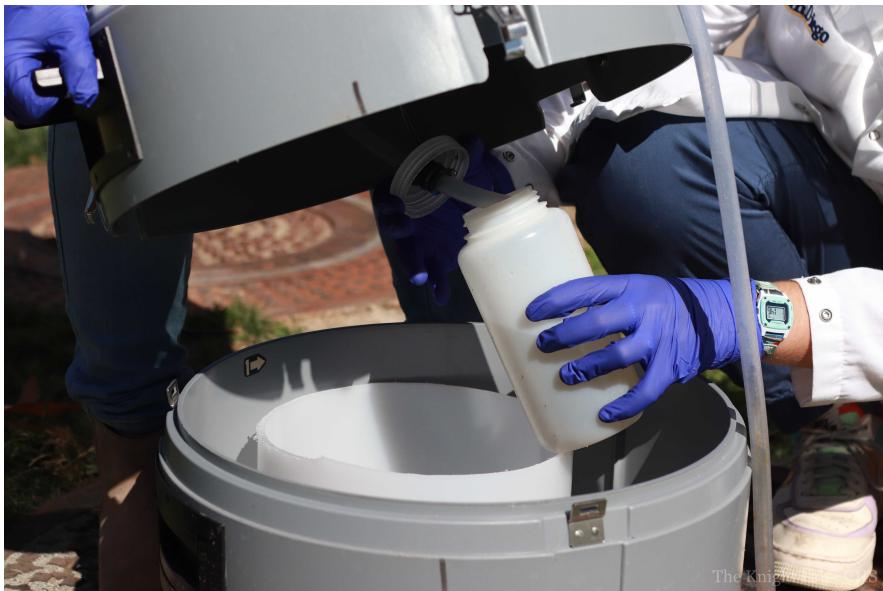
- 4 While Person A holds the middle section, Person B reaches underneath the middle section of the autosampler and takes hold of the bottle that is attached. The bottle will be hanging by the cap and should be full of liquid sample.



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- 5 Twist the bottle to unscrew it from the cap attached to the autosampler. Remove the cap from the clean bottle and screw it onto the bottle filled with sample. Sterilize the full bottle with [70% Ethanol spray Contributed by users](#)
  - . Screw the clean bottle onto the cap attached to the middle section of the autosampler as a replacement for the full bottle just removed.

If the bottle in the sampler is empty, do not remove it. Simply continue at the next step.



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- 6 Place the middle section back on the base of the autosampler, aligning the clips. Press the [Run/Halt Button] on the autosampler. It will take you to a screen that says "Start Program". Press the button for "Select". The pump will start running, it is purging. Wait for the pump to stop and then start running again. Look at the tube to make sure it is drawing up wastewater. Replace the top of the autosampler, aligning the clips.



### Concentration

- 7 Raw sewage samples should only be handled/plated inside a certified Biosafety Cabinet (BSL 2+). Prepare three 24-well plates under a biosafety cabinet (BSL 2+), two of which are used as identical Sample Plates and one which serves as the Elution Plate. Sanitize the work area by wiping down all surfaces with 70% Ethanol and RNase free spray. Bleach the remaining samples inside the BSC. Turn on the UV in the BSC for 20 minutes after you finish working and cleaning the BSC.



- 7.1 Draft an excel spreadsheet with a plate map of the sample ID numbers and organize the wastewater bottles in a way that will ensure the samples in the sample plate will match the plate map.

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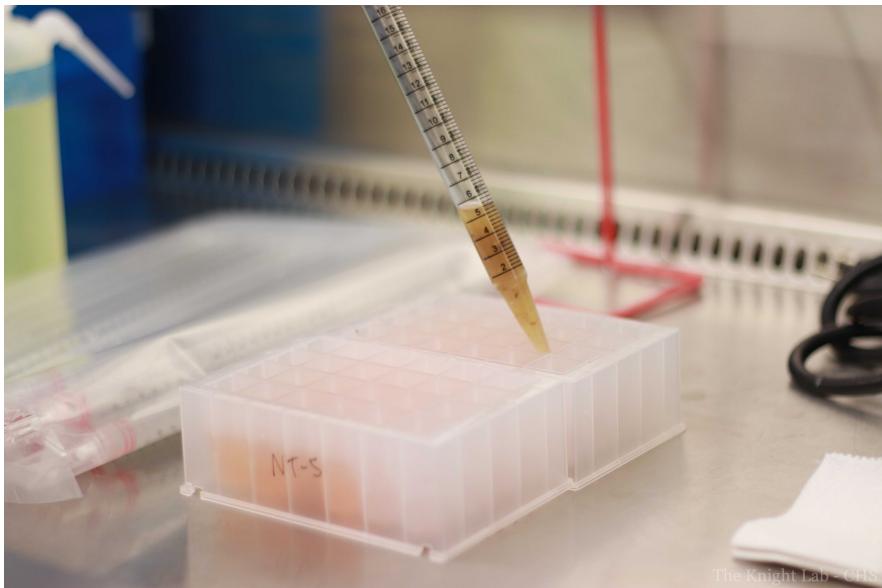
☒ Nanotrap Magnetic Virus Particles (10) Ceres

Add  75  $\mu$ L of **Nano Catalog #44202** per well into each of the two 24-well sample plates.

