MANUAL FOR DETECTION OF SARS-CoV-2 RNA IN WASTEWATER

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1. INTRODUCTION

The worldwide epidemic of novel coronavirus infections (COVID-19) has caused not only loss of life and damage to health, but also tremendous damage to socioeconomic activities. The main routes of transmission of the new coronavirus (SARS-CoV-2) are human-to-human droplet and contact infections, but since viral genes can also be detected in human feces, detection of the virus in sewage has been attempted worldwide. The measurement of the SARS-CoV-2 gene in sewage may be able to reflect the prevalence of the disease in the treated area.

In this paper, we describe a method for the determination of SARS-CoV-2 in sewage. In addition to the accuracy and sensitivity of the method, the cost of consumables, human resources, equipment, speed, and efficiency should be taken into consideration. Therefore, the purpose of this paper is to give examples of various effective measurement methods so that they can be referred to when selecting a measurement method. For this reason, we have listed the methods that are currently recommended based on the results of previous studies.

Note that the last step in the detection of SARS-CoV-2 is assumed to be the PCR method, which detects viral genes and does not mean the presence of infectious viral particles. Therefore, it should be noted that the results can be used as information on the prevalence of the disease in the treated area, but they do not represent the risk of infection in sewage.

2. COLLECTION AND STORAGE OF SAMPLES

2.1 Sample Collection

The required volume of a wastewater sample depends on the applied concentration method (see details in Chapter 3). The typical volume of a sample is 100-250 mL for untreated wastewater; 1.0-10 L for secondary treatment effluent or final effluent of a wastewater treatment plant (WWTP). In collection of treated effluent after chlorine, residue chlorine should be eliminated to prevent degradation of nucleotides in the sample. A concentrated sodium thiosulfate ($Na_2S_2O_3$) solution should be added to the sample container in advance, then mixed well with the sample to the final concentration at approximate 50 mg/L.

A sealable container made of plastic or glass can be used as a sample container. The container should be sterile or autoclaved before sample collection. If a sterilized container or autoclave is not available, rinse the inside of the container with 1% sodium hypochlorite, leave it for a few minutes, and then rinse very well with a sufficient amount of pure water at least five times to eliminate chlorine completely.

When collecting samples, wear the appropriate personal protective equipment (PPE). Wearing masks and gloves is mandatory in most cases. If there is a possibility of splash exposure, consider necessity of face protection (safety goggles or face shields) and/or protective clothing. After the sample collection, wash the surfaces of the sample container with tap water, then disinfect it with ethanol or 5% chlorine bleach. Wash and disinfect hands immediately after removing protective equipment. (see details in Chapter 4).

2.2 Transport and Storage

A sample should be stored and transported in a sealed container. If the sample is to be analyzed within 24 hours, it should be transported on ice and stored in a refrigerator (below 10°C). If the sample is to be analyzed later, it should be stored in an ultralow freezer at -80°C. If an ultralow freezer is not available, store it in a laboratory freezer at -20°C or in a kitchen freezer, then analyze as soon as possible.

When a samples is transported from the sampling site to an external facility, it is recommended to be transported in a triple-layer package (**Figure 1**) in a cool or freezing condition. In the triple-layer packaging, the sealed primary container (= the sample container) should be placed in an airtight secondary container (e.g. sealed plastic bag, Bio-pouch, Ziplock, etc.) with cushioning and leak absorbent material (e.g. paper towels, Kimtowel etc.). The secondary container should then be placed in a tertiary container (e.g., foam box or cardboard box) that has sufficient physical strength durable for the transport.

In order to ensure the safety during transportation and storage, a sample can be heat-inactivated in a thermostatic bath at 56-60°C for 30-60 minutes[1]. The heat inactivation treatment is effective to reduce the potential risk of infection; however, it does not guarantee complete inactivation. Hence, it is highly recommended to take the same safety management regardless of application of inactivation, for sample handling during transport, storage and sample analysis. Decrease of SARS-CoV-2 RNA by heat inactivation under the above temperature and time conditions is reportedly limited [1].

[1] Pastorino, B., Touret, F., Gilles, M., de Lamballerie, X., Charrel, R.N., 2020. Heat Inactivation of Different Types of SARS-CoV-2 Samples: What Protocols for Biosafety, Molecular Detection and Serological Diagnostics? Viruses 12, 735. doi:10.3390/v12070735

Figure 1 Triple-layer packaging for transporting wastewater samples

3. ANALYTICAL METHODS

3.1 Virus Concentration

3.1.1 Process control

In the process of viral enrichment and detection, some effects cause inhibition, which prevents There are several well-known Inhibitory effects on the virus recovery and detection from sewage samples, which prevents accurate quantification of the viral gene. The inhibitory effect includes the loss of viral particle/genome during the virus recovery and viral gene extraction, decreased efficiency of the reverse transcription reaction and gene amplification in PCR. If significant inhibition is detected, the obtained data are not available and virus recovery needs to be repeated. Some surrogate viruses are available as process control viruses to detect the inhibition. Of the four viruses listed below, all but pepper mild mottle virus (PMMoV) and F-phage are prepared in advance in the laboratory, and a known amount is added to the sample before the virus recovery process (or the amount present is quantified), and the final percentage recovered is calculated. In case of using PMMoV or F-phage, the recovery rate is calculated by measuring the concentration before and after the recovery process, since the concentration of PMMoV or F-phage is originally high in sewage (note that inhibition after RNA extraction cannot be detected when using F-phage). A recovery of 1% or higher may be considered acceptable (i.e., the recovery process will not be repeated or the data will not be rejected). In general, the recovery rate of the process control virus is not used to convert the viral gene concentration of the target.

The following is a list of four viruses that can be used as process control viruses. Results obtained without using at least one of these viruses or a virus equivalent to one of these viruses as a process control virus may include a false negative result (a negative result due to inhibition even though the result is not really negative). If a process control virus is not used, the possibility that the negative result is a false negative cannot be denied, and therefore the quantified value should not be adopted as an official result. Even if the methods of recovery, RNA extraction, reverse transcription, and real-time PCR are not completely consistent with this manual, there is no problem in adopting the results obtained if the recovery rate of a process control virus is obtained without any problems (as for PMMoV, confirmation of the detected concentration from sewage is acceptable).

(1) Murine hepatitis virus

Murine hepatitis virus (MHV) is a positive-sense single-stranded RNA virus belonging to the genus *Betacoronavirus*, the same genus as SARS-CoV-2, which infects mice but is not pathogenic to humans and can be handled in the BSL2 facility. Several MHV strains are available from ATCC (e.g., A59, VR-764), and MHV can be cultured in L2 cells or DBT cells[2].

Several qPCR systems for MHV have been reported, and RNA can be quantified by RT-qPCR. Here, we introduce the system of Besselsen et al. (2002)[3], which is relatively widely used, as an example. The sequences of primers and probe are as shown in **Table 1**.

Table 1 Sequences of primers and probe for qPCR of MHV[4]

Primers and probes	Sequence (5'–3')
Primer (+)	GGAACTTCTCGTTGGGCATTATACT
Primer (-)	ACCACAAGATTATCATTTTCACAACATA
Probe	FAM-ACATGCTACGGCTCGTGTAACCGAACTGT-BHQ1

As a standard for calibration, use artificial synthetic DNA with the following sequence, which is the target region of real-time PCR in MHV-A59 strain (ATCC accession number: X00509).

GGAACTCTCGTGTGGGCATATATACTCTACTCTATATACATACATACATACATACA
GCCGGTAGCATGTGTATATATATGTTGTTGTGAAAATGATAATCTTGTGGT

For the qPCR reaction conditions, as an example, Besselsen et al. (2002)[3] used 40 cycles (95°C, 15 s → 60°C, 1 min).

