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Ground Truth in Molecular Diagnostics™

The Molecular App Company™

GT-Digital Wastewater Surveillance Guide

An industry guide for sample collecting, transporting, processing, and analyzing of wastewater samples for SARS-CoV-2

221 East Lincoln Avenue, Fort Collins, CO 80524 USA
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Contents

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Glossary of Wastewater Terminology

The following terms and abbreviations may be encountered throughout this guide.

Term or Abbreviation	Definition
BCoV	Bovine Coronavirus from attenuated vaccine used as a process control
PMMoV	Pepper Mottled Mosaic Virus used as fecal indicator control
SARS-CoV-2	Severe Acute Respiratory Syndrome, Coronavirus, Type 2; previously called 2019-nCoV
COVID-19	Coronavirus Disease – 2019; clinical disease caused by infection from SARS-CoV-2 virus; previously called 2019-nCoV
PCR	Polymerase Chain Reaction
dPCR	Digital PCR
NWSS	National Wastewater Surveillance System
CDC	US Centers for Disease Control and Prevention
GTM	GT Molecular
SOP	Standard Operating Procedure
WWTP	Wastewater Treatment Plant

Symbols and Icons

The NWSS requests that all data be provided in a .csv-format file with appropriate field names, titling, formatting, and values to allow for automated upload and analysis. Through this document you will see this reporting icon with tips on how to convey the appropriate information to NWSS when reporting. Please see the 'Data Dictionary' for final formatting and other instructions before submitting data:
https://www.cdc.gov/healthywater/surveillance/wastewater-surveillance/data-reporting-analytics.html

This symbol is used to call attention to critical details that may affect the performance of the analysis and/or bias data.

This symbol is used to highlight tips and tricks that GT Molecular has identified through development and implementation of our wastewater surveillance program.

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Overview of Wastewater Monitoring

Several methods and validated molecular workflows are currently in use to quantify SARS-CoV-2 in wastewater. Most workflows consist of the seven key steps shown in Figure 1 in which a sample is collected at a primary influent, sewer basin, or facility and transferred to a testing lab for analysis. Once received by the testing laboratory, analysis workflows include the following steps: addition of a matrix recovery control, solid removal, viral concentration, RNA extraction, and viral RNA quantification. This surveillance guide will provide recommendations for each step in the procedure as well as details on the validated, molecular workflow utilized by GT Molecular to test over 7,000 samples for over 100 communities across the United States.

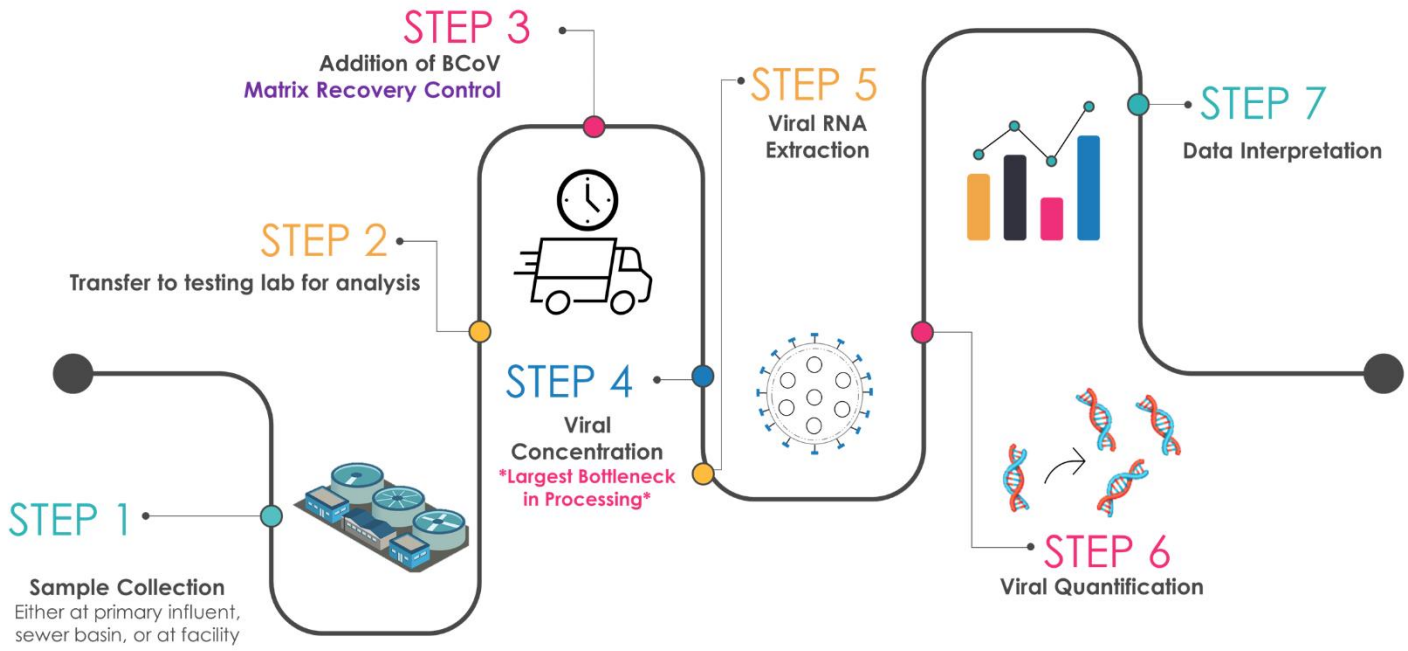

Figure 1. Wastewater surveillance workflow. Throughout the document we will review each step in greater detail and provide detailed explanations and protocols in the referenced appendices.

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Developing a Wastewater Surveillance Sampling Strategy

According to the CDC recommendations, *"A COVID-19 wastewater surveillance sampling strategy should be driven by state, tribal, local, and territorial public health needs with strong engagement from wastewater treatment plants. Wastewater surveillance data are intended to complement other COVID-19 surveillance indicators that inform public health actions. No interventions or public health actions should be based solely on wastewater data. A sampling strategy should balance available resources and testing capacity with public health data needs, and it may need to be updated over time with changing scientific knowledge and public health needs."* Therefore, we recommend communicating with public health experts to establish the site locations and sample frequency.

Developing a Sampling Plan

To develop a sampling plan, the CDC recommends addressing the following questions:

- WHERE TO SAMPLE? Choosing where to sample depends on the goals of your surveillance program. Listed below are the most common types of monitoring in use currently.
    - Community Wastewater Surveillance is achieved by sampling wastewater after entrance to a wastewater treatment facility. This location is also referred to as the 'primary influent' and provides the ideal sampling location to evaluate SARS-CoV-2 levels and trends across the entire upstream community that contributes to the facility. The CDC recommends consulting with wastewater engineers and utilities managers for each treatment facility to understand:
        - Geographic area and population served by the utility
        - Relative contribution of the types of waste inputs (industrial, commercial, residential)
        - Operating factors that could influence the detection of SARS-CoV-2 (e.g., pre-treatment of incoming wastewater or diversion of wastewater to adjust flow upstream of the sampling site)
        - Available sampling locations at the treatment plant
        - Utility capacity for sample collection, documentation, and shipping
        - Availability of utility meta-data needed for public health interpretation (e.g., influent flow measurements, chemical/physical water quality measurements, service area shapefile)
    - Targeted Wastewater Surveillance entails sampling wastewater from upstream of the community wastewater treatment facility as to target neighborhoods or areas, also referred to as 'sub sewersheds', and even individual buildings. This type of monitoring allows communities to better understand and track the area of town that is contributing to the levels seen at the treatment facility.
- HOW OFTEN TO SAMPLE? Choosing how often to sample any given location depends on the goals of your surveillance program. According to the CDC guidelines, *"If the goal of wastewater surveillance is to screen for the presence of SARS-CoV-2 in wastewater, sampling once per week may be adequate. If the goal is early indication of infection trends, at least three sampling points are needed within a trend period of interest for surveillance."* Consider the following when determining sample frequency at a specific location:
    - A minimum of three samples is required to detect wastewater trends over time. The time between consecutive wastewater samples determines the minimum length of time over which a trend may be detected. For example, if samples are collected twice per week, 8 days is the minimum timespan over which a trend can be confirmed.
    - Laboratory testing capacity and supply chain shortages may limit the maximum sampling frequency.

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  - One-time sampling will not provide actionable data beyond presence of SARS-CoV-2 infection within the sewershed."
- WHAT TO SAMPLE? There are two types of wastewater samples that can be tested for SARS-CoV-2: untreated wastewater and primary sludge.
  - Untreated wastewater is the primarily liquid component of the sewage that feeds into the primary influent of wastewater treatment facility or the sewage collected upstream of the plant. Several studies have shown that SARS-CoV-2 RNA levels in untreated wastewater correlate with trends in reported cases (Karthikeyan et al. 2021 and Weidhaas et al. 2021). GT Molecular tests untreated wastewater and is the focus of this surveillance guide.
  - Primary sludge consists of the suspended solids that settle out of untreated wastewater during sedimentation within the wastewater treatment facility. Like untreated wastewater, changes in SARS-CoV-2 RNA levels from primary sludge correlate with trends in reported cases (Peccia et al. 2020). While some studies suggest that the viral concentrations are higher in primary sludge, the CDC notes, *"…the extent of SARS-CoV-2 RNA concentration in sludge is not well characterized. Sludge samples may also present challenges that must be evaluated for each wastewater treatment plant, such as chemicals added at the treatment plant, increased concentrations of compounds that can interfere with laboratory methods, or the addition of recycled waste streams from other parts of the treatment plant."* The GT Molecular sampling workflow is likely incompatible with primary sludge analysis.
- HOW MUCH SAMPLE TO COLLECT? The amount of sample needed for analysis depends on the sensitivity of the molecular workflow and the robustness to inhibitors found in wastewater. When using the GT-Digital SARS-CoV-2 Wastewater Surveillance Kit with the recommended protocol in Step 7, we recommend collecting 120mL of wastewater in three 50 mL conical tubes in which 40mL are processed and the remaining two conical tubes containing 80mL is retained for sample archiving or reprocessing if needed. See the Appendix A: Standard Operating Procedure for Wastewater Sample Collection for more information on sample collection and transportation.

Data Reporting for Public Health

The CDC requires the following minimum set of data to interpret SARS-CoV-2 wastewater measurements for use in public health response. These data are collected during multiple steps of the sample collection and testing processes.

WASTEWATER TREATMENT PLANT: Information on the wastewater treatment plant service area, number of people served by the utility, and treatment processes is needed to understand the wastewater source.

SAMPLING: The sample collection time, date, and location, as well as the sample type (grab or composite) and wastewater flow rate during sample collection are needed to understand sample collection conditions.

TESTING: Information about sample concentration, extraction, and quantification methods, as well as viral recovery efficiency and molecular inhibition measurements are needed to compare wastewater collected from multiple locations and analyzed by different testing laboratories.

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DATA SUBMISSION TO NWSS

The National Wastewater Surveillance System (NWSS) is a collaboration between the CDC and the US Department of Health and Human Services (HHS) and additional agencies through the federal government. The data collected by NWSS will be used to better understand the extent of SARS-CoV-2 infections in communities. To participate in NWSS, testing laboratories should coordinate with their state health department.

Data is submitted to NWSS through the NWSS DCIPHER platform using a standard collection format specified in the NWSS Data Dictionary available for download at the following site:

HTTPS://WWW.CDC.GOV/HEALTHYWATER/SURVEILLANCE/WASTEWATER-SURVEILLANCE/DATA-REPORTING-ANALYTICS.HTML

Within the platform, you will find the 'Data Dictionary', a tool describing how to format the data file prior to submission to the NWSS. We will reference this 'Data Dictionary' multiple times throughout this guide. The provided tables are accurate as of the 20210621 revision of the 'Data Dictionary'.

We encourage users to periodically review the Data Dictionary for revisions to ensure data formatting is appropriate as future revisions to the dictionary are made.

This surveillance guide has been written with the goal of being compliant with all CDC recommendations for NWSS data submission. Table 1 below outlines CDC recommendations for the acquisition and reporting of wastewater surveillance data and references the corresponding Steps in this guide that address these requirements.