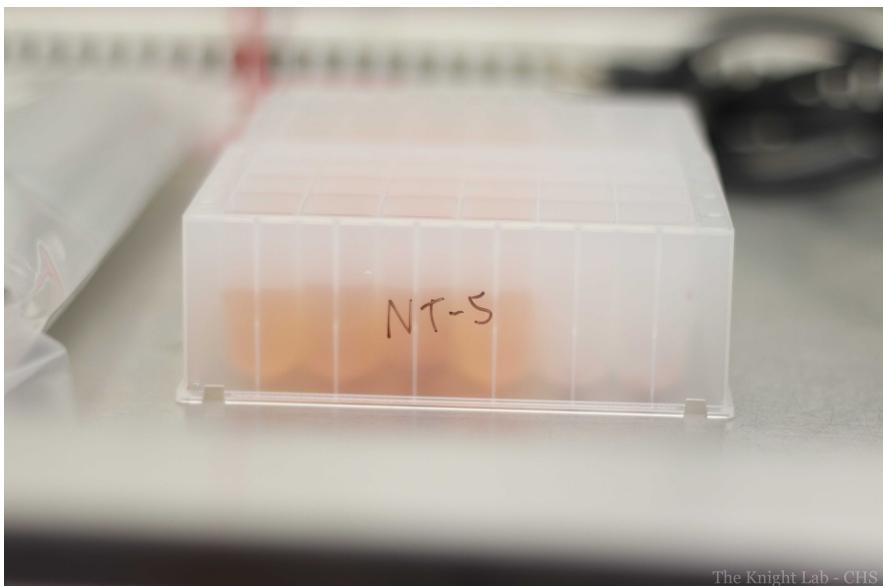


- 9 Using  10 mL wastewater sample drawn up with a serological pipette, add  5 mL wastewater sample per well into each duplicate 24-well plate. Seal the sample plates until they are taken to the KingFisher machine.





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After each sample is loaded into the wells, add bleach to the remaining sample in the Collection Bottle and homogenize to disinfect the sample. Sample disposal should be in accordance with EHS guidelines. The waste generated from the concentration step is collected separately and disposed of via regular EHS pickup. Any remaining raw sewage sample is disinfected by adding concentrated bleach (final sodium hypochlorite % should be 10%) for a contact time of 30 minutes. The disinfected samples are then discarded, bottles rinsed with water, then acid (5% HCl in DI water). The bottles are then washed in a bottle washer and heat sterilized before next use.



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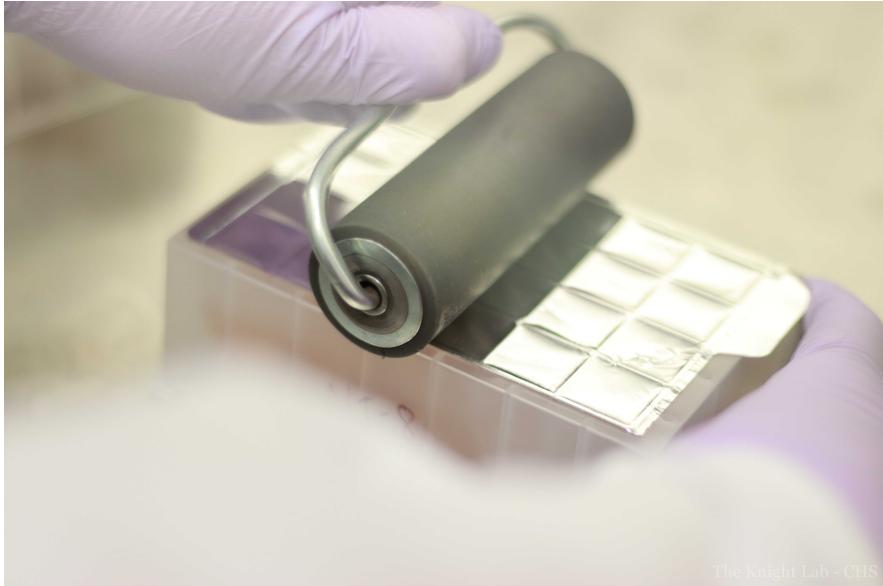
**MagMAX™ Microbiome Lysis Solution Thermo**

Add **500 µl** of the **Fisher Catalog #A42361** to each well in the elution plate.



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Seal the elution plate and the sample plates until they can be taken to the KingFisher machine.



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#### KingFisher Concentration Protocol

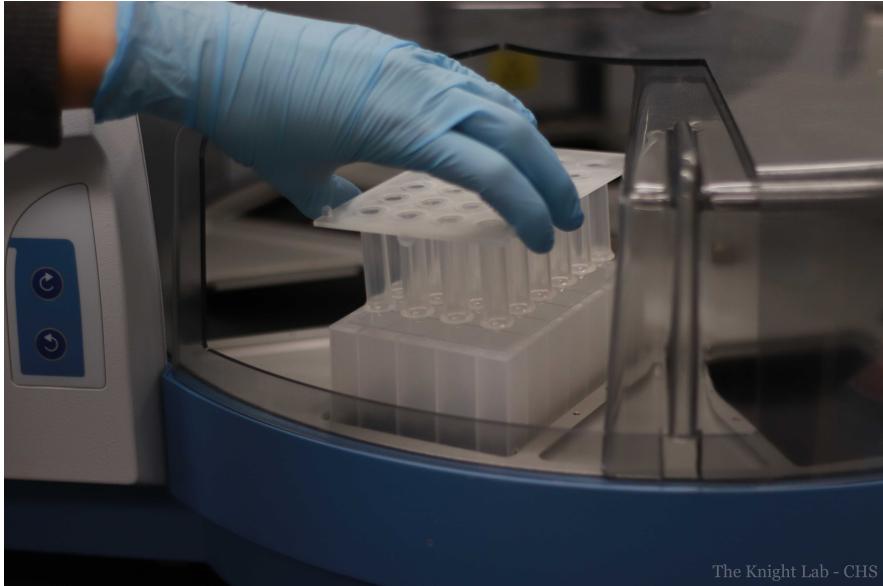
- 12** Load the correct protocol (see the Appendix of this protocol for the appropriate file) into the KingFisher Machine. The KingFisher used in this protocol is specifically designated for the processing of only sewage samples.