(2) Bacteriophage φ6

Bacteriophage φ6 is an enveloped double-stranded RNA virus, with a diameter of 85 nm. Bacteriophage φ6 belongs to the family *Cystoviridae* and infects gram-negative *Pseudomonas syringae*. This virus has been frequently used as a surrogate for pathogenic enveloped viruses (i.e., human coronavirus and influenza virus). A recent study adopted bacteriophage φ6 as a MPC. Moreover, φ6 and its host, *Pseudomonas syringae*, are not pathogenic to humans and require minimal biosafety level (i.e., BSL1). Bacteriphage φ6 can be propagated within one day with high titers (up to 10^{10} PFU/mL); thus, φ6 is practically adoptable as a surrogate for the human pathogenic enveloped viruses.

However, φ6 has double-stranded RNA and an envelope derived from *Pseudomonas syringae*, which do not fully reflect the characteristics of SARS-CoV-2. The applicability of φ6 as a surrogate virus for process control needs further investigation.

[2] Leibowitz et al. (2011) Coronaviruses: Propagation, quantification, storage, and construction of recombinant Mouse Hepatitis Virus. Current Protocols in Microbiology, 15E.1.1-15E.1.46.

[3] Besselsen et al. (2002) Detection of rodent coronaviruses by use of fluorogenic reverse transcriptase- polymerase chain reaction analysis. Comparative Medicine, 52(2), 111-116.

[4] Pastorino et al. (2020). Heat inactivation of different types of SARS-CoV-2 samples: What protocols for biosafety, molecular detection and serological diagnostics? Viruses, 12(7), 735.

Table 2 Advantages and limitations of the application of the φ6 virus as a surrogate virus for process control

Advantages	Limitations
Morphological similarity to SARS-CoV-2 Possess an envelope and thus exhibit similar adsorption properties to mouse hepatitis virus (MHV) compared to MS2, a non-enveloped virus. **Can be used in BSL1-compliant facilities.** Not required for BSL2 facilities (BSL2 is required for handling envelope surrogate viruses such as MHV). **Easy to culture** *Pseudomonas syringae* and φ6 can be easily cultured at high concentrations.	**The presence of double-stranded RNA** Additional steps (e.g., heat denaturation of dsRNA before reverse transcription) are required in the process of quantification. **No explicit proof of comparability to SARS-CoV-2.** The structural differences between φ6 and SARS-CoV-2 may affect the recovery efficiency.

a) Reagents and instruments

Culture

- *Pseudomonas syringae*: Available at the National Institute of Technology and Evaluation (NITE). (NBRC 14084)
- Bacteriophage φ6: Available at NITE. (NBRC 105889)

PCR

Primer and probe: The sequence is shown in Table 3.

Table 3 Sequences of primers and probes for qPCR of φ6 (Gendron et al. 2010)

Primers and probes	Sequence (5'–3')
Primer (+)	TGGCGGCGGTCAAGAGC
Primers (-)	GGATGATTCTCCAGAAGCTGCTG
Probe	FAM-CGGTCGTCGCAGGTCTGACACTCGC-BHQ1

Dilute the standard with TE buffer, etc. as appropriate.

TGGCGGCGGTCAAGAGCAACCCGGTCGTCGCAGGTCTGACACTCGCTCAG
ATCGGAAGCACCGGTTATGACGCCTATCAGCAGCTTCTGGAGAATCATCC

(3) F-phage

F-specific bacteriophages (F-phages) are viruses that infect *E.coli* possessing F-pili. F-phages multiply in humans and other animals, and are excreted with feces. F-phages are classified into FDNA and FRNA phages. The former possess single stranded DNA in a rod-shaped capsid, while the latter possess single-stranded RNA in an icosahedral capsid with a diameter of 20-30 nm, like typical enteric viruses. FRNA phages are classified into four genogroups/serogroups of GI-GIV, which are represented by MS2, GA, Qβ, and SP phages, respectively. Both FDNA and FRNA phages are non-enveloped viruses.

F-phages in sewage can be detected and quantified by a cultural method. Advantages of using F-phage as a process control are that it does not require the addition of virus to the sample, and that with the cultural method, its quantification can be conducted at low cost. In addition, since nucleic acid extraction and RT-qPCR are not required, loss of RNA or RT-qPCR inhibition that potentially occur during these processes do not matter, and only the recovery efficiency during the concentration process can be estimated. On the other hand, disadvantages of using F-phage are that it takes overnight to obtain results and that the recovery efficiency of F-phage does not necessarily reflect that of SARS-CoV-2 because F-phage is a non-enveloped virus. Considering the diversity of F-phage, the recovery efficiency may vary depending on the genotype or strain.

In addition, since the detection is based on the cultural method, F-phage is not applicable when the sample is inactivated. Its concentration may decrease significantly when the sample is stored for a long time.

In the following, we introduce a plate counting method (plaque assay) using *Salmonella typhimurium* WG49 as the host, for the quantitative detection of F-phage. BSL2 laboratory is required for the use of *Salmonella typhimurium* WG49.

a) Reagents and instruments

- Petri dish: Use a sterilized one.
- *Salmonella typhimurium* WG49 (ATCC® 700730™): *Salmonella typhimurium* WG49 should be handled in the BSL2 laboratory. Prepare a glycerol stock and store at -80°C (see "Preparation of a glycerol stock for *Salmonella typhimurium* WG49" below). Medium and petri dish containing *Salmonella typhimurium* WG49 should be discarded after being autoclaved.
- Glycerol: Use for making glycerol stock.
- Trypton: Thermo Fisher Scientific, 211705, etc. Use as a component of culture medium.
- D(+)-Glucose: FUJIFILM Wako Pure Chemical, 047-31161, etc. Use as a component of culture medium.
- $CaCl_2 \cdot 2H_2O$: Use as a component of the culture medium. Prepare 0.3 g/mL calcium chloride solution by mixing 0.40 g of the reagent with 1 mL of purified or ultrapure water.
- $MgSO_4 \cdot 7H_2O$: Use as a component of the culture medium. Prepare 0.15 g/mL magnesium sulfate solution by mixing 0.31 g of the reagent with 1 mL of purified or ultrapure water.
- Agar: Bacto™ Agar (BD, 214010), etc. Use as a component of the culture medium.
- Kanamycin sulfate: Sigma Aldrich, K4000-5G, etc. Use as a component of the culture medium. Prepare an aqueous solution of 20 g/L and keep frozen in advance.
- Nalidixic acid sodium salt: Sigma Aldrich, N4382-5G, etc. Use as a component of the culture medium. Prepare an aqueous solution of 100 g/L and keep frozen in advance.
- Liquid medium: Prepare by mixing the reagents according to Table 4 and autoclave. Use for culture of *Salmonella typhimurium* WG49.
- Agar medium: Prepare by mixing reagents according to Table 4 and autoclave. Can be stored at 50°C without solidification.
- Phosphate buffer: FUJIFILM Wako Pure Chemical, 161-12191, etc. Use for dilution of samples.
- Incubator: Set at about 37°C (as in the case of detection of *E. coli*, etc.).

Table 4 Composition of liquid medium and agar medium used for F-phage detection

Reagents	Unit	Liquid medium	Agar medium
Purified water or ultrapure water	mL	500	500
Trypton	g	5	5
D(+)-Glucose	g	0.5	0.5
NaCl	g	4	4
$CaCl_2$ (aq) (adjusted to 0.3 g/mL)	mL	0.5	0.5
$MgSO_4$ (aq) (adjusted to 0.15 g/mL)	mL	0.5	0.5
Bacto Agar	g	-	5.5
Kanamycin sulfate (aq) (adjusted to 20 g/L)	mL	-	0.5※
Nalidixic acid sodium salt (aq) (adjusted to 100 g/L)	mL	-	0.5※
Salmonella typhimurium WG49 after liquid culture	mL	-	20※

※Kanamycin, nalidixic acid, and *Salmonella typhimurium* WG49 should be added immediately before plating.

b) Procedure

Preparation of a glycerol stock for Salmonella typhimurium WG49.

