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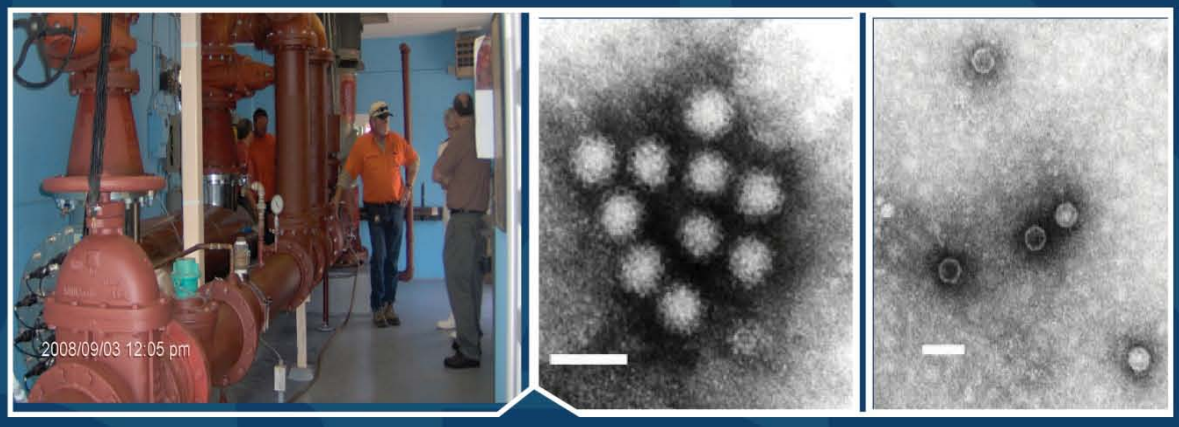
Method 1615
Measurement of Enterovirus and Norovirus Occurrence in Water by Culture and RT-qPCR



Office of Research and Development
National Exposure Research Laboratory, Cincinnati, OH

Cover:

Left picture: Prairie Du Sac, WI Pump house, courtesy of Dr. Mark Borchardt

Middle picture: norovirus, courtesy of Fred P. Williams; Bar = 50 nanometers

Right picture: poliovirus, courtesy of Fred P. Williams; Bar = 50 nanometers

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Method 1615

Measurement of Enterovirus and Norovirus Occurrence in Water by Culture and RT-qPCR

Version 1.1

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Disclaimer

This method has been reviewed by the U.S. Environmental Protection Agency (EPA)'s Office of Research and Development (ORD) and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use. The initial intended use of Method 1615 is to support the nationwide monitoring of enteroviruses and noroviruses under the Unregulated Contaminant Monitoring Regulation (UCMR). The method may however, be adopted in the future for other Safe Drinking Water Act and Clean Water Act purposes.

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ABBREVIATIONS

ACS	American Chemical Society
BGM	Buffalo Green Monkey kidney cells
BSA	Bovine serum albumin
Cat. No.	Catalog number
CCL	Contaminant Candidate List
cDNA	Complementary DNA
CL	Confidence limit
Ct	Cycle threshold
Cp	Crossing point
Cq	Quantitative cycle
CPE	Cytopathic effect
CV	Check valve; Coefficient of variation
D	Volume of original water sample assayed
dH_2O	Deionized or distilled reagent grade water
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotides
DTT	Dithiothreitol
EPA	United States Environmental Protection Agency
EV	Enteroviruses belonging to the genus, *Enterovirus*
FCSV	Final concentrated sample volume
GC	Genome copy
GHT	Garden hose threads
HGV	Hepatitis G virus
ICR	Information Collection Rule
ID	Inner diameter
IV	Inoculum Volume
LIMS	Laboratory Information Management System
LPDE	Low-density polyethylene
MEM	Minimum essential medium
MPN	Most probable number
MSDS	Material Safety Data Sheet
MWCO	Molecular weight cut off
Negative FCSV	Final concentrated sample volume from a negative QC sample
NoV GI	Genogroup I noroviruses belonging to the genus, *Norovirus*
NoV GII	Genogroup II noroviruses belonging to the genus, *Norovirus*
NPT	National pipe thread
NTC	No template control
NTU	Nephelometric Turbidity Units
ORD	Office of Research and Development
OSHA	Occupational Safety and Health Administration
OW	Office of Water
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Performance evaluation
PFU	Plaque forming unit

PSI	Pounds per square inch (15 psi = 1.034 bar)
PTFE	Polytetrafluoroethylene
QA	Quality assurance
QC	Quality control
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RPM	Revolutions per minute
RT	Reverse transcription
RT-PCR	Reverse transcription-polymerase chain reaction
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
S	Assay sample volume
SD	Standard deviation
SOP	Standard operating procedure
TCVA	Total culturable virus assay
TSV	Total sample volume
U.S.	United States

1.0 SCOPE AND APPLICATION

1.1 Background

1.1.1 EPA Method 1615 provides culture and molecular procedures for detecting human enteroviruses, human noroviruses, and mammalian orthoreoviruses (culture procedure only) in water (Table 1). The cell culture procedure detects enterovirus and orthoreovirus species that are capable of infecting and producing cytopathic effects (CPE) in the Buffalo Green Monkey kidney (BGM) cell line (18.16, 18.19). Although this cell line is considered a "gold standard" for detection of infectious waterborne viruses, noroviruses and a number of enteroviruses do not replicate in BGM cells. There is no established cell line for detection of infectious human noroviruses, but a prototype research method is under development (18.42, 18.43). The molecular procedure incorporated into EPA Method 1615 detects the noroviruses and enteroviruses shown in Table 1, including those enteroviruses that do not replicate on BGM cells.

1.1.2 Enteroviruses and noroviruses are enteric viruses that replicate within the gastrointestinal tract and are spread through the fecal-oral route. They cause a variety of waterborne infections through exposure to contaminated drinking and recreational waters. Infections may be asymptomatic or result in mild gastroenteritis, febrile illness, or respiratory symptoms. They can also cause a variety of serious diseases such as aseptic meningitis; encephalitis; flaccid paralysis; hand, foot and mouth disease; hemorrhagic conjunctivitis; myocarditis; neonatal sepsis-like disease; or severe gastroenteritis (18.3, 18.20, 18.21, 18.26, 18.38). Enteroviruses and noroviruses have not only been found in drinking and recreational waters, but have also caused waterborne disease outbreaks (18.4, 18.5, 18.7, 18.18, 18.28, 18.30, 18.35, 18.46). Due to public health concerns, these viruses are on EPA's Contaminant Candidate List (CCL) 3 (http://www.epa.gov/safewater/ccl/ccl3.html). The *Mammalian orthoreovirus* species is not associated with any known waterborne outbreaks and does not usually cause disease in humans (18.13, 18.15). If desired, orthoreoviruses can be assayed using the molecular method found in Fout *et al*. (18.18).

1.1.3 Molecular procedures, such as polymerase chain reaction (PCR) and reverse transcription-PCR (RT-PCR), provide the flexibility to detect all waterborne human enteric viruses for which genome sequence data is available (18.18). The advent of real time quantitative PCR (qPCR) has resulted in additional advantages over conventional PCR in that quantitative results can be obtained in a very short time (18.22). These molecular methods have been widely used to detect viruses in environmental waters (18.7, 18.8, 18.23, 18.30, 18.31, 18.36, 18.44). Despite the advantages, molecular techniques are subject to three main limitations. First, PCR methods assay smaller volumes than culture methods, resulting in lower detection limits. Second, these methods are

sensitive to inhibitors that are present in some environmental samples; to address this problem, controls are used to determine whether negative results are true negative or false negative values. Finally, molecular methods do not distinguish between infectious and noninfectious viruses; therefore, a positive PCR assay for a particular pathogen in drinking water indicates the presence of viral nucleic acid, and does not directly address issues of public health. Research is ongoing on several promising approaches to detect infectious viruses (18.29, 18.37). PCR is still a useful public health tool in spite of these problems. Because there is a strong relationship between indicator measurements by qPCR and health effects in recreational waters, EPA is considering using qPCR to set new criteria for monitoring recreational beaches (18.45). At the very least, positive PCR virus findings provide a warning of possible contamination issues, but recent studies have also indicated a direct relationship between health effects and positive reverse transcription-quantitative polymerase chain reaction (RT-qPCR) findings for human viruses in groundwaters (Borchardt et al., manuscript in preparation).

1.1.4 Development of the ICR Total Culturable Virus Assay – In the 1990s, EPA issued an Information Collection Rule (ICR; Federal Register 61:24353-24388) that required all drinking water utilities serving a population over 100,000 to monitor their source water for viruses monthly for a period of 18 months. The Rule also required that systems finding greater than one infectious enteric virus particle per liter of source water to monitor their finished water on a monthly basis. One of the purposes of the Rule was to obtain national data on virus levels in source waters to determine the adequacy of treatment requirements. To support the Rule, a virus monitoring protocol was developed by virologists at the EPA and modified to reflect consensus agreements from the scientific community and public comments to the draft rule (18.19). This standard ICR Total Culturable Virus method, along with quality assurance and laboratory approval procedures (http://www.epa.gov/microbes/icrmicro.pdf), was incorporated into the ICR by reference. The results of the ICR survey indicated that culturable viruses were present in 24% of source waters throughout the nation. Since the end of the ICR, the ICR Total Culturable Virus method has continued to be used in the U.S. and in international settings for the detection of culturable viruses in surface, ground, and treated waters (18.14, 18.27, 18.40), but the high cost of collecting and analyzing virus samples has limited the method's widespread use.