1. Ensure that the correct Concentration Protocol is loaded into the KingFisher machine.
2. Ensure that the magnetic attachment of the KingFisher is a 24 well attachment (If there is another magnetic attachment, please see Section 5 to change it).

Once the correct protocol is loaded, Press [Start].

- 13** Remove the seal from each plate and load each into the KingFisher. The KingFisher will show prompts for when to load each plate in the following order. (press [START] after loading each plate to move to the next step).
1. Empty 24 well Plate + 24 well Tip Combs
  2. Elution Plate
  3. Sample Plate 1
  4. Sample Plate 2

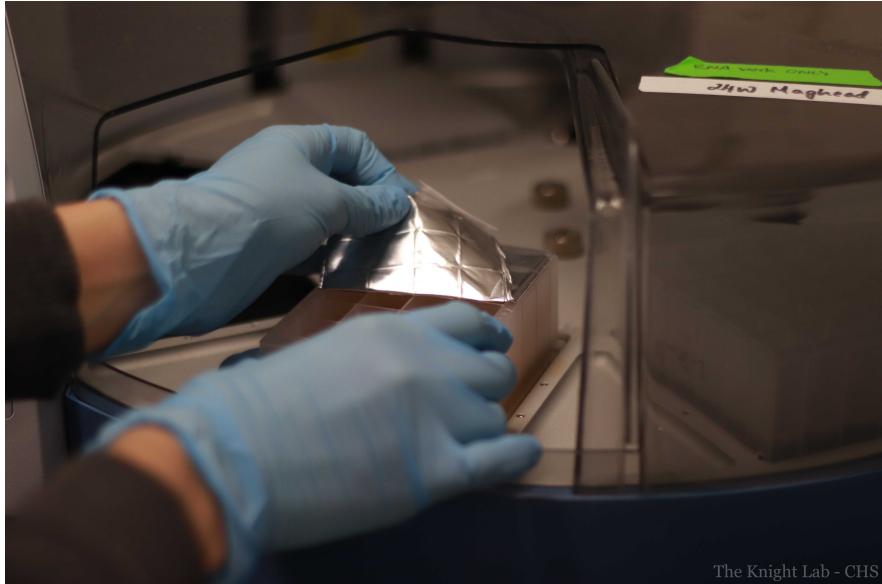


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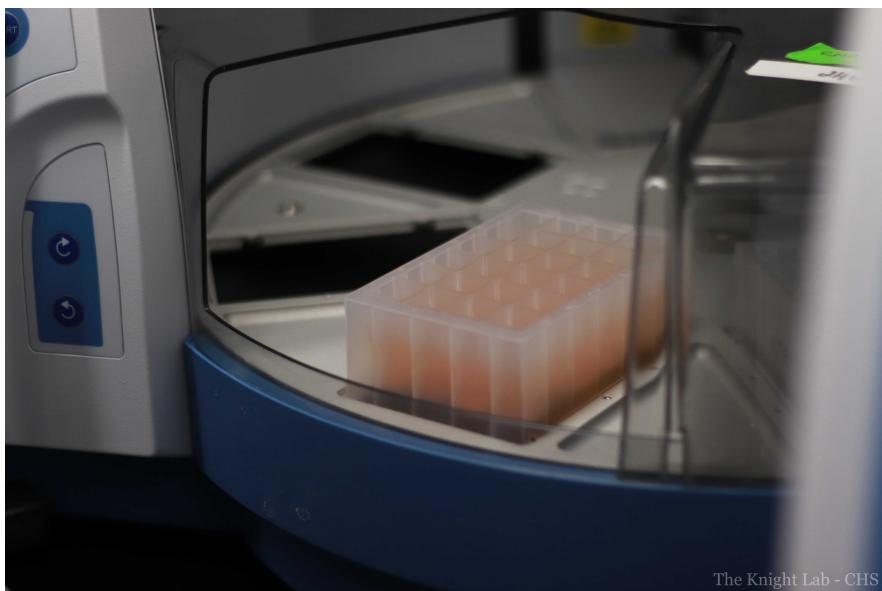
This image shows the loading of 24-well tip combs into an empty 24-well plate.



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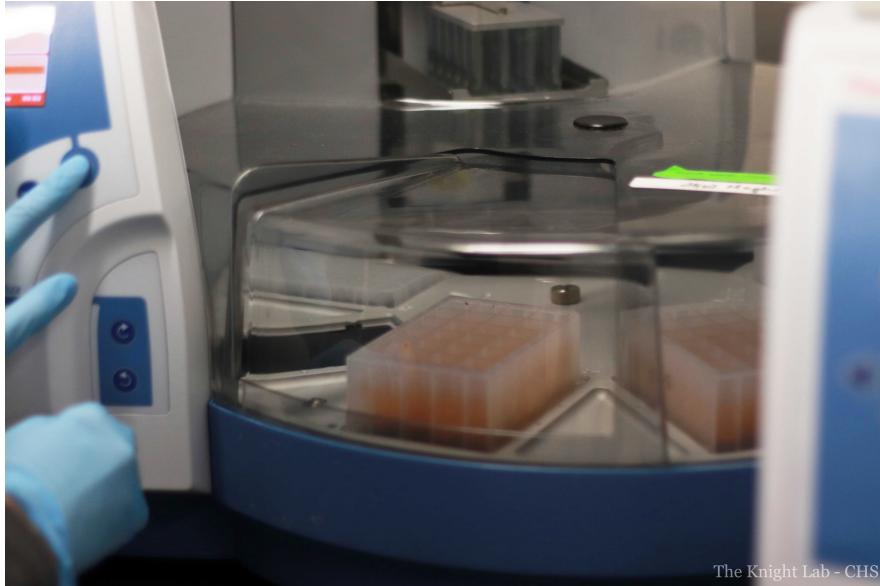
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Ensure that each plate is oriented with the A1 well in the top right corner of the plate holder in the KingFisher. Before loading the elution plate, also double-check that all the wells in the elution plate that correspond to samples in the sample plates contain the lysis buffer (especially if you are running less than 24 samples).