1. Dilute 63 g of 100% glycerol with pure water to be 100 mL of 50% glycerol.
2. Autoclave the 50% glycerol and bring to room temperature.
3. Add a glycerol stock of *Salmonella typhimurium* WG49 to the 100× volume of liquid medium, and incubate in an incubator at 37°C for about 4 hours with shaking.
4. Check the turbidity of the liquid medium visually after the incubation. If there is no turbidity, continue the incubation for another 1-2 hours.
5. Mix 100 μL of the liquid medium after completing the incubation with 100 μL of 50% glycerol in a cryo tube and store in a deep freezer (-80°C).

Detection of F-phage from wastewater samples

1. Prepare agar medium, assuming about 20 mL per Petri dish. Keep the medium at 50°C until just before use to prevent it from solidifying.
2. Prepare 20 mL of the liquid medium per 500 mL of agar medium.
3. Thaw the glycerol stock of *Salmonella typhimurium* WG49.
4. Add a glycerol stock of *Salmonella typhimurium* WG49 to the 100× volume of liquid medium, and incubate in an incubator at 37°C for about 4 hours with shaking. Use a medium in the logarithmic growth phase. The turbidity of the liquid medium can help to determine if it is in the logarithmic growth phase or not.
5. Spread the sample in a Petri dish. Spreading volumes of 100 μL or 1 mL per Petri dish are appropriate. Prepare multiple sample volumes in 10-fold steps in duplicate so that at least one of them results in 10 to 100 plaques (number of F-phages) per Petri dish. If < 100 μL sample volume needs be tested, dilute the sample with phosphate buffer in 10-fold steps. In the case of concentrated solution, the sample volume can be determined based on the volume reduction rate by concentration process and the expected recovery rate (1 to 100%).
6. Add *Salmonella typhimurium* WG49, kanamycin sulfate (aq), and nalidixic acid sodium salt (aq) to agar medium (preferably at 45°C, which is safe to grip with hands).
7. Add an appropriate amount of agar medium to each Petri dish and allow it to solidify at room temperature.
8. Incubate the Petri dish in an incubator at 37°C overnight.
9. Select a Petri dish with a sample volume where 10 to 100 plaques formed and count the plaques.
10. Calculate the concentration of F-phage in the sample based on the plaque count. For example, if 40 and 60 plaques in a sample volume of 100 μL (in duplicate) are obtained, the concentration of F-phage is determined as 500 PFU/mL.
11. Dispose the Petri dish and agar medium after autoclaving.

(4) Pepper mild mottle virus

Pepper mild mottle virus (PMMoV), a plant virus that infects peppers and other peppers of the genus *Capsicum*, is known to be present at extremely high concentrations in human feces and has been detected at high frequencies and concentrations in wastewater and environmental water worldwide[5]. Since the concentrations of PMMoV in wastewater don't show clear seasonal variations and are relatively constant, the measurement of indigenous PMMoV in wastewater samples can be used to confirm that no serious problems are occurring during the detection procedures. The advantage of using this process control is that it does not require adding PMMoV to a sample.

[5] Kitajima et al. (2018) Pepper mild mottle virus as a water quality indicator. npj Clean Water. 1:19.

a) Reagents

- Forward primer, reverse primer, TaqMan MGB probe: Use oligo DNA with the sequences shown in Table 5. Note that the quencher dye of the probe is NFQ-MGB, not TAMRA or BHQ.
- Standard sample: Use artificially synthesized DNA, etc., with the following sequence corresponding to the qPCR amplification region for PMMoV (ATCC accession number: NC_003630). Dilute with TE buffer or other appropriate solution.
  GAGTGGTGTGACCTTAACGTTGTGAGGCCTACGACGAAGCAATGTCGCACTT
  GCATTGCAACCGACAA

Table 5 Sequences of primers and probe for qPCR of PMMoV[6,7]

Primer/probe	Sequence (5'–3')
Forward primer	GAGTGGTTTGACCTTAACGTTTGA
Reverse primer	TTGTCGGTTGCAATGCAAGT
TaqMan MGB probe	FAM-CCTACCGAAGCAAATG-NFQ-MGB

b) Procedure

1. Obtain viral RNA extract by virus concentration and RNA extraction steps, without adding PMMoV to the sample.
2. In two-step RT-qPCR, obtain cDNA sample by RT, following the components of RT mixture and thermal conditions described in the protocol of the reagent.
3. In two-step RT-qPCR, prepare qPCR mixture, while in one-step RT-PCR, prepare RT-qPCR mixture. Mix the mixture with cDNA or RNA extract for the detection of PMMoV. Use the primers and probe listed in **Table 5**. Follow the components of the mixture and thermal conditions shown in the protocol of the reagent; however, the conditions may need to be optimized in case that amplification efficiency is not high.
4. In both one- and two-step RT-qPCR, instead of cDNA or RNA extract, use a standard sample diluted with TE buffer, etc., to be detected with a Ct value of approx. 25–30 as a positive control and PCR-grade water as a negative control. For quantification, prepare 10-fold serial dilutions of the standard sample diluted with TE buffer, etc., and draw a standard curve within the range of 10^1 to 10^6 copies/reaction.
5. Determine a Ct value of the tested sample using the software. If there is no significant difference from previous qPCR results, it is judged that there was no serious problem in the detection procedure.
6. Recovery of PMMoV can be calculated by storing approx. 1 mL of the wastewater sample before the concentration step, quantifying PMMoV in both the sample and the concentrated sample, and then calculating a ratio of the amount of PMMoV in the original wastewater sample to that in the concentrated sample by considering several factors, such as the volumes of the samples.

[6] Haramoto et al. (2013) Occurrence of pepper mild mottle virus in drinking water sources in Japan. App. Environ. Microbiol. 79 (23), 7413–7418.

[7] Zhang et al. (2006) RNA viral community in human feces: prevalence of plant pathogenic viruses. PLoS Biol. 4 (1), 0108–0118.

3.1.2 Concentration method

(1) Polyethylene glycol precipitation method

Polyethylene glycol (PEG) precipitation method is widely used for the concentration of protein components, including viruses. The method starts from mixing polyethylene glycol (a water-soluble polymer) and sodium chloride with wastewater sample. Incubation of the mixture allows the polyethylene glycol for associating with the proteins in the sample. After centrifuging the mixture, the supernatant is discarded. The remaining pellets are resuspended in a small amount of buffer solution and subjected to downstream processing. This method has been used as a primary concentration method for untreated sewage samples and as a secondary concentration method for other water samples. PEG precipitation method can reduce the volume of liquid from 100 mL to about 1 mL or less. PEG with a molecular weight of 6,000 or 8,000 (PEG6000 and PEG8000, respectively) along with sodium chloride is typically added to the water sample. Several studies optimized the type of PEG, the concentration of PEG and sodium chloride, mixing conditions (temperature and time), and centrifugation conditions for improving the efficiency of virus concentration. Here, the method proposed by Jones and Johns (2009) is introduced as an example.

a) Equipment

- A shaker with a cooling function or a shaker that can be installed in a refrigerator, etc.
- Centrifuge with cooling function: A centrifuge capable of centrifuging 50-mL tubes at 10,000 x g. A fixed angle rotor is preferable.

b) Reagents and instruments

- PEG8000: Wako Pure Chemicals, 593-09765, etc.
- Sodium chloride: Wako Pure Chemical Industries, 191-01665, etc.
- Sterilized 50-mL centrifuge tubes
- Sterilized 50-mL centrifuge tube for PEG precipitation: Add 4.0 g of PEG8000 and 2.35 g of NaCl in advance.
- Phosphate buffer (PB): Wako Pure Chemicals, 161-12191, etc.
- 1.5 mL tube: Used for collecting the concentrated solution.

c) Procedure

The method is shown for concentrating 80 ml of sample using two centrifuge tubes, each of which contains 40 mL of samples.