TABLE 1. CDC WASTEWATER SURVEILLANCE RECOMMENDATIONS

Recommendation by CDC for NWSS data submission	Relevant Section in GT Molecular Surveillance Guide
Storage • Refrigerate samples at 4°C immediately after collection and, if possible, process them within 24 hours to reduce SARS-CoV-2 RNA degradation and increase surveillance utility.	Step 1: Sample collection (pg 8) Step 2: Transfer to testing lab for analysis (pg 9) Appendix A, Appendix B
Homogenization • Both liquid wastewater and primary sludge samples should be well-mixed prior to removing portions of collected wastewater for downstream processing. Mix by inverting samples several times (for liquid samples) or by mechanical mixing	Step 4: Viral Concentration (pg 18) Appendix F
Sample clarification: • Clarifying liquid wastewater samples by removing large solids can aid subsequent filtration-based concentration steps if they are used for sample concentration	Step 4: Viral Concentration (pg 18) Appendix F
Viral Concentration • Concentrating wastewater samples can improve detection of SARS-CoV-2 RNA. • Approved methods: ultrafiltration, electronegative membranes, PEG precipitation, flocculation, ultrafiltration	Step 4: Viral Concentration (pg 18) Appendix F

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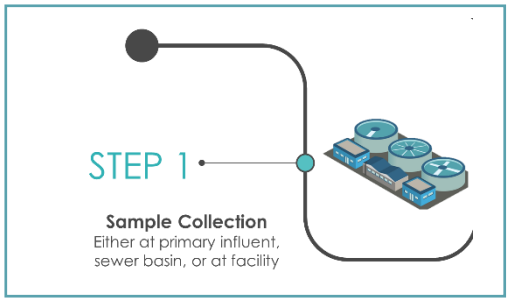
RNA Extraction • Select an extraction protocol designed to produce highly purified nucleic acid extracts from environmental samples. • Avoid degradation of extracted RNA due to multiple freeze-thaw cycles by aliquoting extracts into separate tubes and storing them at -70°C or below.	Step 5: RNA extraction (pg 20) Appendix F
Quantify SARS-CoV-2 RNA in wastewater using either RT-qPCR transcription-quantitative polymerase chain reaction) or RT-dPCR • Primers and probes targeting regions of the SARS-COV-2 N (N1 and N2, published by CDC) or E genes (E_sarbeco) • When possible, compare wastewater measurements using the same target genes.	Step 6: Viral RNA quantification (pg 22) Appendix G
Matrix Recovery Control • You must include a matrix recovery control in method validation and, if possible, include it with each sample to account for unexpected changes in wastewater composition. Always include a matrix recovery control when wastewater conditions (such as from rainwater inflows) or laboratory methods change.	Step 3: Addition of BCoV Matrix Recovery Control (pg 15) Step 7: Data Interpretation (pg 24) Appendix D, Appendix G, Appendix H
Human Fecal Normalization • Normalizing for changes in wastewater dilution and differences in relative human waste input over time is helpful in calculating trends. • Recommended fecal indicators: PMMoV, crAssphage, Bacteroides HF183, Lachnospiraceae	Step 6: Viral RNA quantification (pg 22) Step 7: Data Interpretation (pg 24) Appendix G
Quantitative Measurement Controls • For RT-ddPCR, include a control of known quantity with each instrument run.	Step 7: Data Interpretation (pg 24) Appendix G
Negative Controls • Use these controls to detect molecular reagent contamination and include them with all PCR instrument runs. • Extraction Blanks - These controls are used to detect extraction reagent contamination. Include them with each set of samples extracted.	Step 7: Data Interpretation (pg 24) Appendix F
Inhibition Assessment • Use inhibition testing to determine whether RNA quantification processes (RT and PCR) are performing as expected. Wastewater is a complex and variable mixture, and often contains compounds that can impede accurate measurement by interfering with RNA quantification methods.	Step 7: Data Interpretation (pg 24) Appendix E

Wastewater Surveillance Workflow Guidance

Step 1: Sample Collection



There are two sample collection methods for wastewater surveillance. The type of method should be considered when developing your surveillance strategy.

- Grab Sampling is performed by simply grabbing a sample from a sewer manhole or other location within the sewershed, often by dipping a beaker or bottle into the sewage to collect the sample. The benefits of grab sampling include rapid sampling without the need for specialized equipment. However, grab samples only provide information regarding the singular moment in which the sample was collected. Often grab samples are taken at time of low flow through the system. Throughout our experience collecting samples, low flow through a sewer system associates with high levels of sediment deposits and inhibitor presence. In general, grab samples are less representative of community fecal contributions and are often more susceptible to varied assay performance.

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- COMPOSITE SAMPLING is performed though the use of a 24-hour composite autosampler in which the device collects and pools multiple grab samples at a specified interval or paced on the flow of the system and pools the samples into one large bottle. Composite samples are more representative of community fecal contributions and have been associated with much higher performance in our laboratory.

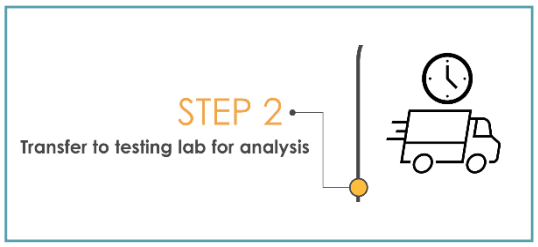
We highly recommend flow-weighted composite sampling for the most representative sample. When possible, use a refrigerated sampler so the sample is held at +2 to +8C during collection.

Most, if not all, wastewater treatment facilities run an autosampler at their primary influent several times a week for required compliance testing. Therefore, when engaging with a wastewater treatment facility ask if they can provide you some of the composite sample they are already collecting.

GT Molecular maintains a fleet of rental autosamplers. Reach out to info@gtmolecular.com if you'd like to discuss your autosampler needs.

Once the sample has been collected via grab sampling or autosampling, mix the sample well. To do this, ensure the sample bottle is capped and sealed properly, then swirl the sample for 60 seconds and gently invert to mix. Try not to overly agitate the sample or introduce unnecessary air bubbles; rather swirl and invert the bottle gently allowing the liquid circle inside the inside of the container to resuspend and homogenize the sample before transferring it to the laboratory submission tubes.

Step 2: Transfer to testing lab for analysis



There are three important components to successfully transferring your sample from the field to the testing lab:

- sample containment
- sample stability
- sampling convenience

SAMPLE CONTAINMENT

While there have been no cases to date of people contracting SARS-CoV-2 from human wastewater due to the likely inactive nature of the virus in waste, there are several other pathogens found in wastewater, such as hepatitis and norovirus. Therefore, the sample should be collected with safety in mind and transferred to the testing lab in secondary containment to reduce the risk of exposure while transporting the samples. For the GT Molecular wastewater surveillance program, we ship wastewater treatment plants 'sampling kits' as shown in Figure 1 and 2. This sampling kits consist of three 50-mL conical tubes (containment level 1) , a plastic ziplock bag with a biohazard label and pocket for sample

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documentation (containment level 2), a Styrofoam cooler (containment level 3) within a cardboard shipping box (containment level 4), 2 icepacks, a return shipping label, and a UN3373 sticker to affix to the outside of the box to comply with US Department of Transportation regulations and to alert FedEx to the biological nature of the shipment. We provide a Standard Operating Procedure for building the shown 'sampling kits' with this appropriate level of containment with the appropriate catalog numbers for each item in Appendix B.

SAMPLE STABILITY

It is critical that immediately upon sample collection, the sample is stored such that the viral particles and viral RNA do not degrade between the time of sample collection and sample analysis. Several studies from our laboratory and others (Wang et al. 2005) demonstrated wastewater sample can be stored at +2 to +8C for two weeks before coronavirus detection sensitivity reduction. However, freezing wastewater samples before analysis is largely detrimental to viral signal and should be avoided. For our wastewater surveillance program, we provide 2 x 8 oz ice packs to keep the samples at +2 to +8C during transportation, following the Standard Operating Procedure in Appendix B. Upon arrival at the testing laboratory, samples are stored at +2 to +8C until immediately before analysis, as temperatures > +20C expedite viral genome degradation.

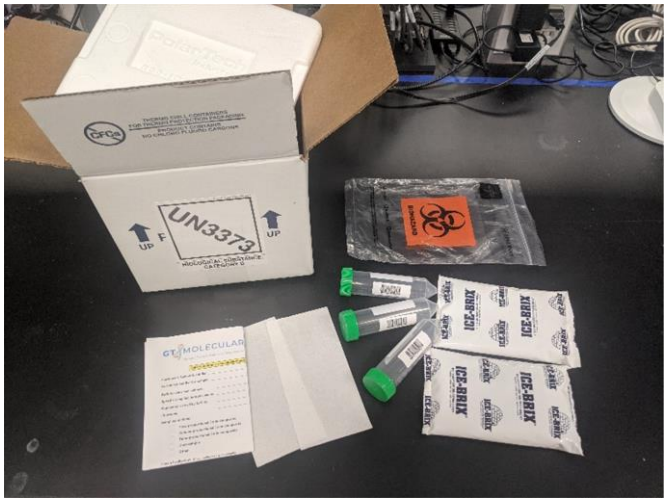

Figure 2. Individual components suggested for assembly of a sample collection kit, including 3 x 50 mL conical tubes, 2 absorbent sheets, 1 biohazard bag (with document pouch), 2 ice packs, a Chain of Custody form, 1 UN3373 sticker, 1 styrofoam shipping cooler.

Figure 3. Fully assembled sample collection kit to be provided to wastewater utility prior to sample collection. Collection kits should be provided with a return shipping label (not shown).

Never freeze wastewater samples. Store samples at +2 to +8C prior to analysis.

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CONVENIENCE FOR PARTICIPATING WASTEWATER TREATMENT FACILITIES

Wastewater treatment facility workers have several responsibilities in treating sewage and water so it can be returned to the environment. In fact, proper treatment of human waste is one of the largest contributions ever made to infectious disease prevention and overall human health. To encourage wastewater treatment plants to participate in your wastewater monitoring programs on top of their other responsibilities, it is important to make the sampling procedure as convenient as possible. For the GT Molecular wastewater testing program, we prioritize sample convenience by 1) providing all materials to the facility as discussed under 'Sample Containment' using the sampling kit Standard Operating Procedure in Appendix B, 2) provide a return shipping label, and 3) provide a simple chain of custody form and 4) provide a straightforward sample collection standard operating procedure for the wastewater treatment professional to follow.

We highly recommend making the sample collection procedure as convenient as possible to encourage participation from wastewater utilities.

REPORTING TO NWSS:

The NWSS requires that information relating to the collection of the sample be included in the .csv sample data file provided to the NWSS for each sample. Use the NWSS Data Dictionary to guide appropriate formatting, acceptable values, and indicate relevant items.

SAMPLING SITE SPECIFIC REPORTING

The lines relevant to reporting on the sampling site can be found within the *Metadata* tab, rows 6 through 32. The following table lists the relevant fields and how to report them for the sampling location.

Note that most items contained in this section are sample-site specific and will not change significantly from sample to sample at the site so most items can be prefilled prior to sample analysis.

Field Name	Acceptable Data Type	Description	Acceptable Values	GTM Provided Example Values*	Units	Required by NWSS?
county_names	List, separated by commas	Names of all counties served by this sampling site (i.e., served by this wastewater treatment plant or, if 'sample_location' is "upstream", then by this upstream location); if there are cities/jurisdictions served that are not within a county (e.g., independent cities), list those in 'other_jurisdiction'	String of text; leave empty if none	[Larimer County]	None	Required

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Field Name	Acceptable Data Type	Description	Acceptable Values	GTM Provided Example Values*	Units	Required by NWSS?
other_jurisdiction	List, separated by commas	Some geographic locations are not contained within a county or counties (e.g., independent cities). Use this field to specify names of jurisdictions that are not within a county that are served by this sampling site (i.e., served by this wastewater treatment plant or, if 'sample_location' is "upstream", then by this upstream location); list counties served in 'county_names'	String of text; leave empty if none	[Northern Colorado]	None	Not Required if county_ names is filled Required if county_ names is empty
zipcode	5-digit integer (#####)	ZIP code in which this sampling site is located	5-digit integer (#####)	[80524]	None	Required
population_served	Integer	Estimated number of persons served by this sampling site (i.e., served by this wastewater treatment plant or, if 'sample_location' is "upstream", then by this upstream location)	≥ 0	[165609]	None	Required
sewage_travel_time	Numerical, with decimal (float)	What is the approximate sewage travel time, on average, from sewage source to this sampling site (i.e., this wastewater treatment plant or, if 'sample_location' is "upstream", then this upstream location)? This should be specified as a duration in hours, not a time of day.	≥ 0 or leave empty	[8.0]	Hours	Not required
sample_location	Specific text string; see Acceptable Values	Sample collection location in the wastewater system, whether at a wastewater treatment plant (or other community level treatment infrastructure such as community-scale septic) or upstream in the wastewater system	[wwtp] or [upstream]	[wwtp]	None	Required
sample_location _specify	Text string	If 'sample_location' is "upstream", specify the collection location in the wastewater system; an arbitrary name may be used if you do not wish to disclose the real name.	Text string, length less than or equal to 40 characters, or leave empty	[influent]	None	Required
institution_type	Specific text string; see Acceptable Values	If this sample represents wastewater from a single institution, facility, or building, specify the institution type; otherwise, specify "not institution specific"	See NWSS Dictionary for allowable strings	[not institution specific]	None	Required
epaid	Permit Number: 2-letter abbrev. followed by 7 numbers	NPDES permit number for the wastewater treatment plant specified in 'wwtp_name'	Permit Number: 2-letter abbrev. followed by 7 numbers	Left empty	None	Not Required
wwtp_name	Text string	The name of the Wastewater Treatment Plant (WWTP) to which this wastewater flows. If this wastewater does not flow to a WWTP, specify an identifiable name for the septic or other treatment system to which this wastewater flows. An arbitrary name may be used if you do not wish to disclose the real name.	Text string, length less than or equal to 40 characters	[City of Fort Collins WWTP]	None	Required
wwtp_jurisdiction	Specific text string; see Acceptable Values	State, DC, US territory, or Freely Associated State jurisdiction name (2-letter abbreviation) in which the wastewater treatment plant provided in 'wwtp_name' is located	See NWSS Dictionary for allowable abbreviations	[CO]	None	Required
capacity_mgd	Numerical, with decimal (float)	Wastewater treatment plant design capacity	Greater than or equal to 0	[3.3]	Million gallons per day (MGD)	Required