- 14 After the last Sample Plate is loaded, Press [Start] to run the concentration procedure and wait the designated time.<sup>53m</sup>  
(approx. **00:53:00** ).



- 15 After the protocol is done, the elution plate (which originally had contained just the lysis buffer) will have the desired lysed sample.



The two Sample Plates and Tip Combs can be discarded in biohazard waste.

- 16 Seal the remaining plate until it can be used in the extraction process (This should be the original Elution Plate). The liquid waste generated from the 2 sample plates are collected and disposed of via EHS waste pickup. The empty plates are then placed in sealed plastic bags and disposed of separately as hazardous waste. The Kingfisher equipment should be wiped clean with 70% ethanol after each use.

#### Extraction

- 17 Extraction Plate Map: Using Excel, map the samples in the 24-well plate to that of a 96-well plate.

- 18 Prepare the use of five 96 well plates, one elution plate, and the following materials:

1. 80% Ethanol

[MagMAX™ Viral/Pathogen Wash Solution Thermo](#)

2. Wash Buffer: [Fisher Catalog #A42360](#)

[MagMAX™ Viral/Pathogen Binding Solution Thermo](#)

3. Binding Solution: [Fisher Catalog #A42359](#)

[MagMAX™ Viral/Pathogen Binding Beads Thermo](#)

4. Binding Beads: [Fisher Catalog #A42362](#)

[MagMAX™ Viral/Pathogen Elution Buffer Thermo](#)

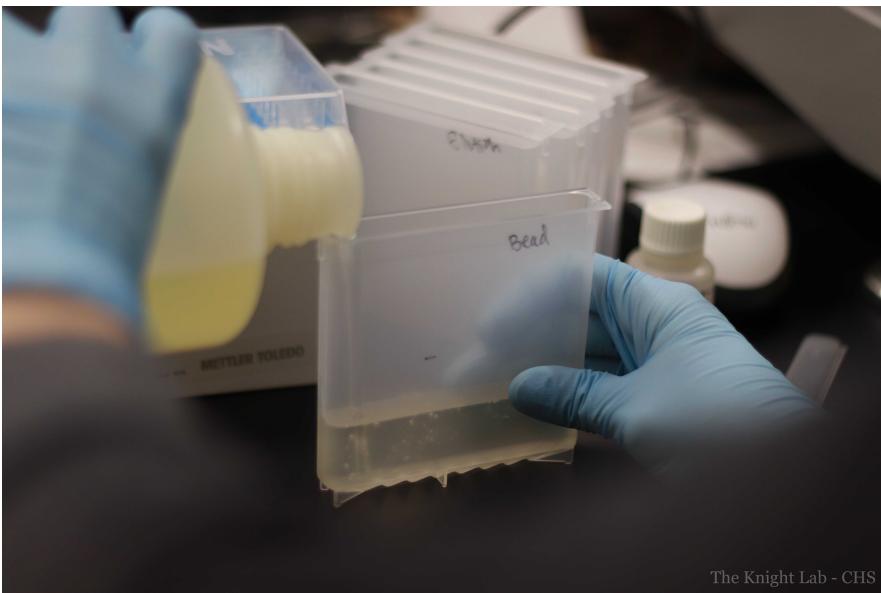
5. Elution Solution: [Fisher Catalog #A42364](#)



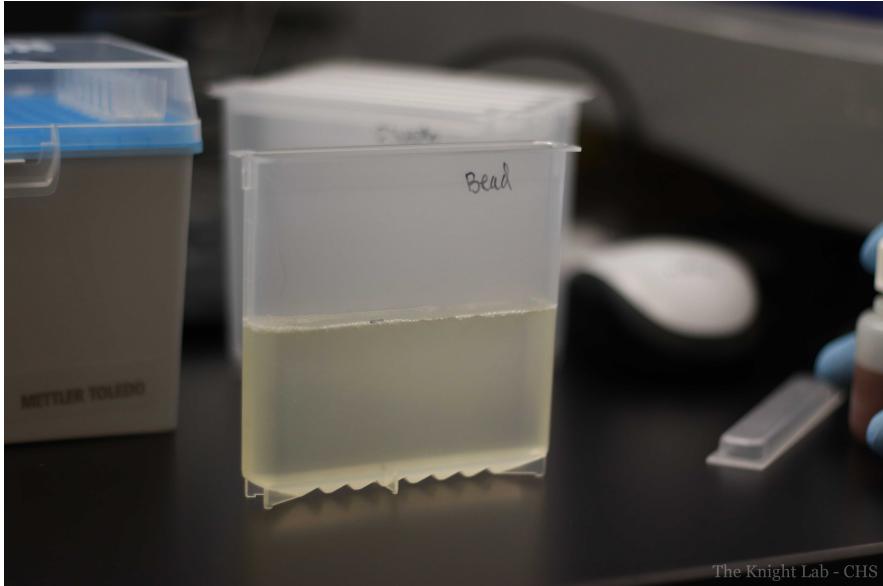
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- 19 Label five reservoirs, for EtOH, Wash Buffer, Binding Solution + Binding Beads, and one for Elution Solution EpMotion Autopipette Protocol for Extraction Plates.