1. Add 4 g of PEG8000 and 2.35 g of NaCl to the centrifuge tube. Close the lid.
2. Add 40 mL of the sample into two empty 50-mL tubes.
3. (In the case of adding a surrogate virus for process control) After adding the surrogate virus, incubate the samples in a refrigerator for at least 1 hour until a solid-liquid equilibrium is reached between the surrogate virus and the sewage components.
4. Centrifuge at 3,500 x g for 5 min and transfer the supernatant to a centrifuge tube containing PEG and NaCl. Shake the tube gently so that PEG and NaCl do not remain undissolved at the bottom. Of note, since a large proportion of SARS-CoV-2 RNA in sewage may exist in solid-phase, the centrifugation (solid-liquid separation) may be omitted. It is also possible to extract RNA from the pellet and analyze it separately from the supernatant.
5. Incubate the mixture overnight at 4 °C in a shaker.
6. Centrifuge the mixture of the sample, PEG, and NaCl at 10,000 × g for 30 min.
7. Discard the supernatant. Resuspend the precipitates in 500 μL of phosphate buffer. Since the precipitate adheres to the wall of the tube, try to resuspend as much as possible by pipetting. Instead of phosphate buffer, purified water, ultrapure water or TRIzol Reagent (Invitrogen, 15596018, etc.) can be used.

8. Centrifuge the tube for a few seconds to collect the suspension at the bottom.
9. Transfer all the suspension to a 1.5 mL tube. Measure the final volume. The final volume is usually around 0.5 to 1 mL.
10. Subject the concentrates to RNA extraction. If no further RNA extraction is to be performed, store the virus concentrate at -20°C or lower.

Note that several other PEG precipitation conditions have been reported. For example, Wu et al. (2020) successfully detected SARS-CoV-2 RNA by using 0.22 μm PES membrane filtration, salt concentration of 0.3 M, shaking time of 15 minutes, and centrifugation time of 2 hours in the solid-liquid separation process.

(2) Electronegative membrane-vortex method

Electronegative membrane-vortex (EMV) method uses an electronegative membrane filter, which has been widely used for the concentration of enteric viruses, to adsorb viruses to the membrane by electrostatic attraction in the presence of cations, and to recover viruses along with turbidity by vigorous vortex mixing of the membrane in an elution buffer[8]. A centrifugal filter unit can be used as a secondary concentration method. The EMV method has been used for the concentration of enteric viruses, such as noroviruses and PMMoV, one of the process control viruses, in various types of water samples, such as wastewater, river water, and groundwater. This method has also been used for the successful detection of SARS-CoV-2 RNA at a wastewater treatment plant in Yamanashi Prefecture, Japan[9]. In addition, this method can simultaneously concentrate not only viruses but also protozoa (*Cryptosporidium*, *Giardia*, etc.) and bacteria by using pellet portions after centrifugation.

a) Equipment

- Suction filtration pump.
- Filtering flask.
- Glass filter holder (diameter, 90 mm): Cover the open parts with aluminum foil when autoclaving. Advantec, cat. no. KG-90, or equivalent.
- Centrifuge: A centrifuge capable of cooling to 4°C is desirable, but a cooling function is not essential.
- Swing rotor: A rotor that can centrifuge 50-mL centrifuge tubes at a maximum of approx. 2,000 · *g*. When using a fixed-angle rotor, it is desirable to consider the optimum conditions for centrifugation prior to the analysis. A fixed-angle rotor is also required if Amicon Ultra-15 is used as a secondary concentration method.

b) Reagents and consumables

- Mixed cellulose membrane (diameter, 90 mm; pore size, 0.8 μm): Mixed cellulose membrane consisting of cellulose ester and nitrocellulose (Merck Millipore, cat. no. AAWP09000, or equivalent). The membrane is placed in a glass filter holder, and the open portions are covered with aluminum foil. After autoclaving, the glass filter holder is stored with the aluminum foil attached.
- $MgCl_2$ solution (2.5 mol/L): Prepare by dissolving $MgCl_2$ $6H_2O$ in (ultra)pure water, autoclave, and store at room temperature. Add 1 mL of this solution per 100 mL of water sample.

[8] Haramoto et al. (2012) Development of a novel method for simultaneous concentration of viruses and protozoa from a single water sample. J. Virol. Methods 182, 62–69.

[9] Haramoto et al. (2020) First environmental surveillance for the presence of SARS-CoV-2 RNA in wastewater and river water in Japan. Sci. Total Environ. 731, 140405.

- Football-shaped stirring bar (25 × φ10 mm): As One, cat. no. 7-217-01, or equivalent.
- Elution buffer (100 ×): 2 g of $Na_4P_2O_7$ $10H_2O$ (Kanto Chemical, cat. no. 37256-00, or equivalent), 3g of $C_{10}H_{13}N_2O_8Na_3$ $3H_2O$ (Wako Pure Chemical, cat. no. 342-01875, or equivalent), and 1 mL of Tween 80 (Sigma-Aldrich, cat. no. P1754-25ML, or equivalent) in approx. 70 mL of (ultra)pure water, adjust pH to 7.2 by adding HCl, scale-up to 100 mL, autoclave, and store in a refrigerator.
- Elution buffer (1 ×): Prepare 100 times dilution of the elution buffer (100 ×) using (ultra)pure water, autoclave, and store in a refrigerator.
- Tweezer: Wrap in aluminum foil and autoclave.
- Transfer pipette: Sterilized and disposable.
- Centrifugal filter units: Centriprep YM-50 (Merck Millipore, cat. no. 4311 (96 units)), Amicon Ultra-15 (Merck Millipore, cat. no. UFC905008 (8 units)), or equivalent.
- Disposable filter unit (pore size, 0.45 μm; diameter, 25 mm): Advantec, cat. no. 25CS045AS, or equivalent.
- Syringe (volume, approx. 20 mL): Terumo, cat. no. SS-20ESZ, or equivalent.
- Electronic pipette: To be used by attaching a plastic pipette. Nichiryo, cat. no. NEO00-PMNEO, or equivalent.
- Plastic pipette: Sterilized. Preparing multiple sizes, such as 10 or 25 mL, is recommended.
- Micropipette: Sterilized.
- Micropipette tip: Sterilized. 20, 200, or 1000 μL, etc. Filter tips are preferable.
- Microtube: Sterilized. 1.5 or 2.0 mL, etc.
- Plastic paraffin film: Parafilm (As One, cat. no. 6-711-01), etc.

c) Procedure

1. Add 1 mL of $MgCl_2$ solution (2.5 mol/L) per 100 mL of water sample, mix by inverting, and incubate it at room temperature for a few minutes.
2. Filter the water sample through a glass filter holder attached with a 90-mm diameter mixed cellulose membrane. Normally, filtration of 200 mL is possible for influent wastewater. However, since filtration may be difficult depending on water quality, adjust the filtration volume while checking for clogging. When testing treated or effluent wastewater, filtration of approx. 5 to 10 liters is possible. When using membranes with a diameter of 47 mm, the standard filtration volume is approx. 50 mL for inflow sewage and 1 to 2 L for treated and effluent wastewater. Meanwhile, when using 47-mm membranes, two or more membranes may be used to filter a single water sample, but no more than four membranes may be used.
3. Remove the filtered membrane from the filter holder with a tweezer, fold it, and transfer it to a 50-mL centrifuge tube containing 10 mL of eluent buffer and a football-shaped stirring bar. When more than two membranes are used for filtration, all membranes should be placed in a single centrifuge tube. When Amicon Ultra-15 is used as a secondary concentration method, the volume of the elution buffer should be 8 mL because there is a possibility of sample leakage during centrifugation. Similarly, when using one 47-mm membrane, the volume of the elution buffer can be set to 8 mL to reduce the volume of the final concentrated solution.
4. Wrap a plastic paraffin film around the cap of the centrifuge tube.
5. Vortex the centrifuge tube vigorously with a vortex mixer to crush the membrane to a few mm square or less. In order to avoid unevenness in mixing, invert and mix as necessary to ensure uniform mixing. The membrane is sufficiently crushed in about 5 min.
6. Recover the whole volume of the solution using a transfer pipette and transfer it to a new 50-mL centrifuge tube. Be careful not to recover the crushed membrane as much as possible.