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Field Name	Acceptable Data Type	Description	Acceptable Values	GTM Provided Example Values*	Units	Required by NWSS?
industrial_input	Numerical, with decimal (float)	Approximate average percentage of wastewater from industrial sources that is received by the wastewater treatment plant specified in 'wwtp_name'	Numerical from 0-100; or leave empty	[18]	Percent	Not required
stormwater_input	Specific text string; see Acceptable Values	Does the wastewater treatment plant specified in 'wwtp_name' treat water from a combined sewer system (i.e., a sewer system that collects both sewage and stormwater)?	[yes]; [no]; or leave empty	[no]	None	Not required
influent_equilibrated	Specific text string; see Acceptable Values	Is influent to the wastewater treatment plant specified in 'wwtp_name' ever stored prior to treatment to equilibrate or modulate the influent flow rate?	[yes]; [no]; or leave empty	[no]	None	Not required
sample_type	Specific text string; see Acceptable Values	Type of sample collected, whether grab or composite. If composite, also provide the duration of sampling and type of composite, as listed in the Value Set (e.g., "24-hr flow-weighted composite"). A grab sample is defined as an individual sample collected without compositing or adding other samples, regardless of whether the sample matrix is liquid wastewater or sludge.	See NWSS Dictionary for allowable string	[24-hr flow-weighted composite]	None	Required
composite_freq	Numerical, with decimal (float)	Frequency of sub-sample collection (for composite samples only): for flow-weighted, the number of sub-samples collected per million gallons of flow; for time-weighted, the number of sub-samples per hour. Flow-weighted example: a value of 5 would indicate 5 sub-samples per million gallons, or 1 sub-sample per 200,000 gallons	Number, greater than or equal to 0; or leave empty	[2]	number per million gallons; or number per hour	Not required
sample_matrix	Specific text string; see Acceptable Values	Wastewater matrix from which the sample was collected	See NWSS Dictionary for allowable string	[raw wastewater]	None	Required
collection_storage_time	Numerical, with decimal (float)	Duration of time the sample was stored after collection and prior to reaching the lab	Number, greater than or equal to 0; or leave empty	[12.0]	Hours	Not required
collection_storage_temp	Numerical, with decimal (float)	Temperature at which the sample was stored after collection and prior to reaching the lab	Number; or leave empty	[4.0]	Celsius	Not required
pretreatment	Specific text string; see Acceptable Values	Was the sample treated with any chemicals prior to reaching the lab? These could include chemicals, such as stabilizers, added to the sample or chemicals, such as chlorine, added to the wastewater treatment train upstream of the sample collection point. Pasteurization should be specified in the 'pasteurized' field.	[yes]; [no]; or leave empty	[no]	None	Not required
pretreatment_specify	Text string	If 'pretreatment' is "yes", then specify the chemicals used	Text string or Leave empty	Left Empty	None	Not required

*GTM Provided Example Values are artificial and should be used as examples only. These values will be different for each sampling site and, for some fields, each different sample collected.

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SAMPLE SPECIFIC REPORTING

NWSS requires that information relating to the collection of the sample be included in the .csv sample data file provided to the NWSS for each sample. Use the NWSS Data Dictionary to guide appropriate formatting, acceptable values, and indicate relevant items. The lines relevant to reporting for this section on sample-specific conditions can be found within the *Metadata* tab, rows 65 through 74. The following table lists the relevant fields and how to report them for the sample-specific sampling conditions.

⚠ Note that most items contained in this section are sample-specific and may change significantly from sampling to sampling. These rows should be filled out uniquely for each sample.

Field Name	Acceptable Data Type	Description	Acceptable Values	GTM Provided Example Values*	Units	Required by NWSS
sample_collect_date	Date in [yyyy]-[mm]-[dd] format	The date of sample collection; for composite samples, specify the date on which sample collection began	Date not after tomorrow's date, in [yyyy-mm-dd] format	[2021-08-31]	None	Required
sample_collect_time	Time, 24-hr in [hh]:[mm]	The local time of sample collection; for composite samples, specify the time at which sample collection began	Time, 24-hr in [hh:mm] format	[10:00]	None	Required
time_zone	Time zone (UTC-[hh]:[mm])	Current local time zone corresponding to the time specified in 'sample_collect_time', represented as a UTC time offset (e.g., UTC-06:00)	Time zone (UTC-[hh:mm])	[UTC-06:00]	None	Not required
flow_rate	Numerical, with decimal (float)	Wastewater volumetric flow rate at the sample collection location over the 24-hr period during which the sample was collected. If only an instantaneous flow measurement is available, it may be reported in units of million gallons per day.	Number, greater than or equal to 0; or leave empty	[3.3]	Million gallons per day (MGD)	Required
ph	Numerical, with decimal (float)	pH of wastewater sample (if sludge, pH of influent at time of collection)	Number; or leave empty	[7.8]	pH units	Not required
conductivity	Numerical, with decimal (float)	Specific conductivity of wastewater sample (if sludge, conductivity of influent at time of collection)	Number, greater than or equal to 0; or leave empty	[1200]	µ-siemen/cm	Not required
tss	Numerical, with decimal (float)	Total suspended solids of raw (or, if unavailable, post-grit removal) wastewater	Number, greater than or equal to 0; or leave empty	[1.0]	mg/L	Not required
collection_water_temp	Numerical, with decimal (float)	Sample temperature at time of collection	Number, greater than or equal to 0; or leave empty	[24.6]	Celsius	Not required
equiv_sewage_amt	Numerical, with decimal (float)	Equivalent unconcentrated volume of wastewater or mass of sludge in PCR reaction	Number, greater than or equal to 0; or leave empty	Left empty	mL waste-water or g sludge	Not required
sample_id	Text string	An ID assigned to a wastewater sample. It must be unique for this NWSS reporting jurisdiction. Wastewater samples that are split and measured by different labs should have the same sample ID but different lab IDs. Wastewater samples for which multiple SARS-CoV-2 PCR targets are measured should also have the same sample ID. Note: do not include PII in this field.	A string 20 characters or less, containing only numbers, English alphabetic characters, underscores, and hyphens; white space is not allowed; not case sensitive	[GTID15021]	None	Required

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Field Name	Acceptable Data Type	Description	Acceptable Values	GTM Provided Example Values*	Units	Required by NWSS
lab_id	Text string	An ID assigned to a testing lab. It must be unique across labs used for this NWSS reporting jurisdiction's testing. If the same lab is used across multiple NWSS reporting jurisdictions, each NWSS reporting jurisdiction may assign that lab a different lab ID. Note: including PII in this field is discouraged.	A string 20 characters or less, containing only numbers, English alphabetic characters, underscores, and hyphens; white space is not allowed; not case sensitive	[GT-Molecular]	None	Required

*Example values provided are artificial and should be used as an example only. These values will be different for each sampling site and, for some, each different sample collected.

ASSOCIATED DOCUMENTS FOR STEP 2: TRANSFER TO TESTING LAB FOR ANALYSIS

Appendix A – Wastewater Sample Collection Procedure

See Appendix A for a standard operating procedure which can be provided to your participating community treatment facilities for consistent and safe packaging of sample for transport.

Appendix B - Standard Operating Procedure for Wastewater Sample Collection Kit Preparation

See Appendix B for a standard operating procedure for preparing wastewater sample collection kits to share with your participating wastewater utilities.

Appendix C - Chain of Custody Form

It is critical for metadata to accompany each sample so the testing laboratory understands when the sample was collected, from where, as well as parameters required to calculate viral load. We have adapted the chain of custody form that we use for our wastewater surveillance program to be fully compliant with the NWSS required information for data reporting. See Appendix C for Chain of Custody form. This form can be provided with each sample in the plastic pouch of the biohazard bag in which the sample tubes are stored (containment level 2) as referenced in 'Sample containment'.

Step 3: Addition of BCoV Matrix Recovery Control

The matrix recovery control, also referred to as a process control, is used to understand the amount of coronavirus lost during the sample processing workflow. As recommended by the CDC and NWSS, a matrix recovery control should be used in method validation and if possible, with each sample to account for changes in wastewater composition. Historically, viruses biologically similar to SARS-CoV-2 have been used to most closely mimic the behavior of the SARS-CoV-2 viral particles in wastewater. The CDC and NWSS recommend the use of the following viruses to be used as the matrix recovery control: murine coronavirus (also called murine hepatitis virus), bovine coronavirus, or bovine respiratory syncytial virus.

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GT Molecular has used a matrix recovery control in every sample tested to date within our wastewater testing program. In validating the use of, in our case, a live attenuated Bovine Coronavirus to serve as a matrix recovery control, we assessed the impact of the control material on reproducibility and error across replicates. We hypothesized that if the matrix recovery control was able to normalize data to remove effects of variation of sample to sample due to differences in processing that we would observe lower relative standard deviations (RSDs) across replicates when samples were normalized to recovery vs when samples were not normalized to recovery.

To test this hypothesis, we split a liter of raw wastewater collected from a northern Colorado treatment facility into 10 sample replicates. Each sample replicate was spiked with the matrix recovery control and processed using our standard procedure. The results in terms of SARS-CoV-2 copies/L are presented in Figure 4 for both non-adjusted and recovery-adjusted measurements. Recovery adjustment reduced the relative standard deviation across the 10 replicates from 28.5% without normalization to 12.6% with normalization. This finding supported our hypothesis that matrix recovery normalization could be used reduce variation introduced in sample processing and thus we implemented this control in our workflow.

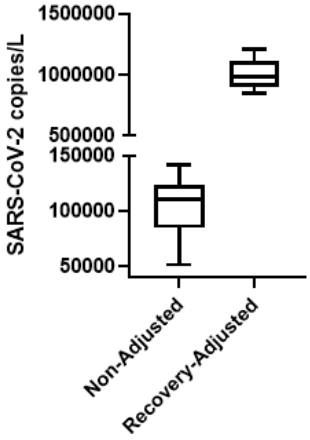

Figure 4. Effects of Matrix Recovery Control Normalization on Sample Result Variation. F test to compare variance revealed statistical differences ($p<0.0001$).

We highly recommend using the Bovilis® Bovine Coronavirus vaccine for use as a matrix recovery control. This practice dramatically reduces variation from sample to sample introduced in sample processing.

USING A MATRIX RECOVERY CONTROL

The GT Molecular wastewater surveillance uses Bovine Coronavirus (BCoV) as a matrix recovery control. This viral material, sourced as an attenuated bovine vaccine, is reconstituted, diluted, aliquoted, stored at -70 to -90C, and quantified via digital PCR. See Appendix D (within North America) and Appendix H (outside North America) for a full protocol for preparation, qualification, and use of BCoV as a matrix recovery control. Once a batch of BCoV Matrix Recovery Control has been quantified, aliquoted, and stored appropriately, a single vial is thawed on ice on the day of sample processing and 50 µL is added to each sample then mixed gently. Samples are concentrated, RNA extracted, and the BCoV that is recovered during this process is measured using the GT-Digital SARS-CoV-2 Wastewater Surveillance Kit for QIAcuity®.

CALCULATING VIRAL RECOVERY

Matrix Recovery Control calculations require known concentration of spike material and all concentration factors entailed in the workflow.

Percent recovery can be calculated using the following equation:

$$Viral\ Recovery_{\%} = \frac{[BCoV_{measured}]}{[BCoV_{expected}]} \text{x } 100$$

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In which the following variables are used:

[$BCoV_{measured}$] = measured dPCR concentration of BCoV, expressed in copies/µL from the QIAcuity® instrument.

[$BCoV_{expected}$] = expected dPCR BCoV concentration, expressed in copies/µL.