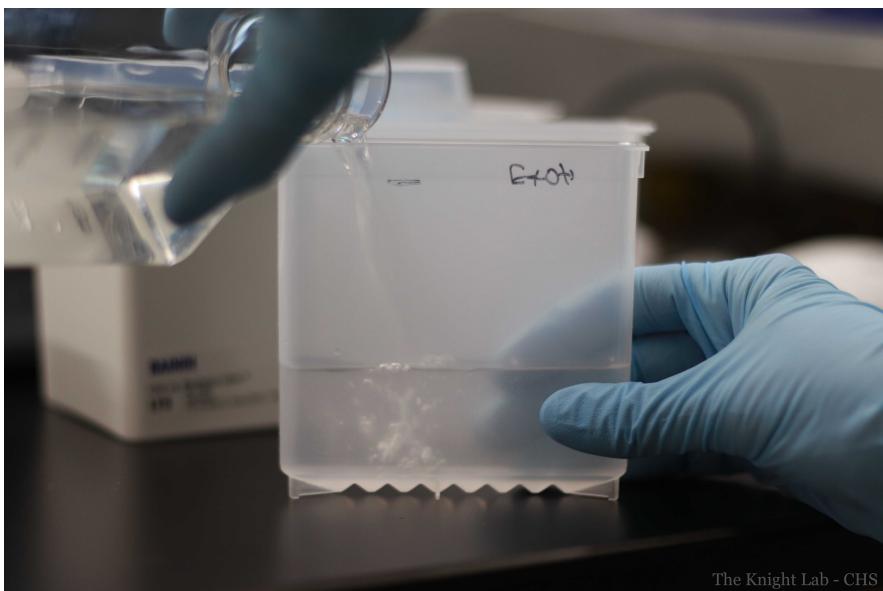
Load the labeled reservoirs with their respective materials into the EpMotion.



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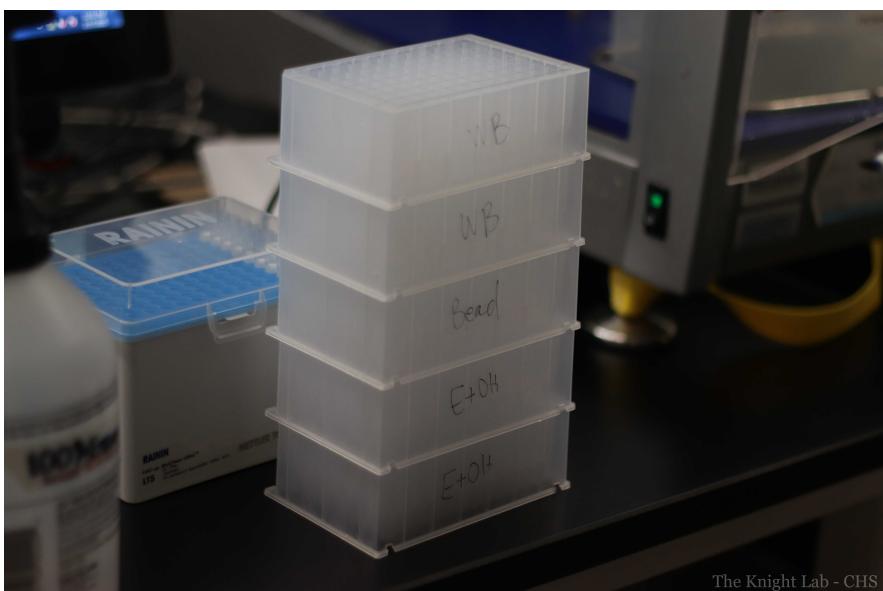


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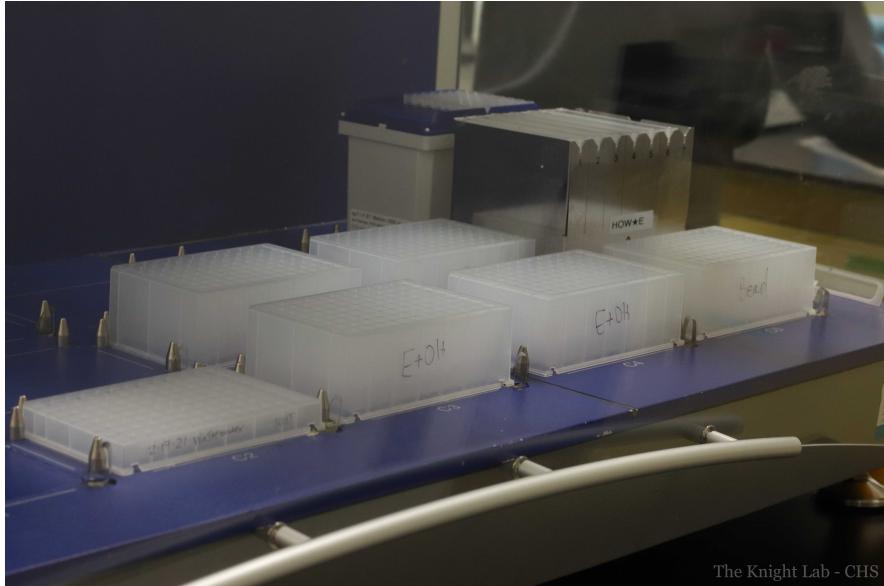
- 20 Label the five 96 well plates and one elution plate. Load the labeled plates into the EpMotion in the order indicated by the program. :
1. Sample Plate
  2. 2 Wash Buffer Plates
  3. 2 Ethanol Plates