7. Add 5 mL of the elution buffer (4 mL if 8 mL was used in the first elution step) to the original tube, agitate with a vortex mixer for ~30 sec (plastic paraffin film is not necessary), and transfer the whole volume of the solution with a transfer pipette to the above mentioned tube. At this step, recover as much of the solution as possible from the inner wall of the tube and from the inside of the cap. The final volume of the solution in the new tube will be ~15 mL (~12 mL if 8 mL was used in the first elution step).
8. Centrifuge the centrifuge tube using a swing rotor (2,000 · *g*, 10 min, 4°C), and recover the supernatant with a transfer pipette. The temperature should be set at 4°C, but it is not necessary. The brake of the centrifuge should be turned off.
9. Transfer the collected supernatant to a syringe equipped with a disposable filter unit and filter under pressure to collect the filtrate. For collection of the filtrate, use the outer unit when using Centriprep YM-50 or the inner unit when using Amicon Ultra-15.
10. The following is the procedure when Centriprep YM-50 or Amicon Ultra-15 is used as the secondary concentration method. When using Centriprep YM-50, perform Steps 11–14, and when using Amicon Ultra-15, perform Steps 15–16. If a suitable centrifugal filter unit is not available, it is possible to skip the secondary concentration and use the concentrated sample obtained in Step 9, although the detection sensitivity will be decreased due to insufficient reduction of volume of the concentrate.
11. [Centriprep YM-50] Attach the inner unit of Centriprep YM-50 to the outer unit, and centrifuge it using a swing rotor (2,000 · *g*, 10 min, 4°C).
12. [Centriprep YM-50] Confirm that the inner and outer units are firmly attached, remove the cap of the inner unit, turn it upside down, and discard the solution in the inner unit.
13. [Centriprep YM-50] Centrifuge Centriprep YM-50 again (2,000 · *g*, 5 min, 4°C) to further reduce the sample volume in the outer unit.
14. [Centriprep YM-50] Remove the inner unit (the solution in the inner unit is not necessary), and recover the solution in the outer unit into a microtube (1.5 or 2.0 mL) using a micropipette, by recording the volume of the sample. Record the sample volume in units of 10 μL. If the sample volume exceeds 1 mL, store only 1 mL as a concentrated sample and discard the remaining sample.
15. [Amicon Ultra-15] Centrifuge Amicon Ultra-15 (5,000 · *g*, 15 min, 4°C) with the inner unit attached to the outer unit.
16. [Amicon Ultra-15] Recover the remaining sample in the inner unit into a microtube (1.5 or 2.0 mL) using a micropipette, by recording the sample volume. Record the sample volume in units of 10 μL. If the sample volume exceeds 1 mL, store only 1 mL as a concentrated sample and discard the remaining sample. Note that the total volume of the concentrated sample should be recorded because it is necessary for calculating the concentration.
    If no further RNA extraction is performed, store the virus concentrated sample at -20°C or lower.

(3) Ultrafiltration membrane method

The ultrafiltration membrane method concentrates virus particles by a "sieving effect" using an ultrafiltration membrane with a molecular weight cut-off of 10 to 100 kDa. The ultrafiltration method has been used for the detection of SARS-CoV-2 in wastewater in the Netherlands[10] and the U.S.[11], and has been successful in detecting the virus. The advantage of this method is that it is relatively simple to operate because it uses commercialized ultrafiltration membrane units (e.g.,

[10] Medema et al. (2020) Presence of SARS-Coronavirus-2 RNA in sewage and correlation with reported COVID-19 prevalence in the early stage of the epidemic in The Netherlands. Environ. Sci. Technol. Lett. 7, 511–516.

[11] Sherchan et al. (2020) First detection of SARS-CoV-2 RNA in wastewater in North America: A study in Louisiana, USA. Sci. Total Environ. 743, 140621.

Centricon Plus-70 from Merck Millipore). On the other hand, the cost of disposable ultrafiltration units is high (about 4,000 JPY/unit for Centricon Plus-70), and they tend to be suffered by inhibition in the subsequent molecular detection process because they simultaneously concentrate inhibitors in the wastewater. Here, we introduce a method using the Centricon Plus-70 from Merck Millipore.

a) Required equipment

- Filter holder for decompression filtration
- Aspirator
- Centrifuge with swinging rotor
- Tweezers

b) Reagents and instruments

- Centricon Plus-70 100 kDa (Merck Millipore, model number: UFC710008)
- Hydrophilic PTFE membrane (pore size: 0.2 μm)

c) Procedure

1. Filter the wastewater sample through a hydrophilic PTFE membrane (pore size: 0.2 μm). (However, since a significant proportion of SARS-CoV-2 in wastewater may be associated with suspended solids, the filtration (solid-liquid separation) step may be omitted unless the suspended solids interfere with the subsequent molecular detection process.)
2. Using an ultrafiltration membrane (Centricon Plus-70), centrifuge 120 mL of the PTFE membrane at 1,900 x g for 8 minutes twice, and then centrifuge it upside down at 800 x g for 2 minutes to collect the concentrate. This method enables concentration of 120 mL of wastewater sample to about 400-700 μL.
3. If RNA extraction is not performed immediately, store the concentrate at -20°C or lower.

3.2 Detection of viral RNA by quantitative PCR

In order to confirm that there is no contamination at a level that affects the detection results in the operations after RNA extraction, when RNA extraction is performed, add water for molecular biology (RNase free water, e.g., Water for molecular biology (Merck, model number: 95284-100ML)) in the same volume as the wastewater concentrate as a negative control, and perform RT-qPCR for SARS-CoV-2 and a surrogate virus for process control. If either or both of SARS-CoV-2 and the surrogate virus for process control are detected, contamination will have occurred in the operation after RNA extraction, and the results of SARS-CoV-2 RNA detection will not be obtained. The detection result of SARS-CoV-2 RNA should not be used. Even when this negative control is used, it is necessary to include a negative control for qPCR in order to determine whether or not there is contamination in the qPCR process.

3.2.1 RNA extraction

(1) Method using the nucleic acid extraction kit QIAamp Viral RNA Mini Kit

a) Necessary equipment

- Vortex mixer
- Microtube centrifuge (usable at 14,000 x g)

b) Reagents and instruments

- QIAamp Viral RNA Mini Kit (Qiagen, model number: 52906)
- TRIzol Reagent (Thermo Fisher Scientific, model number:15596026)
- Ethanol (96-100%)

c) Procedure

Dispense 140 µL wastewater concentrate and 560 µL Buffer AVL into a 1.5 mL tube, pulse vortex, and incubate at room temperature for 10 min. When the precipitate obtained by PEG precipitation is resuspended in TRIzol Reagent, mix 140 µL of the concentrated wastewater solution after pulse vortexing with 560 µL Buffer AVL, pulse vortex again, and incubate at room temperature for 10 minutes. Add 560 µL ethanol to the sample and pulse vortex. Centrifuge at 6,000 x g (8,000 rpm) for 1 minute and discard the collection tube containing the filtrate. After all the samples have been applied to the column, add 500 µL of washing buffer (Buffer AW1) to the column, centrifuge at 6,000 x g (8,000 rpm) for 1 minute, and discard the collection tube containing the filtrate. Add another 500 µL of wash buffer (Buffer AW2), centrifuge at 20,000 x g (14,000 rpm) for 3 minutes, and discard the collection tube containing the filtrate. Attach a new collection tube to avoid carryover, and centrifuge at maximum speed for 1 minute. Place the column in a new 1.5 mL tube, add 60 µL Buffer AVE, and centrifuge at 6,000 × g (8,000 rpm) for 1 minute. The filtrate obtained by this operation is the RNA extraction solution. If the RT reaction is not performed subsequently, store the RNA extract at a temperature below -20°C (or -80°C if possible).