1.1.5 Development of Method 1615 – In the past few years, an alternative sampling protocol that significantly reduces the cost of sampling has been found to be equivalent in performance to the ICR method (18.25). Method 1615 is a modification of the ICR protocol. It incorporates the alternative sampling procedure and reduces the number of cell culture replicates required by the ICR protocol. It also includes a molecular procedure that is a modification of a method used to survey groundwaters for enteric viruses in Wisconsin (18.9, 18.30).

1.2 Method Constraints

1.2.1 This method is for use by analysts skilled in virus concentration, elution, cell culture, and molecular techniques.

1.2.2 Analysts must not deviate from any of the procedures described in this method if the data are being generated to fulfill EPA regulatory requirements. For example, alternative procedures for elution, secondary and tertiary concentration, and analyses by culture and RT-qPCR must not be used without prior approval by EPA.

2.0 SUMMARY OF METHOD

Viruses that may be present in environmental or finished drinking waters are concentrated by passage through a electropositive filter. Viruses are eluted from the filter with a beef extract reagent and concentrated using organic flocculation. A portion of the concentrated eluate is then inoculated onto replicate flasks of BGM cells to measure infectious viruses. Cultures are examined for the development of cytopathic effects for two weeks and then re-passaged onto fresh cultures for confirmation. Virus concentration in each test sample is calculated in terms of the most probable number (MPN) of infectious units per liter using EPA's MPN calculator. For molecular assays, the concentrated eluate is further concentrated by centrifugal ultrafiltration. The viral ribonucleic acid (RNA) is extracted from the concentrate and tested for enterovirus and norovirus RNA using RT-qPCR. Virus concentrations for the molecular assay are calculated in terms of genomic copies of viral RNA per liter based upon a standard curve.

3.0 DEFINITIONS

3.1 Analysis batch – All virus test samples processed by an analyst within one week shall be considered a "batch"; a week is defined as a 7-day period. Each test sample result must be associated with an unique batch number.

3.2 Buffalo Green Monkey kidney (BGM) cells – This is a stable cell line of monkey kidney cells that were originally developed at the University of Buffalo for clinical isolation of enteroviruses and later adapted for use in detecting infectious viruses in environmental samples (18.16). BGM cells form a monolayer of cells when propagated in tissue culture vessels. Figure 1 is a micrograph of uninfected BGM cells growing as a monolayer.

3.3 Contaminant Candidate List (CCL) – A list of chemicals and microbial agents under consideration for regulatory action by EPA. The current list may be obtained at: http://water.epa.gov/scitech/drinkingwater/dws/ccl/.

3.4 Cytopathic effect (CPE) – The degeneration of cells caused by virus replication. It often involves the complete disintegration of cells but also may be identified through changes in cell morphology. However, care must be taken in using changes in cell morphology as evidence of CPE, because uninfected BGM cells change morphology during mitosis. True CPE is always progressive and can be rated on a 0–4 scale, with the values 0, 1, 2, 3, and 4 indicating that 0% (Figure

1), 25% (Figure 2), 50%, 75%, and 100% of the monolayer is showing CPE, respectively. Additional examples of CPE can be found in Malherbe and Strickland-Cholmley (18.32).

3.5 **Cytotoxicity** – The development of CPE from toxic components in the water matrix. Cytotoxicity can be distinguished from viral CPE by its early development after test sample inoculation or by the failure to observe CPE in the second passage required by this method. Unlike viral CPE, which begins as small clusters of killed cells (see Figure 2) after two or more days of incubation, cytotoxicity usually develops uniformly in all inoculated cell culture replicates or in non-uniform areas of cell disintegration within 24 hours of inoculation.

3.6 **Detection limit** – The number of virus particles or genome copy numbers that can be detected in a given volume by a method with 95% confidence.

3.7 **Enteric viruses** – Viruses that primarily infect and replicate in the gastrointestinal tract are known as enteric viruses. These include enteroviruses, noroviruses, rotaviruses, hepatitis A virus, adenoviruses, and reoviruses, among others. Enteric viruses can be present in human and animal feces, which can contaminate recreational and drinking water sources.

3.8 **Enterovirus** – Enteroviruses are a genus in the *Picornaviridae* family. These viruses are among the most common viruses infecting humans worldwide. Enteroviruses are small (approximately 30 nm), nonenveloped, single-stranded, positive sense RNA viruses with an icosahedral capsid. Traditionally, human enterovirus serotypes have been classified into echoviruses, coxsackieviruses group A and B, and polioviruses. Current taxonomy based on molecular typing divides human enteroviruses into four species, *Human enterovirus A, B, C,* and *D*.

3.9 **Field sample** – Any surface, ground, or drinking water sample analyzed by this method.

3.10 **Inoculation** – The placement of concentrated test samples onto a monolayer of cells in a culture vessel for growing or replication of viruses in the cells.

3.11 **Material Safety Data Sheets (MSDS)** – Sheets containing written information provided by vendors concerning a chemical's toxicity, health hazards, physical properties, fire, and reactivity data, including storage, spill, and handling precautions.

3.12 **Matrix spike** – A field sample containing Sabin poliovirus 3 at a known concentration. The matrix spike provides a measure of overall method performance.

3.13 **Monolayer** – A single confluent layer of cells covering the bottom of a tissue culture dish or flask (Figure 1).

3.14 **Norovirus** – Noroviruses constitute a genus in the *Caliciviridae* family. The genus is divided into five genogroups (GI-GV) and 29 genetic clusters (18.47). Noroviruses are recognized as a leading cause of non-bacterial gastroenteritis in humans. Noroviruses are small (approximately 27 nm) and the genome consists of a positive sense, single-stranded RNA in a nonenveloped icosahedral capsid. Due to the absence of a standardized and validated infectivity assay for human noroviruses, the presence of noroviruses in environmental waters must be measured using molecular methods.

3.15 **Performance evaluation sample (PE)** – A test sample containing Sabin poliovirus type 3 at a concentration unknown to analysts. The purpose of the PE sample is to demonstrate on-going analyst approval/on-going demonstration of capability (see Section 8.3.1.3).

3.16 **Performance test sample (PT)** – A test sample containing Sabin poliovirus type 3 at a concentration unknown to analysts. The purpose of the PT sample is to demonstrate initial analyst approval/initial demonstration of capability (see Section 8.3.1.2).

3.17 **Quality control sample (QC)** – This is a test sample containing Sabin poliovirus type 3 at a concentration known to the analysts. The purpose of the QC sample is to give laboratories a standard test sample for training new analysts and to give EPA and laboratory quality assurance officials a tool to evaluate method performance for all laboratory analysts.

3.18 **Quantitative cycle (Cq) [also called cycle threshold (Ct) or crossing point (Cp)]** – The cycle at which the fluorescence of a quantitative PCR assay crosses the threshold that defines a positive reaction or at which the second derivative maximum is reached (18.10, 18.11).

3.19 **Quantitative polymerase chain reaction (qPCR)** – This is a procedure for quantitatively detecting the levels of specific deoxyribonucleic acid (DNA) in a test sample.

3.20 **Reagent water** – This is deionized or distilled reagent grade water (dH_2O) with a resistivity greater than 1 Siemens per meter (S/m; i.e., 1 megohms-cm at 25 ºC). If available, reagent grade water with a resistivity greater than 0.1 S/m (10 megohms-cm) is preferred (18.1).

3.21 **Reverse transcription-qPCR (RT-qPCR)** – This is a procedure for quantitatively detecting the levels of specific RNA (e.g., viral) in a test sample following reverse transcription (RT; e.g., the synthesis of complementary DNA [cDNA] from RNA).

3.22 **Standard operating procedure** – A set of written instructions that document a routine or repetitive activity followed by an organization. The development and use of SOPs are an integral part of a successful quality system as they provide individuals with the necessary information to perform a job properly, and facilitate

consistency in the quality and integrity of data. EPA guidance on developing SOPs can be obtained at http://www.epa.gov/quality/qs-docs/g6-final.pdf**.**

3.23 Test sample – Any sample that is analyzed by this method, including field samples, matrix spikes, quality controls, performance test samples, and performance evaluation samples.

4.0 INTERFERENCES

4.1 Reagents

To minimize cross contamination, Analytical Reagent or American Chemical Society (ACS)-grade chemicals (unless specified otherwise) and reagent water should be used to prepare all media and reagents. It is recommended that water, media, and other reagent solutions be purchased from commercial sources and that tissue culture grade water be used for preparation of tissue culture media not purchased in liquid form.

4.2 Matrix Interference

4.2.1 Matrix interferences may lead to false negative results and are caused by colloidal, suspended, or dissolved substances that are present in the water. Matrix interference can vary across different water sources and even across time in the same source.

4.2.2 Matrix interference due to colloidal or suspended solids may reduce the water volume that can be passed through the positively charged filters used in this method. Prefilters (Item 6.1.6) or more than one electropositive filter must be used to overcome this type of interference.