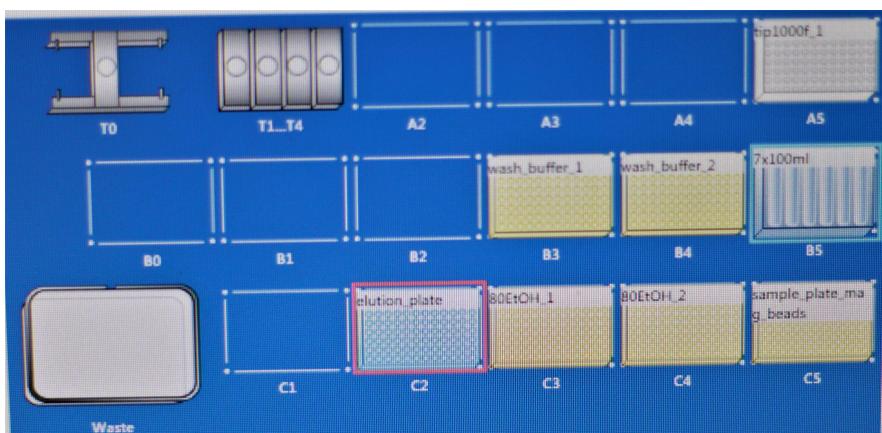
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The elution plate will contain your sample after running the extraction, so ensure that it is labeled with the appropriate information to identify the plate for future use (e.g. Date, Initials).

- 21 Run the EpMotion Program to completion.
- 22 Sample Plate Prep: After the EpMotion has loaded the Sample plate with the Binding Solution + Binding Beads, add 450  $\mu$ l of concentrated wastewater sample per well (from the 24-well Elution Plate) to 96-well Sample Plate.



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Each sample-filled well from the 24-well Elution Plate (obtained from the concentration step) should correspond to one well in the 96-well Sample Plate used in the extraction step (1:1 ratio).

Ensure that the location of each sample on the 96-well plate reflects the location indicated on the Extraction Plate Map described in Step 1.

Change pipette tips between each sample to avoid cross-contamination. NOTE: When filling the reservoirs, try not to overuse materials i.e. prepare only enough wells in each plate to match the number of samples being run.

#### KingFisher Extraction Protocol

- 23 Once the correct protocol is loaded, Press [START]. The KingFisher will show prompts for when to load each plate in the following order (press [START] after loading each plate to move to the next step).



##### Machinery Check:

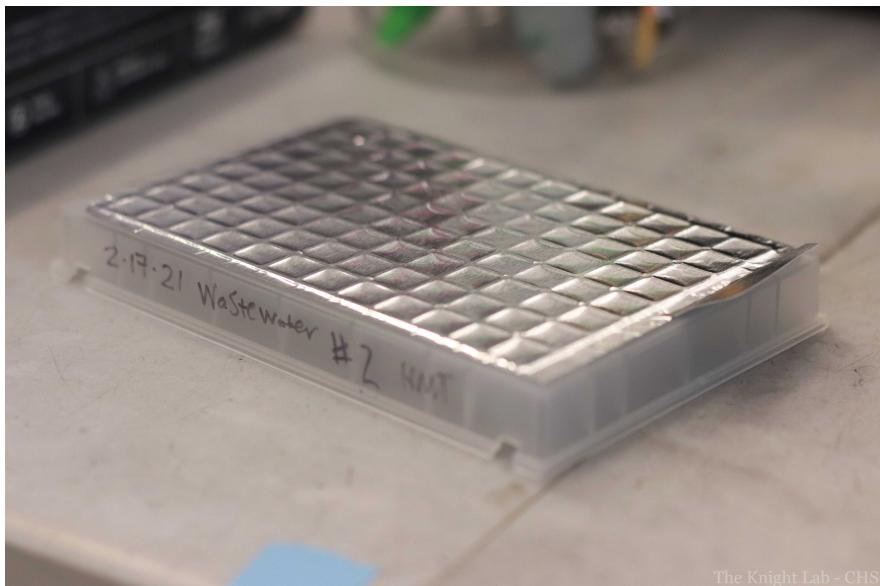
1. Ensure that the correct Extraction Protocol is loaded into the KingFisher machine.
2. Ensure that the magnetic attachment of the KingFisher is a 96 well attachment (If there is another magnetic attachment, please see Section 5 to change it).
3. Ensure that the heating block (the piece on the bottom of the machine located beneath the magnet) is the 96 well heating block. To change the heating block refer to this link: [thermo fisher website/video link]

1. Empty 96-well Plate + 96-well Tip Combs
2. Elution Plate
3. Ethanol Plate 1
4. Ethanol Plate 2
5. Wash Plate 1
6. Wash Plate 2
7. Sample Plate

Ensure that each plate is oriented with the A1 well in the top right corner of the plate holder in the KingFisher. Before loading each plate, double-check that all the wells in the given plate correspond to samples in the sample plate (especially if you are running less than 96 samples).

24 Press start one last time to start the extraction and wait the designated time (approx. **00:35:00**). 35m

25 After the protocol is completed, the elution plate will contain the sample to be used in the PCR protocol. Seal the elution plate until it can be used in the PCR process.



The Wash, Ethanol, and Sample Plates, and Tip Combs can be discarded in plastic waste after their remaining contents have been discarded in a separate biohazardous waste container.