(2) AGPC (acid guanidinium thiocyanate-phenol-chloroform extraction) method

The AGPC method is an RNA extraction and purification method developed for isolating total RNA from cells and tissues. TRIzol (Thermo Fisher Scientific) and TRI Reagent (Sigma Aldrich), which contain phenol and guanidine isothiocyanate, are widely used to perform the AGPC method. A slightly modified method, using TRIzol and a commercially available RNA extraction kit, has also been proposed. Here, we introduce the TRIzol + RNeasy Mini Kit method using TRIzol and the RNeasy Mini Kit.

Note that TRIZOL® LS contains phenol (toxic, corrosive) and guanidine isothiacyanate (irritant). The extraction should be performed with appropriate equipments.

1. [Addition of murine norovirus (MNV) as an option] Add 10 µL of MNV to 250 µL of PEG concentrate. As a control sample, prepare 250 µL of MilliQ water spiked with 10 µL of MNV.

2. Add 750 µL of TRIzol® LS Reagent and homogenize the mixture for about 30 seconds using Fast-prep 24. Vigorous vortex is also acceptable. Incubate at room temperature for 5 minutes.
3. Add 200 µL of chloroform and homogenize again for about 15 seconds. Incubate at room temperature for 2-15 minutes.
4. Centrifuge at 12,000 x g for 15 minutes at 4°C.
   The solution after centrifugation is separated into a dark red phenol-chloroform phase, an intermediate phase, and a colorless aqueous phase. The volume of the resulting aqueous phase will be approximately ~70% of the TRIZOL® LS reagent used.
5. While waiting for centrifugation, prepare a 1.5 mL tube and dispense 650 µL of 70% ethanol.
6. [Ethanol precipitation of RNA] Tilt the tube at 45 degrees and carefully collect the aqueous phase, and transfer it to the prepared tube containing 70% ethanol. Mix well by pipetting.
7. [Adsorption of RNA to silica membrane] Put 700 µL of the mixture into a spin column. Close the lid, and centrifuge at 8,000 × g for 15 seconds.
8. Discard the liquid in the collection tube, and add the remaining mixture (600-700 µL) to the spin column again. Close the lid, and centrifuge at 8,000 × g for 15 seconds.
9. [Washing] Discard the liquid in the collection tube, and add 700 µL of Buffer RW1 to the spin column. Close the lid and centrifuge at 8,000 x g for 15 seconds.
10. [Washing] Discard the liquid in the collection tube and add 500 µL of Buffer RPE to the spin column. Close the lid and centrifuge at 8,000 x g for 15 seconds.
11. [Washing] Discard the liquid in the collection tube, and add 500 µL of Buffer RPE to the spin column. Close the lid and centrifuge at 8,000 x g for 2 minutes.
12. [Drying] Discard the liquid in the collection tube and centrifuge at 12,000 × g for 1 minute.
13. [RNA extraction] Place the spin column to a newly prepared 1.5 mL tube and add 30 µL of RNase-free water to the silica membrane, incubate at room temperature for 1 min, and centrifuge at 8,000 × g for 1 min.
14. [RNA extraction] Open the lid of the spin column again, add 30 µL of RNase-free water, and perform incubation as in step 13. Then, centrifuge the column at 8,000 x g for 1 minute to obtain a total of 60 µL of RNA extract. If the RT reaction is not performed subsequently, store the RNA extract below -20°C (or -80°C if possible).

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3.2.2 Detection by RT-qPCR

a) Necessary equipment

- Real-time PCR system (e.g., ABI 7500, Light Cycler 480)
- PCR system (thermal cycler)

b) Reagents and instruments

- Reverse transcription kit (e.g., High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific))
- Real-time PCR master mix (e.g. QuantiTect Probe qPCR kit (QIAGEN))
- 96 well real-time PCR reaction plate
- 8-strip cap or plate seal
- Primers (**Table 6**)
- TaqMan probe (**Table 6**)
- RNase free water

Table 6 Sequences of primers and probes of qPCR assays for SARS-CoV-2[12]

Assay	Primers / Probe	Name	Sequence
CDC N1	Primer (+)	2019-nCoV_N1-F	GACCCCAAAATCAGCGAAAT
	Primer (-)	2019-nCoV_N1-R	TCTGGTTACTGCCAGTTGAATCTG
	Probe	2019-nCoV_N1-P	FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1
CDC N2	Primer (+)	2019-nCoV_N2-F	TTACAAACATTGGCCGCAAA
	Primer (-)	2019-nCoV_N2-R	GCGCGACATTCCGAAGAA
	Probe	2019-nCoV_N2-P	FAM-ACAATTTGCCCCCAGCGCTTCAG-BHQ1

In past domestic and international studies, the qPCR assays of CDC N1 and CDC N2 have been reported to be the most widely used systems for the detection of SARS-CoV-2 RNA from wastewater samples and to show high detection sensitivity, so we recommend the use of these qPCR assays. However, the use of other qPCR assays may be acceptable if a qPCR assay with superior detection sensitivity is announced with the progress of future research. In that case, the primers and probes will be changed, but the basic detection procedure will be the same as the method described in this manual.

c) Procedure

Reverse transcribed viral RNA into cDNA (complementary DNA) using reverse transcriptase, and then quantify the cDNA by real-time PCR. As examples, the reaction conditions for reverse transcription using Thermo Fisher Scientific's High Capacity cDNA Reverse Transcription Kit and real-time PCR using QIAGEN's QuantiTect® Probe PCR kit are shown below. The reaction conditions are shown below. For details, refer to the manual attached to the kit. All dispensing operations of reagents should be performed on ice.

Reverse transcription reaction

The RNA extract is mixed with the same volume of reverse transcription reaction solution and used for the reverse transcription reaction. cDNA synthesis is carried out at 25°C for 10 minutes, followed by 120 minutes at 37°C. The reverse transcriptase is inactivated by heating at 85°C for 5 minutes, and the reaction is cooled to 4°C. The prepared cDNA is stored at 4°C if it is to be used up within 24 hours, or cryopreserved if it is to be stored for a long time. The composition of the reaction solution is as shown in **Table 7**.

[12] CDC (2020) Research use only 2019-novel coronavirus (2019-nCoV) real-time RT-PCR primers and probes. (https://www.cdc.gov/coronavirus/2019-ncov/lab/rt-pcr-panel-primer-probes.html)

Table 7 Preparation of Reverse Transcription Reaction Solution

Sample (RNA extract)	10.0 µL
10× Reverse Transcription buffer	2.0 µL
25× dNTPs	0.8 µL
10× Random Primers	2.0 µL
MultiScribe™ Rverse Transcriptase (50 U/µL)	1.0 µL
RNase inhibitor (20 U/µL)	1.0 µL
H_2O	3.2 µL
Total	20.0 µL

Real-time PCR reaction

1. Make a 10-fold dilution of the positive control.
2. Prepare the reaction solution shown in **Table 8** using the sample from which the reverse transcription reaction was performed.

Table 8 Preparation of real-time PCR reaction solution

2×Master mix	12.5 µL
Forward primer (10 µM)	1.0 µL
Reverse primer (10 µM)	1.0 µL
TaqMan probe (10 µM)	0.75 µL
H_2O	7.25 µL
Template (cDNA)	2.5 µL
Total	25.0 µL

3. Add 22.5 µL of the reaction solution to each well of a 96-well plate, with a minimum of two wells per sample.
4. Add 2.5 µL each of positive control and sample cDNA. Add 2.5 µL H_2O to the wells of the negative control. In order to prevent contamination of the positive control, sealing or covering with aluminum foil is recommended.
5. Set the reaction conditions and start the reaction. However, since the optimal reaction conditions vary depending on the real-time PCR system, reagents, and reaction vessel used, be sure to check the detection sensitivity, etc. in advance. **Table 9** shows the reaction conditions when the QuantiTect® Probe PCR Kit from QIAGEN is used as the reagent and the Applied Biosystems 7500 or 7500 Fast real-time PCR system is used as the real-time PCR system.