The number of BCoV genomes spiked into the sample is first calculated by multiplying the concentration of the working BCoV stock determined in the lot qualification, [$BCoV_{working}$], by the volume added to the sample, Vol_{spiked} (50 µL).

$$BCoV\ Genomes_{spiked} = \left[BCoV_{working}\right] x\ Vol_{spiked}$$

The expected BCoV concentration in the final PCR reaction needs to account for all variables and concentration factors from processing, including: a) wastewater volume, b) concentration factor achieved from viral concentration; and c) concentration factor achieved from RNA extraction.

REPORTING TO NWSS:

Viral recovery must be calculated for each sample analyzed and reported to NWSS. Use the NWSS Data Dictionary to guide appropriate formatting, acceptable values, and indicate relevant items. The lines relevant to reporting for this section on matrix recovery control can be found within the *Metadata* tab, row 88. The following table lists the relevant fields and how to report them for the sample-specific recovery of spike control. The item contained in this section is sample-specific and may change significantly from sampling to sampling. These rows should be filled out uniquely for each sample.

Field Name	Acceptable Data Type	Description	Acceptable Values	GTM Provided Example Values*	Units	Required by NWSS?
Rec_eff_percent	Numerical, with decimal (float)	Percent of spiked recovery control, specified in 'rec_eff_target_name', that was recovered	Number, greater than or equal to 0; -1 (if not tested)	[12.2]	Percent	Required

*Example values provided are artificial and should be used as an example only. These values will be different for each sampling site and, for some, each different sample collected.

ASSOCIATED DOCUMENTS FOR STEP 3: ADDITION OF BCOV MATRIX RECOVERY CONTROL

Appendix D and Appendix H – Preparation and Use of Bovine Coronavirus (BCoV) Matrix Recovery Control

This standard operating procedure outlines preparation, use, and storage of BcoV matrix recovery control material for use as a proxy for viral recovery.

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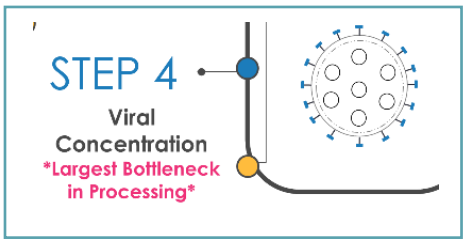
Step 4: Viral Concentration



Due to the dilute viral load in wastewater, viral concentration plays a crucial role in sample processing. When not in rampant spread, the SARS-CoV-2 virus is present as such low levels that the resulting detection is often below the detection limit of even the most state-of-the-art PCR-based technologies used in forensic laboratories for trace DNA analysis. Concentration methods reported to date include:

- Ultrafiltration
  - Innovaprep Concentrating Pipette or Spin concentrators
- Electronegative membrane filtration Polyethylene glycol (PEG) precipitation
- Skim milk flocculation
- Ultracentrifugation
- Viral bead capture

The key features we examined when selecting a method for our testing program were:

- SAMPLE PROCESSING TIME
  - Different methods can vary dramatically on sample processing time. For example, the PEG precipitation method requires a 2-hour centrifugation step while the Innovaprep Concentrating Pipette takes ~3 minutes per sample from start to finish.
  - In order to return results for 100+ samples per day, we put a strong emphasis on sample processing time in our selection criteria. However, if your laboratory does not plan to test more than 10-20 samples per day, this could be less of a driving component for your selection.
  - This decision should also be based on availability of laboratory personnel.
- SUPPLY CHAIN
  - Many critical laboratory components suffered from supply chain issues since the beginning of the pandemic. Methods that require commercial filters or ultrafiltration cartridges may be sensitive to supply chain issues. We recommend reaching out to each vendor you are evaluating for this process to understand their supply chain and to discuss a monthly supply agreement to guarantee your supply of critical reagents.
  - Evaluating and validating a backup concentration method can also alleviate this concern. By using the BCoV matrix recovery control, different methods can produce equivalent result through normalization to viral recovery. The appropriate bridge study must be performed to demonstrate equivalent results across several studies.
- LABORATORY EQUIPMENT REQUIREMENTS
  - As noted by the CDC, centrifuge volumes and force capacity, as well as availability of membrane filtration units, will also constrain method selection.

We highly recommend avoiding protocols that require centrifugation due to the often-limited space within a centrifuge and the limited number of centrifuges within common laboratories.

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GT MOLECULAR RECOMMENDED PROCEDURES FOR VIRAL CONCENTRATION

All samples listed above have shown to be adequate at concentrating virus within wastewater; however, when considering the key features listed above, the Innovaprep Concentrating Pipette and Ceres Nanobeads represent the two methods which are provided as off-the-shelf solutions from high quality manufacturers for rapid integration.

INNOVAPREP CONCENTRATING PIPETTE

This one-pass method works by end-point filtration in which the wastewater is pulled through a high-flow single-use pipette tip containing a hollow filter that captures virus on its surface. Next, the virus is eluted from the hollow filter using a patented Wet Foam Elution™ process. Because the elution fluid is a foam, which has a larger surface area than liquid, the virus is eluted from the hollow filter in a low volume allowing for a high level of sample concentration.

STRENGTHS:

- Throughput – each sample takes approximately 3 minutes to be concentrated and eluted
- Performance – In validation, we observed high viral concentration and viral recoveries.

The GT Molecular wastewater surveillance program has relied exclusively on the Innovaprep® Concentrating Pipette™ (CP) for processing wastewater. Our complete workflow utilizing the Innovaprep® Concentrating Pipette™ is provided in Appendix F.

CERES NANOTRAP® VIRAL CAPTURE BEADS:

An alternative method for viral concentration that has gained traction recently is the method relying on Ceres Nanotrap® beads. In this process, magnetic Nanotrap® particles are mixed with wastewater. The Nanotrap® particles capture viruses on their surface, and magnetic separation isolates the Nanotrap® particles and captured virus, enabling removal of supernatant. The particles are then resuspended in detergent solution and releasing viral RNA and the magnet separates out magnetic beads from viral RNA.

STRENGTHS:

- No capital expenses
- Workflow can be automated using the KingFisher Flex platform

GT Molecular is evaluating the Ceres Nanotrap® Viral Capture Beads workflow and will publish an updated surveillance guide with a protocol for use with the GT Digital SARS-CoV-2 Wastewater Surveillance Kit for QIAcuity®. Please inquire if interested at info@gtmolecular.com

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REPORTING TO NWSS:

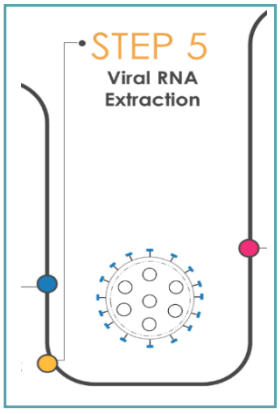
Sample filtration and viral concentration methods must be reported for each sample. Use the NWSS Data Dictionary to guide appropriate formatting, acceptable values, and indicate relevant items. The lines relevant to reporting for this section can be found within the *Metadata* tab, rows 35 and 36.The following table lists the relevant fields and how to report them for the sample-specific filtration and concentration method. The items contained in this section may not be sample-specific and thus may not change significantly from sampling to sampling. These rows can usually be filled out ahead of time barring changes to the procedure.

Field Name	Acceptable Data Type	Description	Acceptable Values	Values to Use for GT Method+	Units	Required by NWSS?
solids_separation	Specific text string; see Acceptable Values	Process used to separate solid and liquid phases of the sample, either prior to or in the absence of the concentration method specified in 'concentration_method'	See NWSS Dictionary for allowable string	[filtration]	None	Not required
Concentration _method	Specific text string; see Acceptable Values	Method used to concentrate the sample prior to analysis of the concentrate	See NWSS Dictionary for allowable string	[innovaprep ultrafiltratio n]	None	Required

ASSOCIATED DOCUMENTS FOR STEP 5: VIRAL CONCENTRATION

Appendix F– GT Molecular Standard Operating Procedure for Wastewater Sample Processing

Step 5: RNA extraction



Upon viral concentration, the next step in sample processing is to extract viral RNA from the concentrated viral material. The CDC recommends considering the following when selected a method:

- Select an extraction protocol designed to produce highly purified nucleic acid extracts from environmental samples. Commercial kits are available for environmental sample extraction.
- Use an extraction kit or a protocol designed specifically to purify RNA and that includes RNase denaturants prior to lysis.

The QIAGEN AllPrep PowerViral DNA/RNA kit meets both recommendations by the CDC in that produces highly purified nucleic acids from environmental samples and includes RNase denaturants in the lysis solution.

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We highly recommend use of the QIAGEN AllPrep PowerViral DNA/RNA kit extracting viral RNA from wastewater samples. This kit produces superior RNA for PCR-based tests due to the inhibitor removal technology that dramatically decreases the concentration of PCR inhibitors that are found in high concentrations in wastewater. Additionally, the AllPrep PowerViral kit has been validated for use with the GT-Digital SARS-CoV-2 Wastewater Surveillance Kit.

NOTES ON RNA STORAGE

Our data demonstrate that the SARS-CoV-2 RNA signal is stable for one freeze thaw cycle but degrades with additional freeze-thaw cycles (Figure 5). If you are considering breaking your workflow across two days, rather than processing start to finish in one, we recommend completing RNA extraction prior to freezing.

Extracted RNA should be stored at -70 to -90C. We recommend avoiding freeze-thaw cycles.

Never freeze Innovaprep® Concentrating Pipette ™ eluates as this will result in dramatic loss of signal.

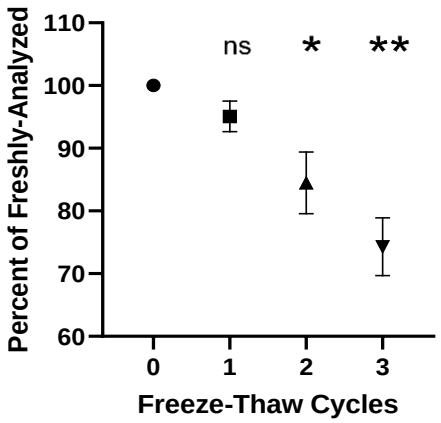

Figure 5. **Effect of Freeze-Thawing RNA on SARS-CoV-2 Quantification**.

REPORTING TO NWSS:

RNA extraction methods must be reported for each sample. Use the NWSS Data Dictionary to guide appropriate formatting, acceptable values, and indicate relevant items. The lines relevant to reporting for this section can be found within the *Metadata* tab, row 37.

Field Name	Acceptable Data Type	Description	Acceptable Values	Values to Use for GT Method[+]	Units	Required by NWSS?
extraction_method	category	Method used for nucleic acid extraction from the sample	See NWSS Dictionary for allowable string	[qiagen allprep powerviral dna/rna kit]	[none]	Required

[+]If following the GT Molecular method exactly, as described in this guide, use these values exactly as listed in the .csv data file for sample upload.

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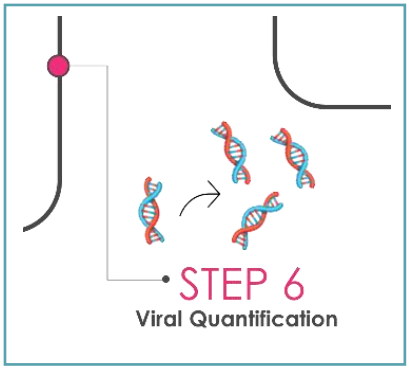
Step 6: Viral RNA quantification



The GT-Digital SARS-CoV-2 Wastewater Surveillance Assay For QIAcuity® comprises a molecular reagent kit containing all primers, probes, and controls for wastewater surveillance of SARS-CoV-2, in compliance with the CDC Wastewater Surveillance Testing Method guidance for reporting to the National Wastewater Surveillance System (NWSS). Assay solutions and appropriate controls are provided for the quantification of 1) SARS-CoV-2 (N1 and N2, published by CDC); 2) Bovine Coronavirus (BCoV), a matrix recovery control (also called a process control) biologically similar to SARS-CoV-2; and 3) Pepper Mild Mottle virus (PMMoV), a fecal indicator that can estimate the human fecal content in a sample. Through use of this kit in conjunction with the QIAGEN QIAcuity® instrument, one can monitor SARS-CoV-2 levels and normalize for changes in viral recovery caused by the variable and complex nature of wastewater, as well as normalize for human waste input, which can change over time due to dilution and variations in human behavior (travel, tourism, etc.).

By following the Instructions for Use of GT-Digital SARS-CoV-2 Wastewater Surveillance Assay for QIAcuity®, the user will generate concentrations (in copies/µL of reaction) for N1, N2, BCoV, and PMMoV targets. These measurements can be converted to copies/L wastewater measurements as describe in Step 7.

REPORTING TO NWSS:

Analysis method information must be reported for each sample. Use the NWSS Data Dictionary to guide appropriate formatting, acceptable values, and indicate relevant items. The lines relevant to reporting for this section can be found within the *Metadata* tab, rows 50 through 63.