4.2.3 Matrix interference may be identified by its effects on the culture or molecular assays. This may be expressed as the development of cytotoxicity in culture assays and or by inhibition in molecular assays.

4.3 Other Interference

4.3.1 Failure to dechlorinate treated tap water test samples during sampling or prolonged exposure to ambient temperatures during test sample transportation or in the laboratory can lead to virus loss.

4.3.2 Inadequate disinfection of the sampling apparatus and contamination of reagents and supplies can lead to test sample contamination. Inadequate disinfection of the sampling apparatus is identified using negative QC samples/equipment blanks (Section 8.4.1).

4.3.3 Inadequate physical separation and controlled workflow may lead to PCR interference due to false positive results from contamination. EPA's guidance for processing and handling environmental samples and quality controls must be followed to minimize this interference (18.41).

5.0 SAFETY

5.1 Safety Plan

5.1.1 The biohazard associated with, and the risk of infection from, human enteric viruses is high in this method because potentially infectious viruses are handled.

5.1.2 This method does not purport to address all the safety issues associated with its use. Each laboratory is responsible for establishing a safety plan that addresses appropriate safety and health practices prior to using this method.

5.1.3 Laboratory staff must know and observe the safety procedures required in a microbiology laboratory that handles pathogenic organisms while preparing, using, and disposing of test sample concentrates, reagents, and materials and while operating sterilization equipment. Minimum requirements have been published by the U.S. Department of Health and Human Services (18.2).

5.2 Shipment of Field Samples

5.2.1 The field samples collected using this method may be shipped as non-infectious materials, unless they are known to contain virus or other infectious materials.

5.2.2 If field samples are known to contain infectious materials, laboratories are responsible for packaging and shipping them according to all Department of Transportation, Centers for Disease Control and Prevention, and State regulations.

5.3 Chemical Safety

Each laboratory is responsible for the safe handling of the chemicals used in this method. Occupational Safety and Health Administration (OSHA) laboratory standards can be found on line at: http://www.osha.gov/SLTC/laboratories/index.html#standards.

6.0 EQUIPMENT AND SUPPLIES

References to specific brands or catalog numbers are included in this method as examples only and do not imply endorsement of the product. These references do not preclude the use of other vendors, equipment, or supplies. However, equivalent method performance as described in Section 14.0 must be demonstrated for any substitutions.

All equipment should be cleaned according to the manufacturers' recommendations, and disposable supplies used wherever possible to reduce the possibility of cross contamination.

6.1 Sample Filtration Apparatus

Figure 3 shows the sample filtration apparatus, which has been modified from that given in Fout *et al*. (18.19) for use with the NanoCeram® electropositive cartridge filter (Item 6.1.2.4); the modification also increases the efficacy for disinfecting the apparatus.

The current configuration does not use a pressure regulator or pressure gauge, as these components are difficult to disinfect and subject to corrosion; however, laboratories are responsible for ensuring that water pressure at sampling sites does not exceed the pressure ratings of the cartridge housings used (125 psi for Item 6.1.2.2).

6.1.1 Intake Module

6.1.1.1 Backflow regulator (Watts Regulator Series 8 C Hose Connection Vacuum Breaker); this component is optional

6.1.1.2 Swivel female insert equipped with garden hose threads (GHT; United States Plastic, Cat. No. 63003)

6.1.1.3 ½-in tubing (Cole-Parmer, Cat. No. 06602-03) and hose clamps (Cole-Parmer, Cat. No. 06403-11)

6.1.1.4 ½-in hose barb quick disconnect body (Cole-Parmer Cat. No. 31307-11)

6.1.2 Cartridge Housing Module for NanoCeram Filters

6.1.2.1 ½-in NPT (M) quick disconnect insert (Cole-Parmer, Cat. No. 31307-31); connected to the inlet port of the cartridge housing

6.1.2.2 Cartridge housing (Argonide, Cat. No. H2.5-5)

6.1.2.3 ½-in NPT (M) quick disconnect body (Cole-Parmer, Cat. No. 31307-06); connected to the outlet port of the cartridge housing

6.1.2.4 5-in NanoCeram cartridge filter (Argonide, Cat. No. VS2.5-5) or 10-in 1MDS Virosorb cartridge filter (Cuno, Cat. No. 45144-01-1MDS)

NOTE: The use of the 1MDS filter requires that the sample filtration apparatus to be modified for use with a 10-in cartridge housing (not shown). See Figure VIII-1 in Fout *et al*. (18.19) for an example.

6.1.3 Discharge Module

6.1.3.1 ½-in NPT (M) quick disconnect insert (Cole-Parmer, Cat. No. 31307-31)

6.1.3.2 ½-in NPT (F) straight connector (Cole-Parmer, Cat. No. 06349-03)

6.1.3.3 Flow meter (Flow Technology, Cat. No. FT6-8NENWULEG-3)

6.1.3.4 Rate totalizer (Flow Technology, Cat. No. BR30-5-A-4)

6.1.3.5 ¾-in NPT (M) x ½-in NPT (M) reduction nipple (Cole-Parmer, Cat. No. 06349-87)

6.1.3.6 ¾-in NPT (F) bronze globe valve (Cole-Parmer, Cat. No. 98675-09)

6.1.3.7 ¾-in NPT (M) x GHT (M) fitting (United States Plastic, Cat. No. 63016)

6.1.3.8 Garden hose of sufficient length to reach a drain

NOTE: An appropriate sized hose connector and ½-in tubing can be substituted for item 6.1.3.7 and the garden hose.

6.1.4 Injector Module

NOTE: This module, prepared using the components below, should only be used when it is necessary to add sodium thiosulfate or HCl to water during sampling.

6.1.4.1 ⅜-in NPT (F) Tee fitting (Cole-Parmer, Cat. No. 06349-52)

6.1.4.2 ⅜-in NPT (M) quick disconnect insert (Cole-Parmer, Cat. No. 31307-30); attached to the left port of the Tee fitting

6.1.4.3 ⅜-in NPT (M) quick disconnect body (Cole-Parmer, Cat. No. 31307-05); attached to the right port of the Tee

6.1.4.4 ⅜-in NPT (M) x ¼-in NPT (M) male reducer (Cole-Parmer, Cat. No. 30623-42); connected to the top port of the Tee

6.1.4.5 ¼-in NPT (F) metallic check valve (CV; Cole-Parmer, Cat. No. 98676-00); connected to the male reducer

6.1.4.6 ¼-in NPT (M) x ¼-in tubing ID male pipe adaptor elbow (Cole-Parmer, Cat. No. 30622-97); connected to the inlet side of the check valve

6.1.4.7 15-gal chemical tank (Pulsafeeder, Cat. No. J63063) equipped with ¼-in tubing

NOTE: The container size can be adjusted to meet the anticipated need.

NOTE: This item is for injecting 2% sodium thiosulfate (Item 7.1.3) into water containing a disinfectant.

6.1.4.8 Metering pump (Pulsafeeder, Cat. No. XP004LAHT)

6.1.5 Double Injector Module

NOTE: This module, prepared using the components below, should only be used when it is necessary to add sodium thiosulfate and HCl to water during sampling.

6.1.5.1 ⅜-in NPT (F) Tee fitting (Cole-Parmer, Cat. No. 06349-52)

6.1.5.2 ⅜-in NPT (M) quick disconnect insert (Cole-Parmer, Cat. No. 31307-30); attached to the left port of the Tee fitting

6.1.5.3 ⅜-in NPT (M) quick disconnect body (Cole-Parmer, Cat. No. 31307-05); attached to the right port of the Tee

6.1.5.4 ⅜-in NPT (M) x ¼-in NPT (M) male reducer (Cole-Parmer, Cat. No. 30623-42); connected to the top port of the Tee

6.1.5.5 ¼-in NPT (F) Tee fitting (Cole-Parmer, Cat. No. 06349-51); connected to the male reducer (Item 6.1.5.4)

6.1.5.6 2-¼-in NPT (F) metallic check valves (CV; Cole-Parmer, Cat. No. 98676-00); connected to each remaining port on the small Tee fitting (Item 6.1.5.5)

6.1.5.7 2-¼-in NPT (M) x ¼-in tubing ID male pipe adaptor elbows (Cole-Parmer, Cat. No. 30622-97); connected to the inlet side of each check valve (Item 6.1.5.6)

6.1.5.8 2-chemical tanks (Item 6.1.4.7) and 2-metering pumps (Item 6.1.4.8)

6.1.6 Prefilter Module

NOTE: This module is for use with waters exceeding 20 NTU for the NanoCeram filter and 50 NTU for the 1MDS filter.

NOTE: The NanoCeram filter is more susceptible to clogging than the 1MDS filter; therefore, a prefilter module may be required for some matrices even when the turbidity is considerably lower than 20 NTU.

6.1.6.1 Prepare the prefilter cartridge housing as described for the cartridge housing module in Steps 6.1.2.1–6.1.2.3.

6.1.6.2 5-in 10-µm polypropylene prefilter cartridge (Parker Hannifin, Cat. No. M19R5A)

6.1.7 Assemble modules using thread tape (Item 6.2.1) on all connections.

6.1.7.1 Sterilize the intake prefilter housing, and cartridge housings with sodium hypochlorite as described in Section 15.2.4.