Table 9 Example of reaction conditions for real-time PCR (Standard mode is selected)

95°C	15 min	
	↓	
95°C	3 sec	×45 cycles
55°C	30 sec (Data collection)	

6. The test is considered to be valid when the amplification curve of the positive control shows a rise and the amplification curve of the negative control does not show a rise.
7. To obtain the quantitative value of the viral gene copy number, construct a calibration curve using the number of cycles obtained for the positive control dilution column. When the normal logarithm of the positive control copy number is taken on the horizontal axis and the number of cycles on the vertical axis, the coefficient of determination of the calibration curve

should be approximately 0.99 or higher, and the slope should be around -3.3. When calculating the amplification efficiency, it should be 90% or more. Confirm that quantifiability is obtained when there are at least 10 copies per well (5 or more copies is more desirable).

8. If a rise in the amplification curve is observed in all wells of the same sample, the average number of cycles obtained in each well shall be used as the number of cycles for that sample. Even if the amplification curve rises in all wells of the same sample, if there is a difference of one cycle or more between the wells, it is recommended that the real-time PCR be redone for that sample.
9. When the amplification curve rises and does not rise among the wells of the same sample, it is recommended that the real-time PCR be redone for that sample. If the results do not change even after redoing the real-time PCR, it is highly likely that the virus-derived gene is present at a very low concentration in the sample, so the sample is judged to be merely positive without calculating the copy number using a calibration curve, and the Ct value is reported as a reference value.
10. If the number of cycles of the sample is less than the number of cycles of the positive control with the highest dilution ratio, the copy number should be calculated using a calibration curve. If the number of cycles of the sample is greater than the number of cycles of the positive control, the sample is judged to be merely positive without calculating the copy number, and the Ct value is reported as a reference value.
11. It is recommended that the PCR plates and tubes containing the samples that tested positive be stored frozen at -20°C or below, since sequencing analysis may be performed in the future to confirm whether the PCR amplification was specific or not. When storing the samples, care should be taken not to cause contamination by peeling off the caps or seals. Autoclaving of plates after PCR should be avoided because of the possibility of contamination.

4. SAFETY MANAGEMENT IN SAMPLE HANDLING

Presence of infectious SARS-CoV-2 in wastewater has not been reported so far[13]. Infectivity of SARS-CoV-2 is reportedly decayed faster than the viral RNA in wastewater[14]. However, a suspicious case of COVID-19 transmission via wastewater route is also reported by a cohort study in unsanitary area[15]. Independent of presence of SARS-CoV-2, wastewater may contain other pathogenic viruses and microbes, such as norovirus and toxigenic E. coli. To avoid these potential risks, it is recommended to take the safety measures which is the appropriate to the actual situations of sample handling and surrounding situations of the laboratory for analysis, as well as the epidemic situation of COVID-19 in the wastewater catchment.

There is no legal standards nor regulations in Japan for the handling of wastewater samples that may contain SARS-CoV-2 (as of December 2021). There is no legal standard for the handling of sewage samples that may contain SARS-CoV-2 (as of February 2021). Hence, it is recommended to follow the guidelines by relevant organization and industry associations if available. For safety management in sample collection, WHO guidelines [13] on "Water, sanitation, hygiene, and waste management for SARS-CoV-2, the virus that causes COVID-19" would be helpful as a reference. In Japan, guidelines for infection prevention in sewage operations[16,17] are published by the Japan Sewer Collection System Maintenance Association and Japan Sewage Facilities Management Association (Both guidelines are published in Japanese only).

Safety management is also recommended in sample handling for laboratory analysis according to the actual situations of the analytical laboratory and the epidemic situation of COVID-19 where the sample has been collected (**Figure 2**). Since the potential risk of SARS-CoV-2 in wastewater is probably lower than clinical specimen, the required level of safety management for handling of wastewater samples in a laboratory is equal to or lower than the required level for clinical specimen. In the highest-level safety management equal to the clinical testing, a sample should be handled wearing personal protective equipment (PPE) in a Class II biological safety cabinet. After analysis is completed, the samples and any containers and microtubes that may have been contaminated should be autoclaved before disposal. (reference, WHO guidelines.) On the other hand, a sample can be handled as usual when the potential risk of SARS-CoV-2 in wastewater is considered to be negligible compared to those of other pathogenic microorganisms in wastewater, regarding the epidemic situation of COVID-19 and the actual situation of sample collection and laboratory. The following sections introduce relevant guidelines from UNICEF/WHO and the National Institute of Infectious Diseases (NIID) as reference information for the development of safety control measures in laboratory for SARS-CoV-2 RNA detection in wastewater.

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[13] WHO (2020) Water, sanitation, hygiene, and waste management for SARS-CoV-2, the virus that causes COVID-19. https://www.who.int/publications/i/item/WHO-2019-nCoV-IPC-WASH-2020.4

[14] Bivins, A., Greaves, J., Fischer, R., Yinda, K.C., Ahmed, W., Kitajima, M., Munster, V.J., Bibby, K., 2020. Persistence of SARS-CoV-2 in Water and Wastewater. Environ. Sci. Technol. Lett. 7, 937–942.

[15] Yuan et al. (2020). Sewage as a possible transmission vehicle during a coronavirus disease 2019 outbreak in a densely populated community: Guangzhou, China, April 2020. Clinical Infectious Diseases, April, 1–14.

[16] Japan Sewer Collection System Maintenance Association (2021) Guidelines for countermeasures against novel coronavirus infection in sewerage pipeline management operations (in Japanese). https://www.jascoma.com/topics/2020/coronavirus_disease/images/20210914/information_1.pdf

[17] Japan Sewage Facilities Management Association (2021) Guidelines for Prevention of Novel Coronavirus Infection in Sewage Facility Operation and Management (in Japanese) https://www.gesui-kanrikyo.or.jp/pdf/news_2021102901.pdf

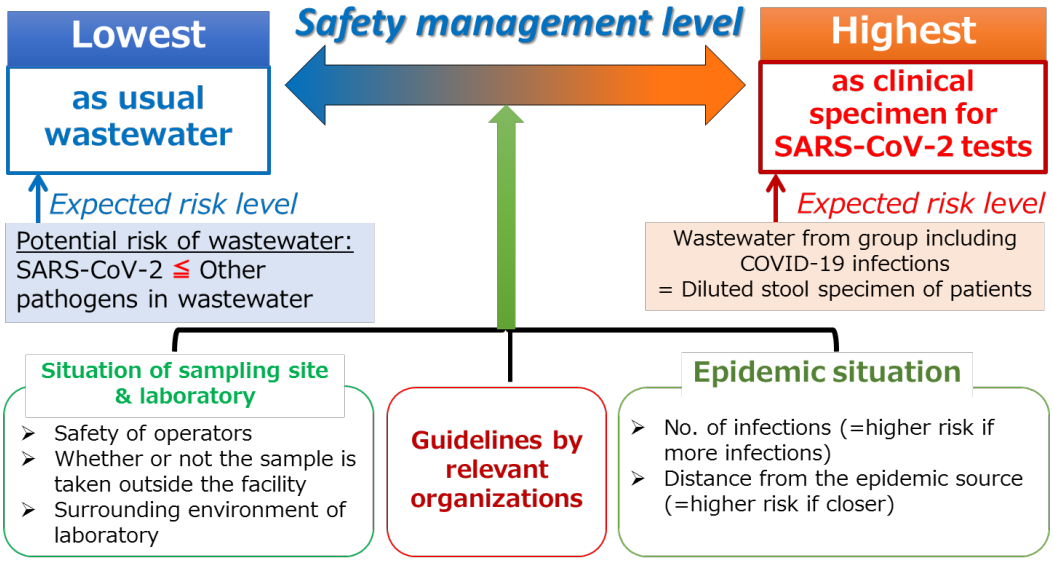

Figure 2 Approach to determine safety management in handling wastewater samples that may contain SARS-CoV-2. It is suggested to take flexible safety management measures according to the situation of the sampling site, COVID-19 epidemic in the target community, and the guidelines of related organizations.