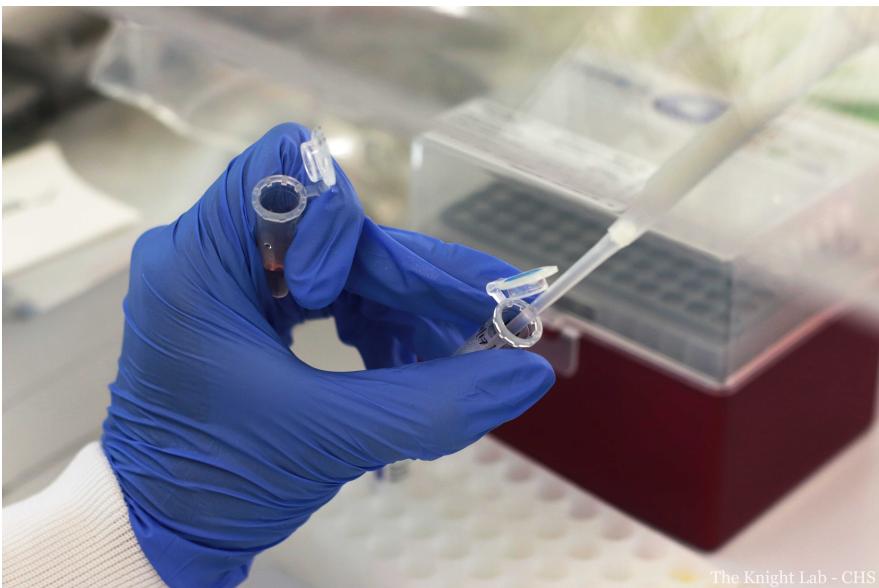
#### Multiplex RT-qPCR

26 Prepare a PCR (384-well) plate under an appropriate hood. Sterilize workspace, obtain appropriate materials/equipment.

27 Mastermix preparation for a **10 µl** reaction volume using the Promega SARS-CoV-2 RT-qPCR Kit for Wastewater (Cat.no. CS317402), is as follows:

A	B	C
RT-qPCR Reaction Master Mix	1X (10 uL Reaction Volume)	100 X (100 Reactions)
GoTaq WW Master Mix (2X)	5 uL	500 uL
GoScript RT (50X)	0.2 uL	20 uL
20X Prime/Probe/IAC Mix	0.5 uL	50 uL
Nuclease Free Water	0.3 uL	30 uL

[Insert names of Reagents with catalog numbers]



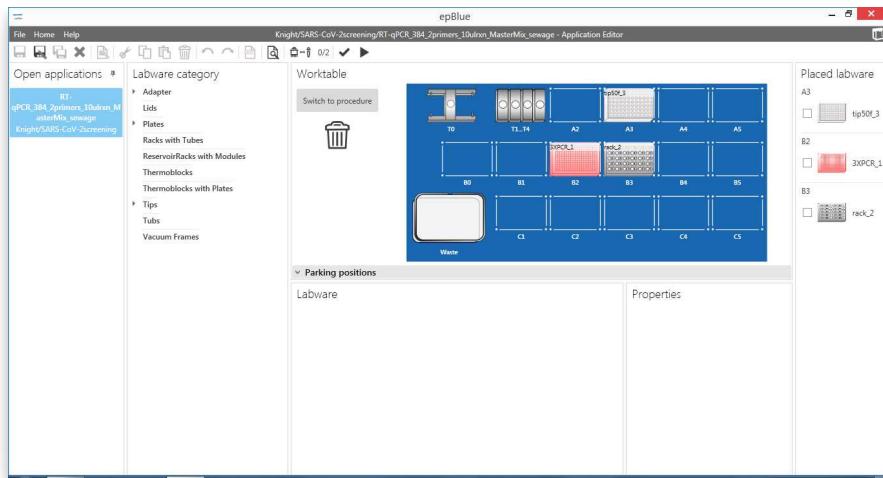
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The presence of RT-qPCR reaction inhibitors is commonly encountered in wastewater matrices (especially those that have a higher degree of solids) and in order to screen for inhibitors.

To detect inhibition specific to sewage samples, the positive control RNA ladder was run with every qPCR run (5 1:10 serial dilutions of a positive control). If Cq values for the no dilution to 1:100000 dilution are not significantly different, then PCR inhibition in the RNA extracts is unlikely. Two-tailed t-tests at a 95% confidence interval can be used to determine if the average Cq values are statistically significant from the spiked-in water control.

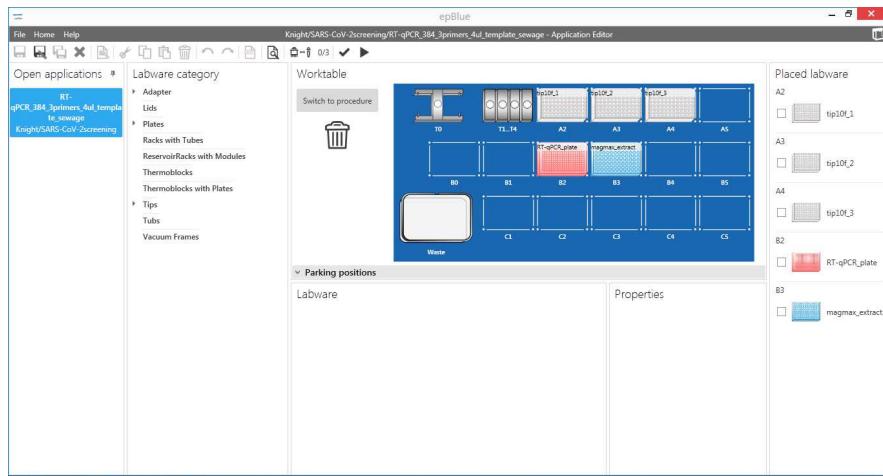
RT-qPCR reactions were run using Promega SARS-CoV-2 RT-qPCR Kit for Wastewater (Cat.no. CS317402, Promega, USA). Primer/probe sets targeting the N1, N2, E gene; primers detecting Pepper Mild Mottle Virus (PMMoV) as an internal process control; and an internal amplification control, IAC (for inhibition assessment) were used.