The following table lists the relevant fields and how to report them for the PCR-based analysis method. The following fields are specific to the PCR assay used. ***If using the GT-Digital SARS-CoV-2 Wastewater Surveillance Assay for QIAcuity® v1.0, use the exact values listed in the 'Values to Use for GT Assay' column***. If reporting on both the SARS-CoV-2 N1 and N2 targets, provide the table twice; once with the N1 target-specific information and once with the N2 target.

Field Name	Acceptable Data Type	Description	Acceptable Values	Values to Use for GT Assay#	Units	Required by NWSS?
pcr_target	Specific text string; see Acceptable Values	The PCR gene target used to quantify SARS-CoV-2	See NWSS Dictionary for allowable string	[N1] or [N2]	None	Required
pcr_target_ref	Text string	A publication, website, or brief description of the PCR gene target used	String of text; leave empty if none	[NC_045512.2]	None	Required
pcr_type	Specific text string; see Acceptable Values	The type of PCR used to quantify SARS-CoV-2	See NWSS Dictionary for allowable string	[QIAGEN dPCR]	None	Required

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Field Name	Acceptable Data Type	Description	Acceptable Values	Values to Use for GT Assay#	Units	Required by NWSS?
lod_ref	Text string	A publication, website, or brief description of the method used to calculate the limit of detection	String of text; leave empty if none	[GT Digital SARS-CoV-2 Wastewater Surveillance for QIAcuity v1.0 Instructions for Use]	None	Required
hum_frac_target_mic	Specific text string; see Acceptable Values	Name of microbial target used to estimate human fecal content	See NWSS Dictionary for allowable string	[Pepper Mild Mottle Virus]	None	Not required
hum_frac_target_mic_ref	Text string	A publication, website, or brief description of the microbial target specified in 'hum_frac_target_mic'	String of text; leave empty if none	[NC_003630.1]	None	Not required
hum_frac_target_chem	Specific text string; see Acceptable Values	Name of chemical compound used to estimate human fecal content	See NWSS Dictionary for allowable string	Leave empty	None	Not required
hum_frac_target_chem_ref	Text string	A publication, website, or brief description of the chemical compound specified in 'hum_frac_target_chem'	String of text; leave empty if none	Leave empty	None	Not required
other_norm_name	Specific text string; see Acceptable Values	Name of a target or compound not specified in 'hum_frac_target_mic' or 'hum_frac_target_chem' used to estimate human fecal content	See NWSS Dictionary for allowable string	Leave empty	None	Not required
other_norm_ref	Text string	A publication, website, or brief description of the target or compound specified in 'other_norm_name'	String of text; leave empty if none	Leave empty	None	Not required
quant_stan_type	Specific text string; see Acceptable Values	The type of nucleic acid used as a standard for SARS-CoV-2 quantification	See NWSS Dictionary for allowable string	[DNA]	None	Required
stan_ref	Text string	A publication, website, or brief description of the quantitative standard material used	String of text; leave empty if none	[double stranded DNA fragment containing SARS-CoV-2 N1 and N2 nucleocapsid gene sequences]	None	Required
Inhibition_method	Text string	A publication, website, or brief description of the method used to evaluate molecular inhibition	String of text; leave empty if none	GT Molecular Inhibition Assessment	None	Required
num_no_target_control	Specific text string; see Acceptable Values	Number of no-template controls (NTC) per instrument run	See NWSS Dictionary for allowable string	[0]; [1]; [2]; [3]; or [more than 3] depending on assay run	None	Required

#If using the GT-Digital SARS-CoV-2 Wastewater Surveillance Assay Kit for QIAcuity® v1.0, regardless of details of other parts of this guide (e.g. filtration, concentration, extraction, etc.), use these values exactly as listed in the .csv data file for sample upload.

ASSOCIATED DOCUMENTS FOR STEP 6

Appendix G – CF-0025 ¬Instructions for Use: GT-Digital SARS-CoV-2 Wastewater Surveillance Assay for QIAcuity®

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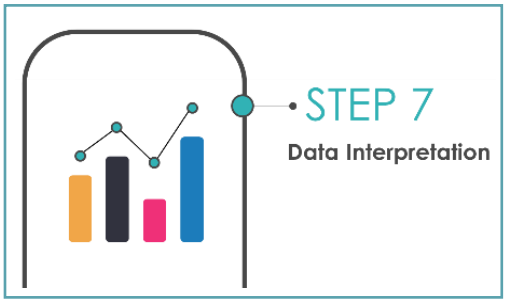
Step 7: Data Interpretation



Digital PCR data will be reported by the QIAcuity® system in copies per µL for each target. To convert digital PCR data to reportable 'copies/L wastewater' values, apply all dilution factors and concentration factors applied to the sample throughout processing. This conversion accounts for the volume of template used in dPCR, the concentration factor of nucleic acid extraction, and sample concentration processes.

Apply the following equation to the raw copies per µL for each target (SARS-CoV-2, N1 or N2) to calculate copies/L from a wastewater sample:

$$[N1\ or\ N2_{wastewater}] = [N1\ or\ N2_{copies\ per\ \mu L}] * \frac{Vol_{reaction}}{Vol_{template}} * \frac{Vol_{extraction\ eluate}}{Vol_{extraction}} * \frac{Vol_{IP\ eluate}}{Vol_{sample}} * 1000$$

Where:

- $[N1\ or\ N2_{wastewater}]$ is the concentration of SARS-CoV-2, N1 or N2 target, in unconcentrated wastewater (native sample), expressed in copies/L
- $[N1\ or\ N2_{copies\ per\ \mu L}]$ is the concentration of SARS-CoV-2, N1 or N2 target, in the dPCR reaction. This is the value measured and reported by the QIAcuity® instrument.
- $Vol_{reaction}$ is the volume of the QIAcuity® reaction. For the GT-Digital SARS-CoV-2 Wastewater Surveillance Assay for QIAcuity® v1.0, this volume is 40 µL.
- $Vol_{template}$ is the volume of pure, extracted RNA used as template for the QIAcuity® reaction in µL.
- $Vol_{extraction\ eluate}$ is the volume of RNA eluted from the RNA extraction column in µL .
- $Vol_{extraction}$ is the volume of concentrated sample upon which RNA is extracted in µL.
- $Vol_{IP\ eluate}$ is the volume of the sample eluted from the Innovaprep Concentrating Pipette tip in µL.
- Vol_{Sample} is the volume of sample added to the Stericup Filtration Device in mL.

PRESENCE

According to the CDC, *"Presence of viral RNA in a wastewater sample is defined for RT-dPCR measurements, as three or more positive partitions."* In other words, the Limit of Detection is defined as 3 positive partitions. To convert this value to an LOD in terms of copies/L wastewater, perform the calculations listed above by applying all dilution and concentration factors for a given sample.

MATRIX RECOVERY CONTROL NORMALIZATION

According to the CDC: *"Viral recovery estimates can be incorporated into SARS-CoV-2 wastewater data by dividing the measured concentration of SARS-CoV-2 by the fraction of matrix recovery control recovered*." The fraction of matrix recovery control

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recovered is the amount of non-SARS-CoV-2 virus measured after processing divided by the amount of non-SARS-CoV-2 virus spiked into the sample before processing.

GT Molecular recommends normalizing all data by the matrix recovery control. This practice greatly reduces sample processing errors and in our hands over the last 7,000 samples has produced the most reliable data.

However, the NWSS requests the non-recovery normalized data and the matrix recovery measurement so that they can perform the normalization of their choosing. When reporting viral concentration per L of wastewater to NWSS, use the non-recovery normalized value.

REPORTING TO NWSS:

Non-normalized raw data must be reported for each sample. Use the NWSS Data Dictionary to guide appropriate formatting, acceptable values, and indicate relevant items. The lines relevant to reporting for this section can be found within the *Metadata* tab, rows 79 through 87. The following table lists the relevant fields and how to report them for the sample-specific data. Note that most items contained in this section are sample-specific and likely will change significantly from sample to sample. These rows should be filled out uniquely for each sample. When reporting on multiple targets, such as for N1 and N2, each target, and the associated data for the target, should be given its own row(s). For example, if measuring and reporting both N1 and N2 values, the sample report should contain two lines for sars_cov2_avg_conc and the other fields below, one for the N1 values, one for the N2 values. Perform similarly for other relevant fields.

Field Name	Acceptable Data Type	Description	Acceptable Values	GTM Example Values*	Units	Required by NWSS?
test_result _date	Date in [yyyy]-[mm]-[dd]	The date on which this SARS-CoV-2 measurement was made	Date, not after tomorrow's date, and not before 'sample_collect_date' (see collection section above), in [yyyy-mm-dd] format	[2021-09-01]	None	Required
sars_cov2 _units	Specific text string; see Acceptable Values	Units of SARS-CoV-2 sample concentration	See NWSS Dictionary for allowable string	[copies/L wastewater]	None	Required
sars_cov2_avg _conc	Numerical, with decimal (float)	Concentration of SARS-CoV-2 back-calculated to unconcentrated sample basis; enter "0" if no amplification occurred, using the definition of amplification described in 'ntc_amplify'; otherwise, enter the estimated concentration; do not adjust for matrix recovery efficiency	Any number (with decimal) other than 0; 0 (if no amplification observed)	[266131]	Units specified in 'sars_cov2 _units' line	Required

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Field Name	Acceptable Data Type	Description	Acceptable Values	GTM Example Values*	Units	Required by NWSS?
sars_cov2_std_error	Numerical, with decimal (float)	Standard error (SE) of SARS-CoV-2 in wastewater sample, or best estimate that is consistently available. If sample replicates are always performed, use SE of sample replicates; else, if processing replicates are always performed, use SE of processing replicates; else, if qPCR is performed, use SE of PCR replicates; else, if digital PCR is performed, use error from multiple replicates if available, and Poisson error if not	Number, with decimal, greater than or equal to 0; -1 (if cannot be calculated, such as when no amplification observed); or leave empty	[512]	Units specified in 'sars_cov2_units' line	Required
sars_cov2_cl_95_lo	Numerical, with decimal (float)	Lower bound of 95% confidence interval of SARS-CoV-2 in wastewater sample, or best estimate that is consistently available. Follow the same hierarchy as described for standard error. (Note: 'cl' stands for confidence limit)	Any number, with decimal, other than -1; -1 (if cannot be calculated, such as when no amplification observed); or leave empty	[220120]	Units specified in 'sars_cov2_units' line	Required
sars_cov2_cl_95_up	Numerical, with decimal (float)	Upper bound of 95% confidence interval of SARS-CoV-2 in wastewater sample, or best estimate that is consistently available. Follow the same hierarchy as described for standard error. (Note: 'cl' stands for confidence limit)	Any number, other than -1; -1 (if cannot be calculated, such as when no amplification observed); or leave empty	[318698]	Units specified in 'sars_cov2_units' line	Required
sars_cov2_below_lod	Specific text string; see Acceptable Values	Was the concentration of SARS-CoV-2 below the limit of detection?	[yes] or [no]	[no]	None	Required
lod_sewage	Numerical, with decimal (float)	SARS-CoV-2 limit of detection back-calculated to unconcentrated sample basis	Any number with decimal	[15200.0]	Units specified in 'sars_cov2_units' line	Required
ntc_amplify	Specific text string; see Acceptable Values	For qPCR, did any no-template controls on this instrument run have a Ct value less than 40? For ddPCR, did any no-template controls on this instrument run have 3 or more positive droplets?	[yes] or [no]	[no]	None	Required

*Example values provided are artificial and should be used as an example only. These values will be different for each sampling site and, for some, each different sample collected.

The Use of CDC Recommended Controls

Laboratory controls and strict quality control measures are critical for reporting reliable measurements and for comparing viral levels in wastewater over time and across locations. The CDC recommends the following types of laboratory controls for monitoring SARS-CoV-2 in wastewater:

- MATRIX RECOVERY CONTROL:
  CDC explanation: "*Use a matrix recovery control (also called a process control) to understand the amount of virus lost during sample processing. This control is important for comparing concentrations resulting from different testing methods and over time. It is important to quantitatively assess recovery because wastewater is chemically and biologically complex and variable, and often contains constituents that can interfere with sample concentration, nucleic*

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acid extraction, or molecular quantification methods. You must include a matrix recovery control in method validation and, if possible, include it with each sample to account for unexpected changes in wastewater composition. Always include a matrix recovery control when wastewater conditions (such as from rainwater inflows) or laboratory methods change."

The use of this laboratory control is explained in the workflow explained herein and the analysis of this control is built into the GT-Digital sARS-CoV-2 Wastewater Surveillance Kit. Step 4 of this document describes the use of this control material and how to report the results of the analysis to NWSS.