6.1.7.2 Using aseptic technique, add a sterile NanoCeram or 1MDS cartridge to the cartridge housing and, if needed, a presterilized polypropylene cartridge to the prefilter housing.

6.1.7.3 Cover the ends with sterile aluminum foil.

6.2 Other Equipment and Supplies for Sample Collection, Preservation, and Storage Procedure

6.2.1 PTFE thread tape (Cole-Parmer, Cat. No. 08270-34)

6.2.2 Peristaltic or chemical resistant pump, capable of pumping water at 4–10 L/min and appropriate connectors (for use where garden hose-type pressurized taps for the source or finished water to be monitored are unavailable and for QC samples).

NOTE: Follow the manufacturer's recommendations for pump priming.

6.2.3 1-L polypropylene wide-mouth bottles (Nalgene, Cat. No. 2104-0032)

6.2.4 Portable pH and temperature probe (Omega, Cat. No. PHH-830)

6.2.5 Portable turbidity meter (Omega, Cat. No. TRB-2020-E)

6.2.6 Portable chlorine (free and total), pocket colorimeter II test kit with reagents (Hach, Cat. No. 5870062).

6.2.7 Commercial ice packs (Cole-Parmer, Cat. No. 06345-20)

6.2.8 iButtons temperature data logger (Maxim, Cat. No. DS1921G), capable of reading temperatures from -40 to 85 °C

6.2.9 Lab-grade insulated container equipped with carrying strap (16 ¾ in x 16 ¾ in x 15 ⅝ in; Cole-Parmer, Cat. No. 03742-00 and 03742-30) or insulated storage and transport chest (Fisher Scientific, Cat. No. 11-676-12)

6.2.10 Aluminum foil (Fisher Scientific, Cat. No. S47271). Sterilize the foil squares as specified in Section 15.2.2.2.2.

6.2.11 Surgical gloves (Fisher Scientific, Cat. No. 19-058-800)

6.2.12 Waterproof marker (Fisher Scientific, Cat. No. 22-290546)

6.2.13 Closable bag (Uline, Cat. No. S-12283)

6.2.14 Closable bag (Fisher Scientific, Cat. No. S31798C)

6.2.15 Packing material: bubble wrap (U.S. Plastics, Cat. No. 50776) or roll paper (U.S. Plastics, Cat. No. 50502)

6.2.16 Packing tape (U.S. Plastics, Cat. No. 50083)

6.2.17 Graduated cylinder, 4-L or larger (e.g., Cole-Parmer, Cat. No. 06135-90)

6.3 Equipment and Supplies for Quality Assurance Measures

6.3.1 Full flow hose Y (DripWorks, Cat. No. HYFFBR), to allow a matrix spike and standard virus field sample to be collected simultaneously

6.3.2 Freezer capable of maintaining a temperature at or below -70 °C (Thermo Scientific, Cat. No. ULT2586-10HD-D), for storing QC stocks

6.3.3 Dispensing pressure vessel (Millipore, Cat. No. XX6700P10) or polypropylene container (Cole-Parmer, Cat. No. EW-06317-53)

6.3.4 Magnetic stirrer (Cole Parmer, Cat. No. EW-04671-82)

6.3.5 Magnetic stirring bar (Fisher Scientific, Cat. No. 14-513-68)

6.3.6 Standard filter apparatus (Item 6.1) with electropositive filter (Item 6.1.2.4) for QC samples

6.3.7 Collapsible 10-L LDPE cubitainer (Cole Parmer, Cat. No. 06100-30) for collecting matrix spike

6.3.8 Duplicate filter apparatus (Item 6.1) with electropositive filter (Item 6.1.2.4), for processing matrix spike

6.4 Equipment and Supplies for the Elution and Organic Flocculation Procedures

6.4.1 Refrigerator (Fisher Scientific, Cat. No. 13-986-152), set at 4±3 ºC, for storing filters prior to elution and eluates prior to further processing

6.4.2 Pressure source, such as laboratory positive pressure airline (equipped with oil filter), compressed nitrogen, peristaltic pump (e.g., Cole-Parmer, Cat. No. 07523-80), or self-priming pump (e.g., Cole-Parmer, Cat. No. 07036-10) and required tubing

6.4.3 Dispensing pressure vessels, 5- and 20-L capacity (Millipore, Cat. No. XX6700P05 and XX6700P20)

6.4.3.1 ⅜-in NPT (M) quick disconnect body (Cole-Parmer Cat. No. 31307-00)

6.4.3.2 Use appropriate fittings to add a quick disconnect body (Item 6.4.3.1) to the outlet of the dispensing pressure vessel

6.4.4 Elution inlet tubing

6.4.4.1 ½-in tubing (Cole-Parmer, Cat. No. 06602-03) and hose clamps (Cole-Parmer, Cat. No. 06403-11)

6.4.4.2 ½-in hose barb quick disconnect body (Cole-Parmer Cat. No. 31307-11)

6.4.4.3 ½-in hose barb quick disconnect insert (Cole-Parmer Cat. No. 31307-46)

NOTE: Connect the quick disconnect body (Item 6.4.4.2) to 1 end of the ½-in tubing and the quick disconnect insert (Item 6.4.4.3) to the other end using the hose clamps.

6.4.5 Elution outlet tubing

6.4.5.1 ½-in tubing (Cole-Parmer, Cat. No. 06602-03) and hose clamps (Cole-Parmer, Cat. No. 06403-11)

6.4.5.2 ½-in hose barb quick disconnect insert (Cole-Parmer Cat. No. 31307-46)

NOTE: Connect the quick disconnect insert (Item 6.4.5.2) to 1 end of the ½-in tubing using the hose clamps.

6.4.6 2-L glass or polypropylene beaker (Fisher Scientific, Cat. No. 02-591-41)

6.4.7 pH meter equipped with combination-type electrode, accuracy of at least 0.1 pH units

6.4.8 Magnetic stirrer and stir bars

6.4.9 Refrigerated centrifuge (e.g., Beckman Coulter, Cat. No. 367501)

6.4.9.1 Centrifuge rotors (e.g., Beckman Coulter, Cat. No. 339080 and 336380), with appropriate accessories

6.4.9.2 Screw-capped centrifuge bottles (Fisher Scientific Cat. No. 05-562-23 or 05-562-26), 250- or 1,000-mL capacity

NOTE: Each bottle must be rated for the relative centrifugal force used.

6.4.10 Orbital shaker (Fisher Scientific, Cat. No. 14-285-729), capable of 160 rpm

6.4.11 Sterilizing filter, 0.22-µm pore-size Acrodisc filter equipped with prefilter (VWR, Cat. No. 28143-295)

6.4.12 Sterilizing filter stack

NOTE: The sterilizing filter stack is optional, but should be used for test samples that are difficult to filter using Item 6.4.11.

6.4.12.1 Place a 0.22-µm pore-size membrane filter (Millipore, Cat. No. GSWP04700) on the bottom of a 47-mm disc filter holder (Millipore, Cat. No. SX0004700).

6.4.12.2 Place an AP15 prefilter (Millipore Cat. No. AP1504700) on top of the 0.22-µm filter and an AP20 prefilter (Millipore, Cat. No. AP2004700) on top of the AP15 prefilter.

6.4.12.3 Assemble the filter holder unit and sterilize as defined in Section 15.2.2.2.

NOTE: Disassemble the filter stack after each use to check the integrity of the 0.22-µm filter. Refilter any media filtered with a damaged stack using another sterile sterilizing filter stack.

6.4.13 50-mL syringe (Thomas Scientific, Cat. No. 8939N37)

6.4.14 Freezer (Thermo Scientific, Cat. No. ULT2586-10HD-D), capable of maintaining a temperature at or below -70 °C

6.4.15 Gauze sponge (Fisher Scientific, Cat. No. 22-415-469) soaked with 0.5% iodine (Item 7.6.4) or 0.525% sodium hypochlorite (Item 7.6.2), for cleaning spills

6.4.16 15-mL polypropylene tubes (Fisher Scientific, Cat. No. 05-539-5)

6.5 Equipment and Supplies for the Total Culturable Virus Assay

6.5.1 Incubator (Thomas Scientific, Cat. No. 1226T31), capable of maintaining the temperature of cell cultures at 36.5±1 °C

6.5.2 Biosafety cabinet (NuAir Laboratory Equipment Supply, Cat. No. Labgard 437 ES)

6.5.3 Tissue culture flasks, 25 cm^2 or 75 cm^2 (Sigma Aldrich, Cat. No. C6481 or C7231, respectively)

6.5.4 Indelible marker (Fisher Scientific, Cat. No. 22-290546)

6.5.5 Appropriate size pipettes and pipetters

6.5.6 Waterbath (Cole Parmer, Cat. No. 12418-60), capable of maintaining a temperature of 37 °C

6.5.7 Freezer (Thermo Scientific, Cat. No. ULT2586-10HD-D), capable of maintaining a temperature at or below -70 °C

6.5.8 Mechanical rocking platform (Daigger, Cat. No. EF4907G)

6.5.9 Sterilizing syringe filter, 0.2-µm (Corning, Cat. No. 431219)

6.5.10 Microcentrifuge (Eppendorf, Cat. No. 022620623), capable of 30,130 x *g*

6.5.11 EPA Most Probable Number Calculator (EPA, http://www.epa.gov/nerlcwww/mpn.html)

NOTE: The MPN program will run on Windows XP and later versions. It has been re-designed for calculation of both standard bacterial and viral MPN values. All entries are saved in a default database and can be viewed to check for data entry errors using the View History selection under the Tools menu. Each program run can also be saved into Word, Excel, or text files for transfer to lab notebooks or to Laboratory Information Management Systems.