This step for preparing the mastermix can be done using an EpMotion auto-pipettor. The following images show the proper setup.



- 28 Pipette **4  $\mu$ l** of sample and **6  $\mu$ l** of mastermix into each corresponding well of the qPCR plate for a total reaction volume of **10  $\mu$ l**.

This step can be done using an EpMotion auto-pipettor. The following images show the proper setup.



Seal the plate with a thermal seal and centrifuge at 1000 rpm for 1 minute.

## 29 RT-qPCR Run

CFX384 Touch  
qPCR machine  
BioRad #1855484

Select "All Channels"

A	B
Fluorophores	Target
FAM	N1, N2, E (SARS-CoV-2), MS2 (spike recovery control)
HEX/JOE	Internal Amplification Control
ROX/CXR	Reference Dye
Cy5	PMMoV

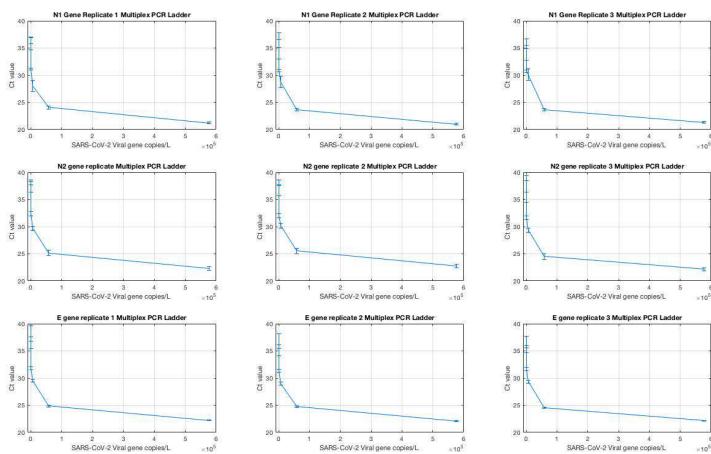
Inactivated viral particles (obtained from the UCSD CFAR BSL3 facility) were quantified using the ORF1ab gene. The viral particles were quantified using 1-step ddPCR. SARS-CoV-2 RNA were quantified as viral gene copies/L of raw sewage by applying a linear regression to the standard curve comprising of nine-fold serial dilution of heat-inactivated SARS-CoV-2 viral particles and calculating the best fit of the standard curve using  $y = mx + b$ , where  $x = \log$  concentration,  $y = Cq$ , and  $m = \text{slope}$  (where  $m = -3.3$  indicates 100% PCR efficiency).

If the IAC Ct is shifted significantly compared to NTC well, PCR inhibitors are present in the experimental sample, and results should be considered qualitative and not quantitative. Repeat purification or clean-up of nucleic acid if necessary.

If a sample yields no detectable amplification for SARS-CoV-2 but exhibits IAC amplification (Ct: 20-25), and PMMoV amplification (Ct: 20-40), SARS-CoV-2 is not detectable with this system.

If IAC fails to amplify or the IAC Ct is shifted >3 Ct compared to NTC wells, no conclusions can be made about absence of SARS-CoV-2 genetic material in a sample. Results may be considered invalid.

30



## APPENDIX A Manual Prep of Extraction Plates

31 Ethanol Plates.

- Load 80% ethanol into a reservoir

2. Add **1 mL 80% Ethanol** per well to each of the Ethanol Plates' columns using an 8 channel multi-channel pipette.
3. Fill both EtOH plates (2 total).

### 32 Wash Plates

1. Load Wash Buffer into a reservoir

**MagMAX™ Viral/Pathogen Wash Solution Thermo**

2. Add **1 mL** of **Fisher Catalog #A42360** per well to each of the Wash Plates' columns using an 8 channel multi-channel pipette.
3. Fill both Wash plates (2 total).

### 33 Elution Plate

1. Load Elution Solution into a reservoir

**MagMAX™ Viral/Pathogen Elution Buffer Thermo**

2. Add **50 µl** of **Fisher Catalog #A42364** per well to each of the Elution Plate's columns using an 8 channel multi-channel pipette.
- When finished loading the wash buffer and ethanol plates, seal and prepare the sample plate before loading into the KingFisher.

### 34 Sample Plate

1. Load Binding Beads into a reservoir by doing

Method 1 (prior to adding sample):

Load the correct ratio of binding beads to binding solution into a reservoir with Binding Solution, so that there is

**MagMAX™ Viral/Pathogen Binding Solution Thermo**

**550 µl** of **Fisher Catalog #A42359** & **20 µl** of

**MagMAX™ Viral/Pathogen Binding Beads Thermo**

**Fisher Catalog #A42362** per well. Add

**570 µl Binding Solution + Beads Mix** per well to each of the columns of the Sample Plate using an 8 channel multi-channel pipette.

Method 2 (prior to adding sample):

Load Binding Solution into a reservoir. Add **550 µl binding solution** per well to each well to each of the Sample Plate's columns using an 8 channel multi-channel pipette. Add **20 µl binding beads** to each well from the original bottle using a single channel pipette or the 8 channel multichannel pipette with only two pipette tips (two wells at a time).

2. Add **450 µl concentrated wastewater sample** per well (from the 24-well Elution Plate) to the 96-well Sample Plate.

### 35 Note:

Each sample-filled well from the 24-well Elution Plate (obtained from the concentration step) should correspond to one well in the 96-well Sample Plate used in the extraction step (1:1 ratio).

Ensure that the location of each sample on the 96-well plate reflects the location indicated on the Extraction Plate Map described in Step 1.

Change pipette tips between each sample to avoid cross-contamination.

Use of multi-channel pipettes instead of single-channel pipettes is not required but is useful for efficiency and preventing human error.