- HUMAN FECAL MATERIAL NORMALIZATION CONTROLS:

  CDC Explanation: "*Normalizing SARS-CoV-2 wastewater concentrations prior to calculating trends is conducted to account for changes in wastewater dilution and differences in relative human waste input over time. If the number of people contributing to the sewershed is expected to change over the surveillance period (due to tourism, weekday commuters, temporary workers, etc.), normalizing SARS-CoV-2 concentrations by the amount of human feces in wastewater can be important for interpreting SARS-CoV-2 concentrations and comparing concentrations between sewage samples over time. Human fecal normalization controls are organisms or compounds specific to human feces that can be measured in wastewater to estimate its human fecal content. Human normalization controls include, but are not limited to Pepper Mild Mottle virus, crAssphage. Bacteroides HF183, and Lachnospiraceae Lachno3. Normalizing SARS-CoV-2 concentrations using human fecal controls (e.g., the ratio of SARS-CoV-2 to human fecal control concentrations) can also account for viral losses that occur anywhere between fecal input into the wastewater system and quantification at the laboratory. However, human fecal normalization cannot replace matrix recovery controls for method performance evaluation.*"

  The use of the PMMoV fecal material normalization control is built into the GT-Digital sARS-CoV-2 Wastewater Surveillance Kit. As described in Appendix G, extracted RNA from wastewater samples are diluted 1:100 before analysis for PMMoV. This dilution is required because the PMMoV material is present at much higher concentrations than SARS-CoV-2. Not diluting the sample before analysis will result in a concentration outside the linear dynamic range achieved by digital PCR technologies.

Always dilute extracted RNA before analyzing for PMMoV Human Fecal Indicator

REPORTING TO NWSS:

Raw data for relevant analysis controls must be reported for each sample. Use the NWSS Data Dictionary to guide appropriate formatting, acceptable values, and indicate relevant items. The lines relevant to reporting for this section can be found within the *Metadata* tab, rows 88 through 97. The following table lists the relevant fields and how to report them for the sample-specific control data. Note that most items contained in this section are sample-specific and likely will change significantly from sample to sample. These rows should be filled out uniquely for each sample.

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Field Name	Acceptable Data Type	Description	Acceptable Values	GTM Example Values*	Units	Required by NWSS?
rec_eff_percent	Numerical, with decimal (float)	Percent of spiked recovery control, specified in 'rec_eff_target_name', that was recovered	Number, with decimal, greater than or equal to 0; -1 (if not tested)	[12.2]	Percent	Required
hum_frac_mic_conc	Numerical, with decimal (float)	Concentration of microbial target specified in 'hum_frac_target_mic'; follow the same guidelines outline for 'sars_cov2_avg_conc'	Number, with decimal; or leave empty	[1200500.0]	Units specified in 'hum_frac_mic_unit' line	Not required
hum_frac_mic_unit	Specific text string; see Acceptable Values	Concentration units of microbial target specified in 'hum_frac_target_mic'	See NWSS Dictionary for allowable string	[copies/L wastewater]	None	Not required
hum_frac_chem_conc	Numerical, with decimal (float)	Concentration of chemical target specified in 'hum_frac_target_chem'	Number, with decimal; or leave empty	Left empty	Units specified in 'hum_frac_chem_unit' line	Not required
hum_frac_chem_unit	Specific text string; see Acceptable Values	Concentration units of chemical target specified in 'hum_frac_target_chem'	See NWSS Dictionary for allowable string	Left empty	None	Not required
other_norm_conc	Numerical, with decimal (float)	Concentration of target specified in 'other_norm_name'	Number, with decimal; or leave empty	Left empty	Units specified in 'other_norm_conc' line	Not required
other_norm_unit	Specific text string; see Acceptable Values	Concentration units of target specified in 'other_norm_name'	See NWSS Dictionary for allowable string	Left empty	None	Not required
quality_flag	Specific text string; see Acceptable Values	Does this observation have quality control issues?	[yes]; [no]; or leave empty	[no]	None	Not required

*Example values provided are artificial and should be used as an example only. These values will be different for each sampling site and, for some, each different sample collected.

- **QUANTITATIVE MEASUREMENT CONTROLS**

  CDC Explanation: *"You must include quantitative measurement controls for all SARS-CoV-2 RNA quantification methods. For RT-dPCR, include a control of known quantity with each instrument run."*

  A quantitative measurement control is provided with each GT-Digital SARS-CoV-2 Wastewater Surveillance for QIAcuity® kit in the form of the N1-N2-BCoV Positive control material. This material is provided with a concentration determined by dPCR. For each dPCR run, include positive control material on your plate and compare the result you obtain to the expected result based on the provided concentration.

- **INHIBITION ASSESSMENT**

  CDC Explanation: *"Use inhibition testing to determine whether RNA quantification processes (RT and PCR) are performing as expected. Wastewater is a complex and variable mixture, and often contains compounds that can impede*

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accurate measurement by interfering with RNA quantification methods. assess inhibition by spiking viral RNA (for example, synthetic SARS-CoV-2 RNA or purified RNA from a non-human coronavirus, as described in Matrix Recovery Controls) into wastewater extracts and comparing the measured concentration to either viral RNA spiked into molecular negatives (no template controls) or to a dilution of the spiked extract.
If you encounter inhibition, it can often be mediated by reducing template volume and/or by diluting extracts. If you frequently encounter inhibition, further optimize sample processing or quantification methods. Potential steps to incorporate include variable wastewater processing volumes and/or wash step incorporation."

At this time, the CDC has not recommended a frequency in which inhibition needs to be assessed. Assessing inhibition for every sample processed would dramatically increase the number of samples that need to be analyzed and therefore also dramatically increase the cost of analysis.

GT Molecular recommends performing an inhibition assessment of your workflow:

1. *during workflow validation before reporting results to NWSS or other stakeholders*
2. *on a quarterly basis*
3. *in the case of repeated failed sample analysis*

See a complete Inhibition Assessment Standard Operating Procedure in Appendix E.

REPORTING TO NWSS:

Inhibition affects, if observed, and how they are accounted for must be reported for each sample. Use the NWSS Data Dictionary to guide appropriate formatting, acceptable values, and indicate relevant items. The lines relevant to reporting for this section can be found within the *Metadata* tab, rows 62, 89, and 90. The following table lists the relevant fields and how to report them for inhibition data. Note that, as recommended above, most items contained in this section are not measured on every sample. These rows can be filled out ahead of time and should be updated whenever inhibition assessment is performed.

Field Name	Acceptable Data Type	Description	Acceptable Values	GTM Example Values*	Units	Required by NWSS?
inhibition_method	Text string	A publication, website, or brief description of the method used to evaluate molecular inhibition	Text string; [none] (if inhibition not tested)	[Inhibition evaluated following GT Molecular Surveillance Guide]	None	Required
inhibition_detect	Specific text string; see Acceptable Values	Was molecular inhibition detected?	[yes]; [no]; or [not tested]	[no]	None	Required
inhibition_adjust	Specific text string; see Acceptable Values	Was inhibition incorporated into the SARS-CoV-2 concentration calculation?	[yes]; [no]; or leave empty	[no]	None	Required

*Example values provided are artificial and should be used as an example only. These values will be different for each sampling site and, for some, each different sample collected.

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- **NEGATIVE CONTROLS**
  CDC explanation: Extraction blanks are made by extracting RNA without the addition of a wastewater sample. These controls are used to detect extraction reagent contamination. Include them with each set of samples extracted. "No template controls" are molecular reaction reagents without added wastewater sample nucleic acid extract. Use these controls to detect molecular reagent contamination and include them with all PCR instrument runs.

For compliance with CDC's guidance, GT Molecular recommends including an extraction blank in which sterile water is extracted in place of a wastewater sample for each of samples extracted and the inclusion of non-template controls as described in the GT-Digital SARS-CoV-2 Wastewater Surveillance Instructions for Use.

REPORTING TO NWSS:

Extraction blanks, if utilized, and how they are analyzed must be reported for each analysis run. Use the NWSS Data Dictionary to guide appropriate formatting, acceptable values, and indicate relevant items. The lines relevant to reporting for this section can be found within the *Metadata* tab, rows 43 and 97.

The following table lists the relevant fields and how to report them for the sample-specific extraction-blank data. Note that most items contained in this section are sample-specific and likely will change significantly from sample to sample. These rows should be filled out uniquely for each sample.

Field Name	Acceptable Data Type	Description	Acceptable Values	GTM Example Values*	Units	Required by NWSS?
ext_blank	category	Are extraction blanks included in the extraction process?	[yes]; [no]; or leave empty	yes	None	Not required
quality_flag	category	Does this observation have quality control issues?	[yes]; [no]; or leave empty	no	None	Not required

*Example values provided are artificial and should be used as an example only. These values will be different for each sampling site and, for some, each different sample collected.

ASSOCIATED DOCUMENTS FOR CDC RECOMMENDED CONTROL REPORTING

Appendix G – CF-0025 ¬Instructions for Use: GT-Digital SARS-CoV-2 Wastewater Surveillance Assay for QIAcuity®

Protocol for quantification of 1) SARS-CoV-2 (N1 and N2, published by CDC); 2) Bovine Coronavirus (BCoV), a matrix recovery control (also called a process control) biologically similar to SARS-CoV-2; and 3) Pepper Mild Mottle virus (PMMoV), a fecal indicator that can estimate the human fecal content in a sample.

Appendix E – Molecular Inhibition Assessment SOP

Standard operating procedure for the assessment of inhibition levels present in wastewater workflow

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References

Karthikeyan S, Ronquillo N, Belda-Ferre P, Alvarado D, Javidi T, Longhurst CA, Knight R. "High-Throughput Wastewater SARS-CoV-2 Detection Enables Forecasting of Community Infection Dynamics in San Diego County." mSystems. 2021 Mar 2;6(2). doi: 10.1128/mSystems.00045-21. PubMed PMID: 33653938.

Peccia J, Zulli A, Brackney DE, Grubaugh ND, Kaplan EH, Casanovas-Massana A, Ko AI, Malik AA, Wang D, Wang M, Warren JL, Weinberger DM, Arnold W, Omer SB. "Measurement of SARS-CoV-2 RNA in wastewater tracks community infection dynamics." Nat Biotechnol. 2020 Oct;38(10):1164-1167. doi: 10.1038/s41587-020-0684-z. Epub 2020 Sep 18. PubMed PMID: 32948856; PubMed Central PMCID: PMC8325066.

Wang XW, Li JS, Guo TK, Zhen B, Kong QX, Yi B, Li Z, Song N, Jin M, Xiao WJ, Zhu XM, Gu CQ, Yin J, Wei W, Yao W, Liu C, Li JF, Ou GR, Wang MN, Fang TY, Wang GJ, Qiu YH, Wu HH, Chao FH, Li JW. "Concentration and detection of SARS coronavirus in sewage from Xiao Tang Shan Hospital and the 309th Hospital." J Virol Methods. 2005 Sep;128(1-2):156-61. doi: 10.1016/j.jviromet.2005.03.022. Erratum in: J Virol Methods. 2005 Dec;130(1-2):210. PMID: 15964082; PMCID: PMC7112879.

Weidhaas J, Aanderud ZT, Roper DK, VanDerslice J, Gaddis EB, Ostermiller J, Hoffman K, Jamal R, Heck P, Zhang Y, Torgersen K, Laan JV, LaCross N. "Correlation of SARS-CoV-2 RNA in wastewater with COVID-19 disease burden in sewersheds." Sci Total Environ. 2021 Jun 25;775:145790. doi: 10.1016/j.scitotenv.2021.145790. Epub 2021 Feb 12. PubMed PMID: 33618308; PubMed Central PMCID: PMC7879159.

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Appendix A: Standard Operating Procedure for Sample Collection

Doc. ID CF-0050 Version R002

Description

For consistent sampling across different wastewater facilities, each site should be provided a standard operating procedure to instruct on best practices to follow. An example standard operating procedure is detailed below.

Sample Description:

Untreated wastewater from Publicly-Owned Treatment Works (POTWs) are considered to be "diagnostic specimens" and are not subject to restrictions on shipping. Nevertheless, untreated wastewater influent may contain a variety of infectious human pathogens, including (but not limited to) norovirus, hepatitis A, and SARS-CoV-2 viruses and should therefore be handled with care.

Chain of custody:

Please fill out the Chain of Custody form prior to shipping the sample.

Sampling Kit Contents:

- 3 x 50 mL conical sample tubes
- 2 Cold packs
- Plastic bag with absorbent pads
- Insulated shipping cooler & box
- Chain of Custody form
- Return shipping label
- UN3373 sticker label

Sampling and Packing Procedure:

Individuals handling sample bottles should wear appropriate PPE, including but not limited to: gloves, lab coat, goggles and/or face shield.