6.6 Equipment and Supplies for the Enterovirus and Norovirus Molecular Assays

6.6.1 UV-Vis spectrophotometer (Thermo Scientific, Cat. No. NanoDrop ND-2000)

6.6.2 Vivaspin 20 centrifugal concentrator units, 30,000 MWCO (Sartorius-Stedim, Cat. No. VS2022)

NOTE: Other centrifugal concentrators with 30,000 MWCO may be substituted for this item, if equivalent recoveries are demonstrated.

6.6.3 50-mL polypropylene centrifuge tubes and multitube carrier (e.g., Beckman Coulter, Cat. No. 362213) for centrifuge (Item 6.4.9)

6.6.4 Microcentrifuge, capable of 30,130 x *g* (Fisher Scientific, Cat. No. 05-406-11)

6.6.5 1.5-mL microcentrifuge tubes equipped with snap caps (Fisher Scientific, Cat. No. 02-682-550)

6.6.6 Vortex mixer (Fisher Scientific, Cat. No. 02-216-100)

6.6.7 Dry bath incubator (Fisher Scientific Cat. No. 11-716-50Q)

6.6.8 Collection tubes, 2-mL (Qiagen, Cat. No. 19201)

6.6.9 Multichannel pipette (Rainin, Cat. No. L8-20)

6.6.10 Various pipettes (e.g., Rainin, Cat. No. PR-2, PR-10, PR-20, PR-200, PR-1000)

6.6.11 Various pipette tips (e.g., Rainin, Cat. No. RT-10F, RT-L10F, RT-20F, RT-200F, RT-1000F)

6.6.12 Reagent Reservoir (Fisher Scientific, Cat. No. 21-381-27E)

6.6.13 Mini-plate spinner (Labnet, Cat. No. C1000)

6.6.14 Thermal cycler (Applied Biosystems, Cat. No. 4314879)

6.6.15 Optical reaction plate (Applied Biosystems, Cat. No. 4314320) or PCR MicroAmp tubes (Applied Biosystems, Cat. No. N8010612)

6.6.16 Quantitative PCR thermal cycler (Applied Biosystems, Cat. No. 4351405)

6.6.17 0.2-µm sterilizing filter (Sigma-Aldrich, Cat. No. F-9768)

6.6.18 Freezers (VWR, Cat. No. 97043-346; Thermo Scientific, Cat. No. ULT2586-10HD-D), capable of maintaining temperatures of -20 °C and at or below -70 °C, respectively

NOTE: Storage of reagents at -20 °C must be done using manual defrost freezers.

6.6.19 Refrigerator (Fisher Scientific, Cat. No. 13-986-152), capable of maintaining a temperature of 4±3 °C

6.7 Equipment and Supplies for Sterilization Techniques

6.7.1 Autoclave, capable of maintaining a temperature of 121 °C and 15 psi (Steris Amsco® Lab Series), for sterilizing solutions and autoclavable laboratory ware and equipment

6.7.2 Dry heat oven, capable of maintaining a temperature of 170 °C (Binder, Cat. No. 9010-0164), for sterilizing glassware

6.7.3 Aluminum foil (Fisher Scientific, Cat. No. 01-213-100)

6.7.4 Kraft or roll paper (U.S. Plastics, Cat. No. 50083)

7.0 **REAGENTS, MEDIA, AND STANDARDS**

References to specific reagents, media, and standards brands or catalog numbers are included in this method as examples only and do not imply endorsement of the product. These references do not preclude the use of other vendors or other reagents, media, or standards. However, equivalent method performance as described in Section 14.0 must be demonstrated for any substitutions.

The amount of reagents, media, and standards prepared for each step of the method may be adjusted proportionally to the number of test samples to be analyzed.

NOTE: For any given section of this method only media, reagents, and standards that are not described in previous sections are listed.

7.1 Reagents for the Sample Collection, Preservation and Storage Procedure

7.1.1 Hype-Wipe (Fisher Scientific, Cat. No. 14-412-56)

7.1.2 0.12-, 1.2-, and 6-M hydrochloric acid (HCl)

7.1.2.1 Prepare 0.12-, 1.2-, and 6-M solutions by mixing 50, 100, or 50 mL of concentrated HCl with 4950, 900, or 50 mL of dH_2O, respectively.

NOTE: HCl at 37% concentration is about 12 M.

NOTE: To adjust the pH of reagents where the HCl concentration is not specified, use the higher concentration initially to reduce the volume of HCl required for pH adjustment and then switch to lower concentration as the pH approaches the target level.

7.1.2.2 Prepare solutions to be used for adjusting the pH of water samples at least 24 h before use.

NOTE: HCl solutions can be stored for several months at room temperature.

7.1.3 2% sodium thiosulfate ($Na_2S_2O_3$) pentahydrate

7.1.3.1 Prepare 2% thiosulfate by dissolving 1 kg of sodium thiosulfate pentahydrate in 49 L of sterile dH_2O.

NOTE: Sodium thiosulfate solutions may be stored for 6 months at room temperature.

7.2 Reagents for Quality Assurance Measures

7.2.1 HEPES (Sigma Aldrich, Cat. No. H4034)

7.2.2 QC stock

7.2.2.1 Prepare a stock of Sabin poliovirus type 3 containing 500±10 MPN/mL and store in aliquots containing about 1.1 mL at or below -70 °C.

NOTE: This stock may be prepared by the analytical laboratory or, if available, obtained from a contractor designated by the EPA or from other sources.

7.2.3 PT/PE stock

7.2.3.1 Prepare stocks of Sabin poliovirus type 3 with various levels between 300 and 5,000 MPN/mL and store in aliquots containing about 1.1 mL at or below -70°C. Several levels each of low (300-500 MPN/mL), medium (1,000-2,000 MPN/mL), and high (3,000 to 5,000 MPN/mL) stocks must be prepared.

NOTE: For studies not conducted by EPA, these stocks may be prepared by the analytical laboratory or obtained from other sources.

7.2.4 Matrix spike

7.2.4.1 Prepare a stock of Sabin poliovirus type 3 containing 1,000±50 MPN/ mL and store in aliquots containing about 1.1 mL at or below -70 °C.

NOTE: This stock may be prepared by the analytical laboratory or, if available, obtained from a contractor designated by the EPA or from another source.

7.2.5 0.1-M sodium hydroxide (NaOH)

7.2.5.1 Prepare a 0.1-M NaOH solution by dissolving 0.4 g of NaOH in a final volume of 100 mL of dH_2O, respectively.

NOTE: NaOH solutions may be stored for several months at room temperature.

7.3 Reagents for the Elution and Organic Flocculation Procedures

7.3.1 1.5% beef extract, pH 9.0

7.3.1.1 Prepare buffered 1.5% beef extract by dissolving 30 g of beef extract, desiccated powder (BD Bacto, Cat. No. 211520) and 7.5 g of glycine (final glycine concentration = 0.05 M) in 1.9 L of dH_2O.

7.3.1.2 Adjust the pH to 9.0 with 1- or 5-M NaOH and bring the final volume to 2 L with dH_2O.

7.3.1.3 Autoclave the beef extract solution at 121 °C, 15 psi for 15 min and use at room temperature.

NOTE: Beef extract solutions may be stored overnight at room temperature, for 1 week at 4 °C, or for longer periods at -20 °C.

7.3.1.4 Screen each new lot of beef extract before use to determine whether virus recoveries are adequate.

CAUTION: Desiccated beef extract lots show considerable variation in virus recovery.

7.3.1.4.1 Perform the screening by spiking 1 L of beef extract solution with 1 mL of a QC stock (Item 7.2.2).

7.3.1.4.2 Process the spiked sample according to the organic flocculation and total culturable virus assay procedures (Sections 11.0 and 12.0, respectively).

NOTE: The mean recovery of poliovirus for three trials should be greater than 50%.

7.3.2 1.5% beef extract, pH 7.0–7.5

7.3.2.1 Prepare 1.5% beef extract by dissolving 7.5 g of beef extract, desiccated powder and 1.88 g of glycine in 0.5 L of dH_2O.

7.3.2.2 Autoclave the beef extract solution at 121 °C, 15 psi for 15 min and use at room temperature.

NOTE: This beef extract solution may be stored for up to 6 months at room temperature, but must be discarded if there is evidence of microbial growth or any other change in appearance.

7.3.3 Antifoam (Sigma, Cat. No. A8311)

7.3.4 1- and 5-M sodium hydroxide (NaOH)

7.3.4.1 Prepare 1- and 5-M solutions by dissolving 4 or 20 g of NaOH in a final volume of 100 mL of dH_2O, respectively.

NOTE: NaOH solutions may be stored for several months at room temperature.