4.1 Handling of wastewater samples

The purpose of personal protective equipment (PPE) is to prevent exposure to wastewater aerosols and to prevent its intrusion into uncontaminated areas. It is important to take off the PPE before entering the uncontaminated area. The appropriate PPE should be selected according to the potential risk of infection based on the epidemic situation of COVID-19 in the wastewater catchment and the location of the sampling site. In general, higher risk is expected when more infections are present in the target population and/or when the sampling site is closer to the epidemic sources. The potential risk of treated wastewater is lower than that of untreated wastewater.

For handling of untreated wastewater under COVID-19 outbreak situations, Japan Sewer Collection System Maintenance Association and Japan Sewage Facilities Management Association recommend the following safety management in their guidelines.

- Wearing of masks and gloves is recommended during on-site work.
- When performing work that may involve direct contact with untreated wastewater, ye protection wear (e.g. safety goggles) is encouraged to prevent direct exposure to splash on their eyes. Wearing the personal protective equipment (e.g. face shield and protective clothing) is encouraged upon necessity.
- Clothing and equipment that have been contacted untreated wastewater should be washed and disinfected.

The UNICEF/WHO Interim Guidance on Water, sanitation, hygiene, and waste management for SARS-CoV-2, the virus that causes COVID-19 (July 29, 2020) recommends the following

> For personnel working with untreated sewage for which there are considerable infectious risks, standard PPE should be worn (protective outerwear, heavy-duty gloves, boots, masks, goggles or a face shield). It should be worn at all times when handling or transporting excreta offsite, and great care should be taken to avoid the splashing and release of droplets. For sanitation workers, this includes pumping out tanks or unloading pumper trucks. After handling the waste and once there is no risk of further exposure,

> individuals should safely remove their PPE and perform hand hygiene before entering the transport vehicle. Soiled PPE should be put in a sealed bag for safe laundering later (see Environmental cleaning and laundry). Workers should be properly trained in how to put on and remove PPE, so that these protective barriers are not breached. If PPE is not available or the PPE supply is limited, the frequency of correct hand hygiene should increase, and workers should keep at least 1m distance from suspected or confirmed cases.

The guideline also recommends the adequate manner for hand disinfections after contact with samples at potential risk for COVID-19. It recommended to perform hand disinfection before and after wearing PPE (personal protective equipment); e.g. when changing gloves, after contact with the environment around untreated wastewater. It also advise to practice disinfection of shoes by using a disinfectant mat, if possible.

> If hands are not visibly dirty, the preferred method is using an alcohol-based hand rub for 20-30 seconds using the appropriate technique. When hands are visibly dirty, they should be washed with soap and water for 40-60 seconds using the appropriate technique.

Wastewater samples spilled during the experiment in a laboratory should be wiped off with paper towel after spraying sufficient amount of disinfection ethanol. To ensure the disinfection, 5% bleach solution can be also used. Sample containers that may have contacted with the sample (including paper towels used to wipe off the sample) should be autoclaved before disposal.

4.2 Safety management in laboratory work for wastewater analysis

When the wastewater sample has potentially high risk from the situation of sampling site and COVID-19 outbreak, the highest level of safety is ensured by applying the safety management as strict as clinical specimen for COVID-19 testing. For the handling of medical specimens that may contain SARS-CoV-2, the provisional rules developed by the National Institute of Infectious Diseases (NIID) are widely used in Japan. (Note that this guideline was developed for handling of clinical specimen including nasopharyngeal swab samples, not for wastewater samples. Since the potential risk of wastewater is smaller than that of clinical specimens, handling of wastewater samples does not require more stringent safety control than this guideline.)

The National Institute of Infectious Diseases (NIID) has established the following internal rules (dated February 21, 2020) [18].

> 1. The pathogen of novel coronavirus 2019-nCoV will be handled as BSL3/ABSL3.
> 2. Clinical specimens derived from patients suspected infection with novel coronavirus 2019-nCoV will be handled as BSL2.

Accordingly, the 2019-nCoV Detection Manual (Ver. 2.9)[19] by the National Institute of Infectious Diseases (NIID) describes about operational precautions as below.

> Specimens should be handled ... in a biosafety cabinet in the BSL2 experimental facility. Personal protective equipment (PPE) such as disposable gowns, gloves (double), masks

[18] National Institute of Infectious Diseases (2020) Handling of the new coronavirus SARS-CoV-2 in the National Institute of Infectious Diseases. https://www.niid.go.jp/niid/ja/byougen-kanri/9367-n-cov-bio.html

[19] National Institute of Infectious Diseases (2020) 2019-nCoV Detection Manual (Ver. 2.9) https://www.niid.go.jp/niid/images/lab-manual/2019-nCoV20200318v2.pdf

(surgical masks are acceptable), and caps should be worn during sample handling. Before opening the microtube lid, centrifuge first and use a tube opener to prevent the generation of aerosols.

The National Institute of Infectious Diseases (NIID) sample handling precautions are in accordance with the WHO post-outbreak biosafety guidelines for handling of SARS-CoV specimens and cultures (December 18, 2003)[20]. The guideline stipulates about handling of specimens suspected of containing SARS coronavirus as below.

<Handlings requiring infection prevention measures>

- Aliquoting and/or diluting specimens
- Inoculation of bacterial or mycological culture media
- Performance of diagnostic tests that do not involve propaganda of viral agents in vitro or in vivo
- Nucleic acid extraction procedures involving untreated specimens
- Preparation and chemical- or heat-fixing of smears for microscopic analysis

<Infection prevention measures to be implemented>

- Any procedure that may generate aerosols or droplets should be performed in a biological safety cabinet (e.g., sonication, vortexing).
- Laboratory workers should wear protective equipment, including disposable gloves, solid-front or wrap-around gowns, scrub suits, or coveralls with sleeves that fully cover the forearms, head covering and, where appropriate, shoe covers or dedicated shoes, eye protection and a surgical mask, or full-face shield, because of the risk of creating aerosols or droplets exposure when performing specific manipulations.
- Centrifugation of specimens should be performed using sealed centrifuge rotors or sample cups. These rotors or cups should be unloaded in a biological safety cabinet.
- Work surfaces and equipment should be decontaminated after specimens are processed. Standard decontamination agents that are effective against enveloped viruses should be sufficient if used according to the manufacturer's recommendations. Generally, 5% bleach solutions are appropriate for dealing with biohazardous spillage. More information on disinfection and sterilization is provided in the WHO Laboratory Biosafety Manual, 3rd revised edition.
- Biological waste contaminated with suspect or confirmed SARS specimens, or with SARS-CoV, should be treated as outlined in the WHO Laboratory Biosafety Manual, 3rd revised edition before disposal.
- When a procedure or process cannot be conducted within a biological safety cabinet, then appropriate combinations of personal protective equipment (e.g. respirators, face shields) and physical containment devices (e.g. centrifuge safety cups or sealed rotors) must be used.

Clinical specimen laboratories are stipulated to be sterilized and disposed as soon as possible after the pathogen is detected. In the case of wastewater samples, it is recommended to autoclave and dispose the sample after SARS-CoV-2 is detected if it is not planned to be used further.

[20] WHO (2003) post-outbreak biosafety guidelines for handling of SARS-CoV specimens and cultures. https://www.who.int/publications/m/item/who-post-outbreak-biosafety-guidelines-for-handling-of-sars-cov-specimens-and-cultures

4.3 Other reference information on pathogen management

- ○ Ministry of Health, Labour and Welfare: Regulations for the Control of Specified Pathogens, etc. under the Infectious Diseases Control Law
  https://www.mhlw.go.jp/stf/seisakunitsuite/bunya/kenkou_iryou/kekkaku-kansenshou17/03.html
- ○ National Institute of Infectious Diseases: Packaging containers for transporting pathogens - Basic triple packaging configuration
  https://www.niid.go.jp/niid/ja/from-biosafe/947-youkisb.html