1. Open the kit and store the provided ice packs in a freezer for at least 60 minutes. This ice packs will be used to return samples to the lab. **DO NOT** use ice cubes or other methods of cold-chain to return the samples.
2. Distribute the wastewater sample to be analyzed into the provided 50 mL conical tubes (3 total per sample).
    a. Gently mix sample prior to sample addition to each tube.
    b. Allow sufficient headspace in all tubes to compensate for pressure and temperature changes. **DO NOT** provide more than 40 mL in each conical collection tube.
    c. **DO NOT** provide samples using your own bottles or tubes. Sample integrity and user safety cannot be guaranteed if alternate materials are used.
3. Close each tube and ensure all lids are tight and will not leak.
4. Wipe the outside of all tubes with a 10% bleach solution.
5. Label tubes clearly with an alcohol-resistant ink. Provide the sample identifier and date of collection.
6. Place filled conical tubes in the provided biohazard bag. Ensure the absorbent pads are in the bag. Seal the bag.
7. Fill out and attach the Chain of Custody form for the sample to the outside of the bag.
8. Insert the sample bag(s) into the provided polystyrene shipping box.
9. Place the provided ice packs, **FROZEN**, into the shipping container with the sample.
10. Ensure a UN3373 label is affixed to a visible location on the outside of the box.
11. Tape the box shut and affix the return shipping label to the box

Appendix B: Standard Operating Procedure for Wastewater Sample Collection Kit Preparation

Doc. ID	Version
CF-0051	R002

Description

Sample collection should be as streamlined, consistent, and easy as possible to facilitate quick collection and delivery of samples with limited issues. Providing each sampling site with each component required to prepare, collect, and ship a sample is the best way to ensure this.

Kit Specifications

Each kit should contain all required consumables necessary for sample collection and delivery. Each kit should be provided pre-assembled to the sampling site with the following:

*Note: The table outlines what GT Molecular uses, but equivalent products/suppliers are available

Material/Reagent	Quantity	Supplier	Catalog #
Styrofoam cooler and box	1	Uline	S-13391
Sterile, nuclease free 50 mL conical tube	3	SPL Life Sciences	50150
Biohazard bag	1	Medline	DYND30261T or LGLIP2AP69B
Absorbent pads	2	Various	Various
Ice Pack	1	Polar Tech Industries, Inc.	IB 3
Instructions for sample collection	1	See Appendix A	NA
Chain of Custody Form	1	See Appendix C	NA

Assemble Kits

1. Prepare an assembly line for kits.
2. In each Styrofoam cooler, place the following:
    a. 2 ice packs (room temperature, not frozen)
    b. 1 biohazard bag
    c. 2 absorbent pads, place inside the biohazard bag
    d. 3 x 50 mL conical tubes
    e. Sample chain of custody form
    f. Standard operating procedure for sample collection
3. Purchase and print a return shipping label for the site to return the sample kit to the lab.
    a. Purchase priority overnight shipping (or equivalent service) to ensure samples are returned quickly.
4. Place the return label (on its sheet; do not remove and sticker) on top of the styrofoam cooler.
5. Tape the boxes shut and ship to the sampling site.
6. Contact the site to notify them to expect and prepare for arrival of sample collection kits

Appendix C:

Chain of Custody Form

SARS-CoV-2 Wastewater Monitoring

Contact: _____ or _____

Customer's Sample Identifier: _____

Location Identifier for sample: _____

Facility name and address: _____

Specific sampling location name: _____

Population served by facility: _____

Time zone: _____

Sampling method:

- ☐ **Flow-proportional 24-hr composite**
- ☐ **Volume-proportional 24-hr composite**
- ☐ **Time-proportional 24-hr composite**
- ☐ **Grab sample**
- ☐ **Other** _____

Time of collection: **(If you collected a grab sample)** _____

If you used an automatic sampler:

Sampling start date & time: _____ **Sampling stop date & time:** _____

Sample collection and processing information:

Sample was maintained at 4°C during sample collection/compositing (required by NWSS):

- ☐ Yes
- ☐ No. Please specify sample storage temperature: _____

Sample was maintained at 4°C during sample storage and transport (required by NWSS):

- ☐ Yes
- ☐ No. Please specify sample storage temperature: _____

Average Influent flow over sampling period (MGD): _____

-- Please use Million Gallons per Day (MGD)

Name: _____

Signature: _____

Notes:

Appendix D: Preparation and Use of Bovine Coronavirus (BCoV) Matrix Recovery Control

Doc. ID	Version
CF-0053	R002

For research purposes only.

Description

The following procedures outline preparation, use, and storage of BCoV matrix recovery control material for use as a proxy for viral recovery. Please note this document describes the use of BCoV available for customers within North America.

Materials Required but Not Provided

- BCoV Internal Process Control - Bovilis® Coronavirus Vaccine, Cattle Vaccine – 10 doses x 20 mL
- Lucigen Quick Extract (catalog # QE905T)

dPCR Materials Required but Not Provided

QIAGEN Catalog #	Product Name	Qty (each)	Storage (°C)
1123145	QIAcuity® One-Step Viral RT-PCR Kit	1	-20
250001	QIAcuity® Nanoplate 26k 24-well	1	+15 to +25

General Laboratory Equipment Required but Not Provided

Description	Source
Single and multichannel adjustable pipettors	Multiple suppliers
Microcentrifuge	Multiple suppliers
Microwell plate centrifuge	Multiple suppliers
Laboratory freezers, -20°C	Multiple suppliers
96-well or 384-well cold block or ice	Multiple suppliers
Nonstick, RNase-free microcentrifuge tubes (1.5 mL and 2.0 mL)	Multiple suppliers
Sterile aerosol barrier (filtered) pipette tips	Multiple suppliers
50 mL conical tube	Multiple suppliers

Equipment

The GT-Digital SARS-CoV-2 Wastewater Surveillance Assay for QIAcuity® kit was formulated and optimized for use with the QIAGEN QIAcuity® Digital PCR system.

Protocol

Re-constitute a single 10 dose vial of Bovine Coronavirus Vaccine Modified Live Virus

1. Obtain ice and maintain each component on ice.
2. Gently swirl to ensure contents settle at the bottom of the vial.
3. Reconstitute lyophilized virus in 5 mL pre-chilled de-ionized water. Swirl and pipette to mix. Do not vortex.
4. Aliquot up to 100 – 500 µL stock in sterile 1.5 mL tubes, label $BCoV^{ND}$
5. Place 1 aliquot on ice.
6. Store remaining aliquots of non-diluted ($BCoV^{ND}$) stock solution at -80°C.

Dilute $BCoV^{ND}$ stock to an intermediate solution ($BCoV^{INT}$)

7. Dispense 540 µL pre-chilled deionized water to a 1.5 mL tube
8. Add 60 µL $BCoV^{ND}$ and pipette to mix. Do not vortex.

Note: volumes can easily scale to prepare larger volumes of this intermediate solution. Store aliquots at -80°C

Prepare $BCoV^{working}$ working solution aliquots

9. Further dilute $BCoV^{INT}$ to achieve $BCoV^{working}$:
   a. Add 49.5 mL pre-chilled de-ionized water to 500 µL $BCoV^{INT}$
   b. Invert and/or pipette with serological pipette to mix.
10. Aliquot 1 mL aliquots in sterile 1.5 mL tubes, label $BCoV^{working}$
11. Store aliquots of $BCoV^{working}$ at -80°C.

Quantify each batch of working $BCoV^{working}$ aliquots

12. Thaw one aliquot of $BCoV^{working}$ on ice and pipette to mix.
13. Prepare RNA for quantification using lysis-based QuickExtract buffer by mixing 50 µL QuickExtract with 50 µL $BCoV^{working}$ in a thin walled PCR tube.
14. Place on thermalcycler programmed to the following:
    a. 60°C for 20 minutes
    b. 98°C for 2 minutes
15. Briefly centrifuge to collect contents at the bottom of the tube
16. Quantify the extracted BCoV RNA by analyzing the sample with digital PCR in 3 replicates using the GT-Digital SARS-CoV-2

Wastewater Surveillance Assay for QIAcuity® kit by following the manufacturer's instructions.

Calculate $BCoV^{working}$ concentration and express in copies/µL

17. Use the following equation to calculate $BCoV^{working}$ concentration using the average concentration of BCoV copies/µL measured in Step 20.

    $BCoV^{M}$ is the average measured concentration in copies/ µL of the three replicates reported by the QIAcuity® dPCR system in step 20.

    V^{R} is the reaction volume for QIACuity® of 40µL.

    D^{Ext} is the extraction dilution factor that compensates for the dilution of the BCov template during RNA extraction in Step 17. The dilution factor for this protocol is 2.

    V^{T} is the template volume of extracted BCoV RNA added to each reaction in µL.

$$\left[BCoV^{working}\right] = \frac{BCoV^{M} * V^{R}}{V^{T}} * D^{Ext}$$

18. Assign this concentration to the lot of $BCoV^{working}$ stock aliquots.

To use this $BCoV^{working}$ working stock material as a matrix recovery control for use in wastewater monitoring:

19. Thaw an aliquot of $BCoV^{working}$ on ice.
20. Add 50 µL of $BCoV^{working}$ spike into each wastewater sample prior to processing and quantify BCoV remaining in each sample using the GT-Digital Wastewater Surveillance Kit by following the manufacturer's instructions.
21. Calculate the number of viral genomes spiked into the sample by multiplying the Lot $BCoV^{working}$ concentration by the volume spiked into each sample.
22. Use the above value to calculate percent recovery. Ensure all concentration factors are accounted for.

Recommendations

Perform this procedure in a biosafety cabinet to minimize contamination with high concentration stock material and decontaminate surfaces upon completion.

Proper aseptic technique should always be used when working with RNA. Always wear powder-free latex, vinyl, or nitrile gloves while handling reagents, tubes, and RNA samples to prevent RNase contamination from the surface of the skin or from dust in the environment. Change gloves frequently and keep tubes closed. During the procedure, work quickly and keep everything on cold blocks when possible, to avoid degradation of RNA by endogenous or residual RNases. Clean working surfaces, pipettes, etc. with 20% bleach or other solution that can destroy nucleic acids and RNases.

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Appendix E: Molecular Inhibition Assessment Protocol for use with GT-Digital SARS-CoV-2 Wastewater Surveillance Assay for QIAcuity™

Doc. ID	Version
CF-0054	R002

For research purposes only.

Description

The CDC recommends the use of inhibition testing to determine whether RNA quantification processes such as digital PCR are performing as expected. Wastewater is a complex and variable mixture and often contains molecules that can impede accurate measurement by interfering with RNA quantification methods. We recommend performing this analysis in the process of validating your workflow, on a quarterly basis, and in the case of failed sample analysis.

According to the CDC recommendations, inhibition assessment can be done by spiking synthetic SARS-CoV-2 RNA into wastewater, extracting RNA as well as a water control and comparing the percent viral RNA recovery from the wastewater extract. The water spiked control serves to determine the baseline concentration of the spike material without inhibitor presence. Assay inhibition can be assessed by calculating the relative percentage of signal obtained from wastewater-spiked samples relative to water-spiked sample. The following guidelines are used by GT Molecular in assessing the level of inhibition in a sample.

Viral Recovery of SARS-CoV-2 RNA in Wastewater Sample	Interpretation
> 90%	Low to No inhibition
80-90%	Mild Inhibition
< 80%	Inhibition present

Inhibition is often mediated by alternate RNA template volumes prior to analysis. If lower template volume is problematic for your workflow due the effects on sensitivity, or if you frequently encounter inhibition, we recommend further optimizing the sample processing method in use.

Materials Required but Not Provided

- Twist Synthetic SARS-CoV-2 RNA Control 1 (MT007544.1), Catalog # 102019
- Lucigen Quick Extract (catalog # QE905T)

Notes on working with synthetic RNA:

RNA is a labile molecule. Once reconstituted, RNA should be aliquoted to avoid freeze/thaw cycles and stored at -80C. Make sure to use the same aliquot of RNA for spiking into both the wastewater extracted RNA and the water control to ensure the water control captures the accurate concentration of the RNA stock and accounts for any concentration changes dues to handling.

dPCR Materials Required but Not Provided

QIAGEN Catalog #	Product Name	Qty (each)	Storage (°C)
1123145	QIAcuity® One-Step Viral RT-PCR Kit	1	-20
250001	QIAcuity® Nanoplate 26k 24-well	1	+15 to +25

General Laboratory Equipment Required but Not Provided

Description	Source
Single and multichannel adjustable pipettors	Multiple suppliers
Microcentrifuge	Multiple suppliers
Microwell plate centrifuge	Multiple suppliers
Laboratory freezers, -20°C	Multiple suppliers
96-well or 384-well cold block or ice	Multiple suppliers
Nonstick, RNase-free microcentrifuge tubes (1.5 mL and 2.0 mL)	Multiple suppliers
Sterile aerosol barrier (filtered) pipette tips	Multiple suppliers
50 mL conical tube	Multiple suppliers

Equipment

The GT-Digital SARS-CoV-2 Wastewater Surveillance Assay for QIAcuity® kit was formulated and optimized for use with the QIAGEN QIAcuity® Digital PCR system.