7.3.5 0.15-M sodium phosphate, pH 9.0

7.3.5.1 Prepare 0.15-M sodium phosphate by dissolving 40.2 g of sodium phosphate, dibasic ($Na_2HPO_4 \cdot 7H_2O$) in a final volume of 1 L dH_2O.

7.3.5.2 Adjust the pH to 9.0 with HCl, if necessary.

7.3.5.3 Autoclave at 121 °C, 15 psi for 15 min.

NOTE: Sodium phosphate solutions may be stored at room temperature for up to 12 months.

7.3.6 0.15-M sodium phosphate, pH 7.0–7.5

7.3.6.1 Prepare by dissolving 40.2 g of sodium phosphate, dibasic (Na_2HPO_4 C $7H_2O$) in a final volume of 1,000 mL dH_2O.

7.3.6.2 Adjust the pH to 7.0–7.5 with HCl.

7.3.6.3 Autoclave at 121 °C, 15 psi for 15 min.

NOTE: Sodium phosphate solutions may be stored at room temperature for up to 12 months.

7.4 Reagents for the Total Culturable Virus Assay

7.4.1 Cell culture media

7.4.1.1 Hank's balanced salt solution (Invitrogen, Cat. No. 14170-112)

7.4.1.2 Minimum essential medium (MEM) with Hanks' salts and L-glutamine (Sigma-Aldrich, Cat. No. M4642)

7.4.1.3 Leibovitz L-15 medium with L-glutamine (Sigma-Aldrich, Cat. No. L4386)

7.4.1.4 Sodium bicarbonate, 7.5% (Sigma Aldrich, Cat. No. S8761)

7.4.1.5 Fetal bovine serum, certified, heat-inactivated (Invitrogen, Cat. No. 10082-139)

7.4.1.6 Penicillin-Streptomycin (Invitrogen, Cat. No. 15140-122)

7.4.1.7 Fungizone (Invitrogen, Cat. No. 15290-018),

7.4.1.8 Prepare growth and maintenance medium as described in the most recent version of the EPA Manual of Methods for Virology, available at: http://www.epa.gov/microbes/about.html.

7.4.1.8.1 Briefly, growth medium consists of a 50/50 mixture of MEM (7.4.1.2) and L-15 medium (Item 7.4.1.3), 1 mL/L of 7.5% sodium bicarbonate (Item 7.4.1.4), 5–10 mL/L of penicillin-streptomycin (Item 7.4.1.6), 1 mL/L fungizone (Item 7.4.1.7), and 100 mL/L of fetal bovine serum (Item 7.4.1.5).

7.4.1.8.2 Briefly, maintenance medium consists of a 50/50 mixture of MEM (Item 7.4.1.2) and L-15 medium (Item 7.4.1.3), 1 mL/L of 7.5% sodium bicarbonate (Item 7.4.1.4), 5-10 mL/L of penicillin-streptomycin (7.4.1.6), 1 mL/L of fungizone (Item 7.4.1.7)and 20 mL/L of fetal bovine serum (Item 7.4.1.5).

NOTE: The amount of 7.5% sodium bicarbonate (Item 7.4.1.4) added is sufficient for incubation of cell cultures in non-CO_2 incubators. The amount should be reduced to 0.47 mL/L for use in CO_2 incubators.

7.4.2 BGM cell culture

7.4.2.1 Trypsin, 0.05% with EDTA (Invitrogen, Cat. No. 25300-062)

7.4.2.2 Trypan blue solution, 0.4% (Sigma-Aldrich, Cat. No. T8154)

7.4.2.3 BGM cells should be passaged and maintained using the standard procedures available in the most recent version of the EPA Manual of Methods for Virology (18.6), available at: http://www.epa.gov/microbes/about.html. Briefly, cells are passaged by removing them from confluent vessels using trypsin with EDTA (Item 7.4.2.1). A portion of the removed cells is stained with trypan blue (Item 7.4.2.2) and counted to obtain the fraction of live cells. Warm growth medium is added to the remaining cells and new vessels prepared using a split ratio of 1:3 to 1:4 based upon the live cell count.

NOTE: BGM cells from various sources and other standard tissue culture techniques and media may be used as long as analysts meet the acceptance criteria listed in Section 14.0.

NOTE: Cell cultures used for virus assay are generally found to be at their most sensitive level 3–6 days after their most recent passage; those older than 7 days must not be used.

7.4.2.4 Prepare cell culture test vessels using Item 6.5.3 and the most recent version of the EPA Manual of Methods for Virology (18.6), available at: http://www.epa.gov/microbes/about.html.

CAUTION: The flask size for the cell culture test vessels must be large enough to ensure that the inoculum volume (Step 12.1.2.2) is ≤0.04 mL/cm^2 of surface area.

7.4.3 Positive assay control

7.4.3.1 Prepare by diluting the QC stock (Item 7.2.2) in 0.15-M sodium phosphate, pH 7.0–7.5 (Item 7.3.6) to give a concentration of 20 MPN per *Inoculum Volume* or, if used, 20 MPN per *Final Inoculation Volume* (see Step 11.2.6.4 for a definition of *Inoculum Volume* and Step 11.2.6.5 for a definition of *Final Inoculation Volume*).

7.5 Reagents for the Enterovirus and Norovirus Molecular Assays

7.5.1 Primers and TaqMan® probes in Table 4 (Applied Biosystems, custom order)

7.5.2 PCR-grade water (Roche, Cat. No. 03315932001)

7.5.3 PBS (Dulbecco's phosphate buffered saline, without $CaCl_2$ and $MgCl_2$; U.S. Biological, Cat. No. D9820)

7.5.4 5% BSA

7.5.4.1 Prepare 5% BSA by dissolving 5 g of albumin/bovine crystalline (United States Biochemical, Cat. No. 10856) in 100 mL of dH_2O.

7.5.4.2 Sterilize by passing the solution through a 0.2-µm sterilizing filter (Item 6.6.17).

7.5.4.3 Store at 4 °C.

7.5.5 PBS, 0.2% BSA

7.5.5.1 Prepare by adding 4 mL of 5% BSA (Item 7.5.4) to 96 mL of PBS (Item 7.5.3).

7.5.5.2 Sterilize by passing the solution through a 0.2-µm sterilizing filter (Item 6.6.17).

7.5.5.3 Store at 4 °C.

7.5.6 QIAamp DNA Blood Mini Kit (Qiagen, Cat. No. 51104 or 51106), with buffer AL, buffer AW1, buffer AW2, buffer AE, and mini spin columns

7.5.7 Buffer AVL (Qiagen, Cat. No. 19073)

NOTE: Carrier RNA is supplied with this reagent

7.5.8 Buffer AVE (Qiagen, Cat. No. 1026956)

7.5.9 Absolute ethanol (C_2H_5OH; Fisher Scientific, Cat. No. BP2818-100)

7.5.10 RNasin® Plus RNase Inhibitor, 40 units/µL (Promega, Cat. No. N2615)

7.5.11 Random primer, 0.5 µg/µL (Promega, Cat. No. C1181)

7.5.12 Armored RNA® Hepatitis G virus (Asuragen, Cat. No. 42024)

7.5.13 10X PCR Buffer II and 25-mM $MgCl_2$ in separate vials (Applied Biosystems, Cat. No. N8080130)

7.5.14 PCR nucleotide mix, 10-mM (dNTPs; Promega, Cat. No. C1141)

7.5.15 Dithiothreitol, 100-mM (DTT; Promega, Cat. No. P1171)

7.5.16 SuperScript II Reverse Transcriptase, 200 units/µL (Invitrogen, Cat. No. 18064-022)

7.5.17 LightCycler® 480 Probes Master kit (Roche Diagnostics, Cat. No. 04707494001)

7.5.18 ROX reference dye, 25-mM (Invitrogen, Cat. No. 12223)

7.5.19 Armored RNA® containing the complete sequences which are amplified by the enterovirus, norovirus GI, norovirus GII assays described in section 13.5 (Asuragen, custom order giving $>10^{10}$ genomic copies at a defined concentration)

7.6 Reagents for Sterilization Techniques

7.6.1 95% ethanol (Sigma Aldrich, Cat. No. 493511)

7.6.2 0.525% sodium hypochlorite (NaClO)

7.6.2.1 Prepare a 0.525% NaClO solution by diluting household bleach 1:10 in dH_2O.

NOTE: Store 0.525% NaClO solutions for up to 1 week at room temperature.

7.6.3 1-M sodium thiosulfate ($Na_2S_2O_3$) pentahydrate

7.6.3.1 Prepare a 1-M solution by dissolving 248.2 g of $Na_2S_2O_3$ in 1 L of dH_2O.

NOTE: Sodium thiosulfate solutions may be stored for 6 months at room temperature.

7.6.4 0.5% iodine

7.6.4.1 Prepare a 0.5% iodine solution by dissolving 5 g of iodine in 1 L of 70% ethanol.

NOTE: Iodine solutions can be stored for 1 year at room temperature.

8.0 QUALITY ASSURANCE

This section describes the minimum quality assurance requirements. Laboratories are encouraged to institute additional QC practices that go beyond these minimum criteria to meet their needs. All laboratories analyzing test samples with this method must adhere to defined QA procedures that ensure analytical data that are scientifically valid and demonstrate acceptable precision and specificity.

8.1 Quality Assurance Plan

Each laboratory must have a written Quality Assurance Plan that addresses the following:

8.1.1 Laboratory organization and responsibility – This section must: 1) include a list that identifies the laboratory QA manager(s) and key individuals who are responsible for ensuring the production of valid measurements and the routine assessment of QC data; 2) specify who is responsible for internal audits and reviews of the implementation of the QA plan and its

requirements; and 3) include a chart showing the laboratory organization and line authority.

8.1.2 Personnel – This section must list each analyst's academic background and experience, describe how each analyst is trained to perform the method, and describe how training is documented.

8.1.3 Facilities – This section must describe the arrangement and size of laboratories, workflow patterns to minimize cross contamination, air system(s); the laboratory reagent water system, and the waste disposal system [see Sen *et al.* (18.41)].

8.1.4 Field sampling procedures – This section must describe the laboratory chain-of-custody procedures, including the sample identification and information recording system, and describe how field samples are collected and transported, including transportation time and temperature.

8.1.5 Laboratory test sample handling procedures – This section must describe test sample-holding times and temperature during analyses and the procedures for maintaining the integrity of the test samples (i.e., logging and tracking of samples from receipt through analyses and disposal).

8.1.6 Equipment – This section must describe the specifications, calibration procedures, preventive maintenance, and maintenance of quality control records for each item used during the performance of the method. All calibrations must be traceable to national standards, when they are available.

8.1.7 Supplies – This section must describe the specifications, storage conditions, and documentation of catalog and lot numbers for chemicals, reagents, and media.

8.1.8 Laboratory practices – This section must describe the preparation of reagent-grade water, glassware washing and preparation procedures, and sterilization procedures. It should also describe the workflow requirements among laboratories to prevent cross contamination, especially for molecular procedures. The workflow and other recommended requirements are described in detail in Sen *et al*. (18.41).

8.1.9 Analytical procedures – This section must reference this method and identify available laboratory SOPs.

8.1.10 Quality control checks – This section must describe all laboratory procedures that are implemented to ensure the quality of each analyst's data.

8.1.11 Data reduction, verification, and reporting – This section must describe any procedures for converting raw data to final data, identify procedures for ensuring the accuracy of data transcription and calculations, and describe the laboratory's procedures for reporting all data to EPA.

8.1.12 Corrective actions – This section must describe how the laboratory will respond to PE and QC failures and failures of its own internal QC

procedures, identify the person(s) responsible for taking corrective action, and describe how the effectiveness of the actions will be documented.

8.1.13 Record keeping – This section must describe how records are maintained (e.g., hard copy, electronic, or laboratory information management system [LIMS], etc.), how long records are kept, and where records are stored.

8.2 Laboratory Personnel

8.2.1 Principal Analyst/Supervisor – Laboratories must have a principal analyst who may also serve as a supervisor if an additional analyst(s) is to be involved. The principal analyst/supervisor oversees or performs the entire analyses and carries out QC performance checks to evaluate the quality of work performed by analysts and technicians. This person must be an experienced microbiologist with at least a B.A./B.S. degree in microbiology or a closely related field. The person must also have a minimum of 3 years continuous bench experience in cell culture propagation, processing and analysis of virus samples, and in performing PCR, along with at least 6 months of experience in performing RT-qPCR. This analyst must meet initial analyst approval/initial demonstration of capability (Section 8.3.1.2) and on-going analyst approval/on-going demonstration of capability (Section 8.3.1.3) requirements. The principal analyst must also demonstrate acceptable performance during any on-site performance audits.

8.2.2 Analyst – The analyst performs at the bench level under the supervision of a principal analyst and can be involved in all aspects of analysis, including preparation of sampling equipment, filter extraction, sample processing, cell culture, virus assay, qPCR, and data handling. The analyst must have 2 years of college lecture and laboratory course work in microbiology or a closely related field. The analyst must have at least 6 months bench experience in cell culture, animal virus analyses, and PCR, including 3 months experience in filter extraction of virus samples and sample processing. Six (6) months of additional bench experience in the above areas may be substituted for the 2 years of college. Each analyst must meet initial analyst approval/initial demonstration of capability (Section 8.3.1.2) and on-going analyst approval/on-going demonstration of capability (Section 8.3.1.3) requirements. The analyst must also demonstrate acceptable performance during any on-site audits. Should laboratories choose to use teams of analysts who specialize in performing the culture or molecular portions of this method; analysts only need to meet the educational requirement of the portion they perform. Laboratories using analyst teams must ensure that all quality controls are analyzed by the appropriate team member.

8.2.3 Technician – The technician extracts filters, processes samples, and performs qPCR under the supervision of an analyst, but does not perform cell culture work, virus detection, or enumeration. The technician must

have at least 3 months experience in filter extraction and processing of virus samples to participate in the cultural portion of this method and 3 months of experience with PCR to participate in the molecular portion of the method.

8.2.4 Samplers – The sampler collects water samples and ships them to the analytical laboratory. The sampler must be familiar with the field sample collection process and have at least training by means of a video or written instructions demonstrating proper sampling technique. Unless specified otherwise by EPA, laboratories are responsible for ensuring that samplers have adequate training.

8.3 Laboratory Performance

8.3.1 Laboratories using this method must evaluate the ability of analysts to perform the method using known quality control (QC) samples, unknown performance test (PT) samples, and unknown performance evaluation (PE) samples, as defined in Sections 8.3.1.2 and 8.3.1.3.

NOTE: EPA may also require laboratories to be approved.

8.3.1.1 Laboratory approval – Laboratories must have a Quality Assurance Plan, adequately trained staff, proper equipment, and at least 1 approved analyst to be approved.

8.3.1.2 Initial analyst approval/initial demonstration of capability – Each analyst must demonstrate the ability to perform the method using QC and PT samples, as part of an initial approval process. New analysts must initially use QC samples to gain method proficiency followed by the analysis of PT samples.

8.3.1.2.1 For initial approval, analysts must analyze 7 PT samples as described in Section 8.5 and meet the method performance characteristics defined in Section 14.0 or in any additional guidance from EPA.

8.3.1.2.2 Any analyst who does not meet the initial demonstration of capability must not process test samples.

8.3.1.3 On-going analyst approval/on-going demonstration of capability – To remain approved, each analyst must analyze 1 QC sample set (Section 8.4) for every analysis batch (see Section 3.1) and 1 PE sample (Section 8.5) per month following initial approval.

8.3.1.3.1 For on-going approval, 1 out of every 7 PE samples must be a negative PE sample. The order in which analysts receive the negative PE sample

and the virus levels on the positive PE samples must be randomized.

8.3.1.3.2 For on-going approval, analysts must meet the method performance characteristics defined in Section 14.0 or in any additional guidance from EPA.

8.3.1.3.3 Any analyst who does not meet the on-going demonstration of capability must not process test samples until the cause of the failure has been identified and corrected.

8.4 QC Sample Set

NOTE: A QC sample set must be associated with each analysis batch (Section 3.1). A QC sample set consists of a negative and a positive QC sample.

8.4.1 Negative QC sample/equipment blanks

8.4.1.1 Place 10 L of reagent grade water in a dispensing pressure vessel or polypropylene container (Item 6.3.3).

8.4.1.2 Adjust the pH to 6.5–7.5 with 0.12-M HCl (Item 7.1.2) or 0.1 M-NaOH (Item 7.2.5), as necessary.

NOTE: It is difficult to obtain an accurate pH on pure water. To compensate, a buffering agent, such as HEPES (Item 7.2.1), may be added to the water at a concentration up to 0.01 M (23.83 g/10-L).

8.4.1.3 Place a magnetic stir bar into the vessel or container and stir for 10 min at a speed sufficient to create a vortex.

8.4.1.4 Pass the water through a sterile standard filter apparatus (Item 6.1) containing a sterile electropositive filter, using a flow rate of approximately 10 L/min.

NOTE: To meet on-going QC requirements, standard filter apparatuses from field or positive QC samples must be used after cleaning and sterilization.

NOTE: Both negative and positive QC samples must use the same filter type (e.g., 1MDS or NanoCeram) that will be used for collecting field samples. If the analytical laboratory is processing field samples using both filter types, the filter types should be separated into different batches, with each batch associated with a QC sample.

8.4.1.5 Process and analyze the filter using the filter elution (Section 10.0), organic flocculation (Section 11.0), total culturable virus

assay (Section 12.0), and enterovirus and norovirus molecular assay (Section 13.0) procedures.

8.4.2 Positive QC sample

8.4.2.1 Place 10 L of reagent grade water in a dispensing pressure vessel or polypropylene container (Item 6.3.3).

8.4.2.2 Adjust the pH to 6.5–7.5 with 0.12-M HCl (Item 7.1.2) or 0.1-M NaOH (Item 7.2.5), as necessary.

NOTE: It is difficult to obtain an accurate pH on pure water. To compensate, a buffering agent, such as HEPES (Item 7.2.1), may be added to the water at a concentration up to 0.01 M.