Protocol

Prepare Wastewater-extracted RNA for Inhibition Assessment. The following steps outline means to assess the impact of wastewater inhibitors on dPCR performance.

1. Process wastewater using your standard workflow through the RNA extraction step to produce wastewater extracted RNA for analysis. Increase sample size to ensure a final RNA volume > 140 µL. Keep on ice. Gently flick tubes and pulse centrifuge to ensure contents settle at the bottom of the vial.
2. Dilute SARS-CoV-2 RNA (Twist Bioscience Catalog 102019) to 5000 copies/µL. Keep on ice.
3. Prepare the templates outlined below in Table 1.

Note: volumes were calculated based on the recommended 10 µL wastewater RNA template volume. If your workflow uses variable template volumes, scale accordingly.

Table 1. SARS-CoV-2 RNA Template Preparation

Tube label	Dilution Factor	Volume Water (µL)	Volume WW RNA (µL)	Volume SARS-CoV-2 RNA (5000 copies/µL)	Total (µL)
WW - spike	1	4	40	0	44
WW + spike	1	0	40	4	44
WW 1:2 - spike	2	24	20	0	44
WW 1:2 + spike	2	20	20	4	44
WW 1:4 - spike	4	34	10	0	44
WW 1:4 + spike	4	30	10	4	44
Water + spike	NA	40	0	4	44
water (NTC)	NA	44	0	0	44

Perform dPCR

4. The dPCR calculations below account for the recommended 10 µL wastewater RNA as well as SARS-CoV-2 RNA spike (or water).
5. Prepare the following mastermix, vortex, and briefly centrifuge.

Table 2. Mastermix preparation

Total Reactions	24	
Safety Reactions (pipette error)	2	
	Volume per reaction	**Volume in Mastermix**
Nuclease-free water	16.6	431.6
4X Master Mix	10	260
100X reverse transcription mix	0.4	10.4
20X N1-N2-BCoV 3-plex Assay	2	52
Template	11	NA
Final Volume	40	754
Volume to add to each rxn		29

6. Dispense 29 µL mastermix to each well (24) of microcentrifuge tubes.
7. Proceed with dPCR setup as described in the GT-Digital SARS-CoV-2 Wastewater Surveillance Assay for QIAcuity® IFU.

Inhibition Analysis

8. Ensure NTC wells are negative and auto-thresholds were applied appropriately
9. Calculate the percent inhibition.

$$\% \text{ Inhibition} = 100 - \left(\frac{(\text{with spike} - \text{no spike})}{\text{water spike}}\ x\ 100\right)$$

Example inhibition assessment and interpretation

- Note: this sample (Table 3) contained baseline SARS-CoV-2. Therefore, these values obtained from the WW -spike samples should first be subtracted from the WW +spike samples.
- Percent expected values are represented as a percent of the water spike. This value serves as the no inhibition reference.
- As expected, wastewater RNA contains inhibitors that prevent the full amplification of the spike material. This percent inhibition decreases as the wastewater RNA dilution factor increases.
- If the standard workflow shows > 20% inhibition, the workflow may require optimization. Suggested optimization parameters include: reducing template input volume per reaction and rinsing steps during wastewater processing procedures, to name a few.

Table 3. Example Inhibition Data

Template	[Observed] (copies/µL)	Reference Subtracted	%Expected	%Inhibition
water (NTC)	0	0	-	-
water + spike	125	125	100	0
WW - spike	5	0	-	-
WW + spike	105	100	80	20
WW 1:2 - spike	4	0	-	-
WW 1:2 + spike	116.5	112.5	90	10
WW 1:4 - spike	3	0	-	-
WW 1:4 + spike	121.75	118.75	95	5

Recommendations

Proper aseptic technique should always be used when working with RNA. Always wear powder-free latex, vinyl, or nitrile gloves while handling reagents, tubes, and RNA samples to prevent RNase contamination from the surface of the skin or from dust in the environment. Change gloves frequently and keep tubes closed. During the procedure, work quickly and keep everything on cold blocks when possible, to avoid degradation of RNA by endogenous or residual RNases. Clean working surfaces, pipettes, etc. with 20% bleach or other solution that can destroy nucleic acids and RNases.

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Appendix F: Standard Operating procedure for Wastewater Sample Processing

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Description

The following procedures outline the filtration, concentration and RNA extraction procedures for processing wastewater samples to use in digital RT-PCR analysis. For instructions on wastewater sample collection process see **Appendix A** and **Appendix B**.

Materials Required Per 40 mL Wastewater Sample

Material/Reagent	Quantity	Supplier	Catalog #
Tween20	400µL (of a 10% solution)	SigmaAldrich	P9416 or equivalent
Prepared Bovine Coronavirus Process Control ($BCoV_{working}$)	50 µL	See Appendix D	
Stericup Quick Release-GP Sterile Vacuum Filtration System (150mL)	1	SigmaAldrich	S2GPU01RE
Ultrafiltration PS Hollow Fiber Concentrating Pipette Tip	1	Innovaprep	CC08004-200
Tris Elution Fluid Can	1	Innovaprep	HC08001
CP strorage fluid	1	Innovaprep	HC08558
AllPrep PowerViral DNA/RNA Kit	1 (kit sufficient for 50 samples)	QIAGEN	28000-50
B-mercaptoethanol	See AllPrep PowerViral Kit instructions	SigmaAldrich	444203 or equivalent
Ultrapure Water	~100 µL	ThermoFisher	10977015 or equivalent
Sterile, nuclease free 15 mL and 50 mL conical tubes	1 x 50 ml 1 x 15 mL/sample	SigmaAldrich	CLS430055 or equivalent
Sterile, nuclease free 1.5 mL Eppendorf tube	2	Genesee Scientific	22-281 or equivalent
Sterile, nuclease free, filter-barrier pipette tips, 2-20 µL	> 5	Various	Various
Sterile, nuclease free, filter-barrier pipette tips, 20-200 µL	> 5	Various	Various
Sterile, nuclease free, filter-barrier pipette tips, 100-1000 µL	> 5	Various	Various
Sterile, nuclease-free filter-barrier serological pipette, 1 mL	1	Various	Various
Personal Protective Equipment (PPE)	Various	Various	Various

General Laboratory Equipment Required

Description	Source
Single and multichannel adjustable pipettors	Multiple suppliers
Vacuum System for filtration	Various
Innovaprep® Concentrating Pipette Select™	Innovaprep
Microcentrifuge	Multiple suppliers
Ultracentrifuge, capable of 13,000 x g	Multiple suppliers
Laboratory freezers, -20°C	Multiple suppliers
Vortexer	Multiple suppliers
96-well or 384-well cold block or ice	Multiple suppliers

Safety

Due to the potential presence of infectious pathogens in wastewater samples, users should work with their organization's occupational safety team to ensure that methods and safety measures are appropriate and approved. Unless working with samples known to be non-infectious, InnovaPrep recommends that CP Select operations be performed in a biosafety cabinet. Additional information is published by the U.S. CDC: Interim Laboratory Biosafety Guidelines for Handling and Processing Specimens Associated with Coronavirus Disease 2019 (COVID-19).

Protocol

Processing Preparation

1. Prepare QIAGEN PowerViral extraction-specific reagents as listed by the manufacturer, including the Solution PM1/β-ME solution.
2. Thaw previously-prepared BCoV Internal Process Control (labeled $BCOV^{working}$)(see Appendix D for initial preparation of reagent) on ice.
    a. Aliquots should be used as single-use only. Throw away any remaining material at the end of the day. Do not refreeze.
3. Prepare a 10% Tween20 solution (if needed).
    a. Combine 45 mL of nuclease-free water with 5 mL of Tween20 in a clean, sterile, nuclease-free 50 mL conical tube.
        i. GT Molecular recommends dispensing Tween20 solution using a 5-10 mL

syringe to fully eject the viscous solution.

b. Vortex well and invert until solution has mixed well.
c. Use for up to 1 month.

4. Remove and stage stericup filtration devices.
5. For each sample you will process in this batch, label each of the following materials with the appropriate sample identifiers:
    a. Stericup Filtration Device
    b. 15 mL sterile conical tube
    c. Extraction materials, such as columns and sample processing tubes
    d. Microcentrifuge tubes (for collection of extracted RNA)
6. Initialize Innovaprep CP Select™
    a. Setup the CP Select as instructed in Section 4 of the CP Select User Guide provided by Innovaprep.
    b. Following the instructions provided in Section 8 of the CP Select User Guide, set up a Custom Protocol using the Advanced Options shown below:

Advanced Options	**Setting**
Valve open (ms)	770
Pulse	1
Foam Factor	10
Valve Closed (ms)	100
Flow Start (sec)	3
Flow End (sec)	10
Flow min start (sec)	40
Ext Delay(sec)	3
Pump (%)	25%
Ext. Pump delay (sec)	1

Filter Samples and Prepare for Concentration

7. Perform all work in a Class II BioSafety Cabinet (BSC).
8. Maintain wastewater samples at 4C until processing.
9. Dispense 400 µL of 10% Tween20 solution into each wastewater sample for every 40 mL of sample.
    a. NOTE: If processing more than one vial per sample (eg. 2 vials of 50 mL were collected for the sample), add requisite volume to *each* vial to achieve 0.1% Tween20 (v/v).
10. Close sample and invert gently twice to mix.
    a. NOTE: Avoid introducing air bubbles at this step
11. Dispense 50 µL of pre-thawed BCoV Internal Process Control ($BCoV^{working}$)to each sample for every 40 mL of sample.
12. Cover and invert twice to mix.
13. Pour sample vial(s) into filter and process according to manufacturer's instructions.
14. Record the total volume of sample added to filter.

Viral concentration and elution

15. Perform all work in a Class II BioSafety Cabinet (BSC).
16. After filtration is complete, remove filter from collection container.
17. Connect a fresh Innovaprep Ultrafiltration PS Hollow Fiber Concentrating Pipette Tip (now referred to as CPT for Concentrating Pipette Tip) to the Innovaprep CP Select™.
18. Lower CPT into sample.
19. On the CP Select™, select ***Start Run***.
    a. NOTE: If the Innovaprep halts the run prior to uptaking all of the sample, select Return and ***Start Run*** again. Repeat running until all of the sample has been taken up into the tip. This does not affect performance.
20. Lift the CP Select™ head, remove the collection bottle and dispose of according to local biosafety regulations. Place a sterile, nuclease-free 15 mL conical tube under the CPT.
21. Select ***Elute*** and collect elution foam in conical tube.
    a. NOTE: You can gently lift the CP Select™ head during elution process to avoid elution foam 'overflowing' the tube if overflow is a concern.
22. Cap the 15 mL conical tube and transfer to ice. Allow solution to settle. Proceed with all other samples.
23. Measure and record the volume of eluate and transfer solution to a clean, sterile, nuclease free 1.5 mL Eppendorf Tube.
    a. NOTE: We use 1mL serological pipettes to measure the volume and usually observed 300 to 600 µL volumes.
24. Store eluate on ice and extract RNA within an hour.
    a. NOTE: Do not freeze the eluate for RNA extraction on a later date. This causes a severe reduction in viral signal.
25. Dispose all reagents and consumables according to local biosafety regulations.

Viral RNA Extraction

26. Perform initial steps in a Class II BioSafety Cabinet (BSC). Once Solution PM1 + βME has been added to the virus sample, further work can be completed outside the BSC.
27. Extract viral RNA using the QIAGEN AllPrep Power Viral DNA/RNA Kit (Cat# 28000-50).
28. Follow manufacturers instructions for extraction processing.
    a. NOTE: Wastewater samples are generally not in a solid-matrix and thus do not require bead beating prior to extraction. It is not recommended to use solid-matrix wastewater samples as the levels of inhibitor present are likely too high to provide reliable results.
29. Load 200 µL of the viral concentration eluate to the tube and process according to the manufacturer's instructions.
30. Elute RNA from the column using 100 µL of ultrapure water into a clean, sterile, nuclease free 1.5 mL Eppendorf tube (or equivalent).
31. Keep RNA on ice until ready for use. Freeze the remainder at -70 to -90°C for long term storage. Avoid freeze/thaw cycles.

Digital PCR by QIAcuity®

32. Perform digital PCR on the extracted RNA samples using the GT-Digital SARS-CoV-2 Wastewater Surveillance Assay Kit for QIAcuity®.
33. Follow the manufacturer's instructions for sample set up, assay set up, and data analysis.

Recommendations

Proper aseptic technique should always be used when working with RNA. Always wear powder-free latex, vinyl, or nitrile gloves while handling reagents, tubes, and RNA samples to prevent RNase contamination from the surface of the skin or from dust in the environment. Change gloves frequently and keep tubes closed. During the procedure, work quickly and keep everything on cold blocks when possible, to avoid degradation of RNA by endogenous or residual RNases. Clean working surfaces, pipettes, etc. with 20% bleach or other solution that can destroy nucleic acids and RNases.

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