8.4.2.3 Add 1.0 mL of a QC stock (Item 7.2.2) to the water.

8.4.2.4 Place a magnetic stir bar into the vessel or container and stir for 10 min at a speed sufficient to create a vortex.

8.4.2.5 Pass the water through a sterile standard apparatus (Item 6.1) containing a sterile electropositive filter, using a flow rate of approximately 10 L/min.

8.4.2.6 Process and analyze the filter using the elution (Section 10.0), organic flocculation (Section 11.0), total culturable virus assay (Section 12.0), and enterovirus molecular assay (Section 13.0) procedures.

8.4.3 QC sample results must meet the method performance characteristics defined in Section 14.0.

8.4.3.1 A positive result on a negative QC sample constitutes a failure of all test samples associated with the analysis batch.

8.4.3.2 A recovery result on positive QC samples outside the performance criteria specified in Section 14.0 or a positive norovirus assay constitutes a failure of all test samples associated with the analysis batch; however, laboratories may use a rolling average of 6 positive QC samples to determine the pass/fail status. The rolling average shall be done by averaging the first 6 positive QC samples run by an analyst and then, for each new QC sample, dropping the oldest and adding the new result to the average.

8.5 PT and PE Samples

8.5.1 Laboratories using this method for non-EPA studies shall prepare their own PT and PE samples internally or through an external contract or other mechanism.

NOTE: For EPA studies, PT and PE samples will be prepared by an EPA designated contractor and sent to participating laboratories in a randomized fashion.

8.5.1.1 Prepare negative PT and PE samples as described for negative QC samples (Section 8.4.1).

8.5.1.2 Prepare positive PT and PE samples as described for positive QC samples (Section 8.4.2), except substitute the appropriate PT/PE stock (Item 7.2.3) for the QC stock (Item 7.2.2).

8.5.1.3 Prepare a Sample Data Sheet (Section 17.1) for each PE sample with a derived sample volume and data typical of the type of samples an analyst would expect to see and ensure that the analyst is unaware that the sample is a PE control.

8.5.2 Process and analyze the PT and PE filter using the elution (Section 10.0), organic flocculation (Section 11.0), total culturable virus assay (Section 12.0), and enterovirus and norovirus molecular assay (Section 13.0) procedures, in accordance with any additional requirements supplied with the samples.

8.5.3 PT and PE sample results must meet the method performance characteristics defined in Section 14.0.

8.5.3.1 A positive result on a negative PT or PE sample constitutes a failure.

8.5.3.2 A recovery result based on a rolling average of 6 positive PT and PE samples that is outside the performance criteria specified in Section 14.0 constitutes a failure.

8.5.3.2.1 A mean recovery value shall be calculated using the 6 positive PT samples from each analyst's initial analyst approval/initial demonstration of capability (Section 8.3.1.2) test.

8.5.3.2.2 For on-going approval, a new average shall be calculated each month by dropping the analyst's oldest PT or PE sample from the average and adding the month's ongoing PE sample.

8.6 Matrix Spike

8.6.1 Run a matrix spike for every field sample location initially and then after every 10^{th} field sample from the same location.

8.6.2 Matrix spike duplicates are performed by collecting 2 field samples at the sampling location.

NOTE: A full flow hose Y (Item 6.3.1) may be used to collect both samples simultaneously.

8.6.2.1 Collect the first of the 2 field samples using the specified volume (see Table 2).

8.6.2.2 Collect the second of the 2 field samples using the duplicate field apparatus (Item 6.3.8) and the specified volume minus 10 L.

8.6.2.2.1 Collect an additional 10 L in a 10-L cubitainer (Item 6.3.7).

NOTE: The cubitainer can be shipped at ambient temperatures.

8.6.2.2.2 After arriving at the analytical laboratory, seed the 10-L cubitainer with 1 mL of the matrix spike (Item 7.2.4).

8.6.2.2.3 Pass the seeded 10 L through the duplicate filter apparatus containing the second field sample.

8.6.2.3 Process and analyze both field samples using the elution (Section 10.0), organic flocculation (Section 11.0), total culturable virus assay (Section 12.0), and enterovirus and norovirus molecular assay (Section 13.0) procedures.

8.6.3 The results of the analysis of the matrix spike must meet the performance measures in Section 14.0.

8.7 Record Maintenance

Laboratories shall maintain all records related to data quality. This shall include a record of the analyst name, date, and results of all QA controls performed, records of equipment calibration and maintenance, and reagent and material catalog and lot numbers used for all analytical procedures.

9.0 SAMPLE COLLECTION, PRESERVATION, AND STORAGE

9.1 Field Sample Collection

9.1.1 Preliminary procedures

9.1.1.1 Filter sampling apparatus sterilization

9.1.1.1.1 Before each use, analytical (or contract) laboratories must wash and then sterilize the intake and cartridge housing modules, any necessary injector modules, and the pumps, as described in Section 15.2.4.

9.1.1.1.2 Cover the filter sampling apparatus module ends and the injector port(s) with sterile aluminum foil (Item 6.2.10).

9.1.1.1.3 Place the injector module and tubing into a sterile bag or wrapping in such a way that they may be removed without contaminating them.

9.1.1.1.4 Record a unique sample number of a Sample Data Sheet (Section 17.1).

9.1.1.1.5 Take or ship the filter sampling apparatus components and the Sample Data Sheet to the individual who will be collecting the field sample, along with any necessary instructions on where to collect the sample.

9.1.1.2 Calibrate flow meter

9.1.1.2.1 Confirm the flow meter calibration at the flow rates used for sampling before the first use and at least after every month of use.

9.1.1.2.2 Perform the calibration check by measuring the time required to fill a 4-L or larger graduated cylinder (Item 6.2.17). The time required to reach the 4-L mark on the graduated cylinder must be 24±1 sec for the 10 L/min rate or 60±1 sec for the 4 L/min rate (Table 2).

9.1.2 Preparation for field sample collection

CAUTION: Individuals collecting field samples for virus analysis must wear surgical gloves and avoid conditions that could contaminate a sample with virus. Gloves should be changed after touching human skin or handling components that may be contaminated (e.g., water tap**s**, other environmental surfaces, etc.).

CAUTION: Care must be taken to ensure that cartridge filters are properly seated in the housings. Housings with properly seated filters will not leak and the filter will not move within the housing when shaken. Upon opening housings at the analytical laboratory, filters should be checked for proper seating by examining the gaskets for depressions that do not extend beyond the edge of the gasket. Samples from housings with improperly seated filters should be recollected rather than processed.

9.1.2.1 Wipe the outside of the water tap thoroughly with a Hype-Wipe pad (Item 7.1.1). If the discharge module (Item 6.1.3) is stored at the sampling site (see Note for Section 9.2.3), wipe the outside surface of the quick disconnect with another Hype-Wipe pad. Wait 2-min before proceeding.

9.1.2.2 Purge the water tap to be sampled before connecting the filter apparatus. Continue purging for 2–3 min or until any debris that has settled in the line has cleared.

NOTE: If it is necessary to use a garden hose (Item 6.1.3.8) to reach a drain during the purge step, wipe the inside threads of the hose with a Hype-Wipe pad and wait 2-min before connecting the hose to the tap.

NOTE: If a pump is being used instead of a water tap, purge the pump with the water to be sampled for 10 min before proceeding.

9.1.2.3 Connect the Intake Module to the water tap or pump.

9.1.2.3.1 Remove the foil from the backflow regulator, if used. Loosen the swivel female insert slightly to allow it to turn freely, and connect the backflow regulator to the tap or pump. Retighten the swivel female insert.

9.1.2.3.2 Connect the swivel female insert directly to the water tap or pump, if a backflow regulator is not used.

9.1.2.4 Disconnect the cartridge housing module (Item 6.1.2) at the quick connect, if connected, and cover the open end with sterile foil.

9.1.2.5 Remove the foil, if present, from the ends of the discharge module (Item 6.1.3) and connect it to the intake module (Item 6.1.1).

9.1.2.6 Place the end of the discharge module or the tubing connected to the outlet of the discharge module into a 1-L polypropylene wide-mouth bottle (Item 6.2.3).

9.1.2.7 Slowly turn on the tap and adjust the globe valve of the discharge module until the flow meter/totalizer reads 10 L/min.

NOTE: If the tap is incapable of reaching this flow rate, adjust the valve to achieve the maximum flow rate. Slower flow rates will result in longer sampling times.

9.1.2.8 Flush the apparatus assembly with at least 75 L of the water to be sampled.

9.1.2.8.1 While the system is being flushed, measure the chlorine residual (Item 6.2.6), pH and temperature (Item 6.2.4), and the turbidity (Item 6.2.5) of the water collecting in and overflowing from the 1-L polypropylene bottle.

9.1.2.8.2 Record the pH, temperature, turbidity, and free chlorine values on the Sample Data Sheet.

9.1.2.9 Turn off the water at the tap and disconnect the discharge module from the intake module.

9.1.3 Injector module adjustment

9.1.3.1 If the water to be sampled does not contain a disinfectant and if the water pH is ≤9.0 (if using a NanoCeram filter) or ≤8.0 (if using a 1MDS filter), skip to Section 9.1.4.

9.1.3.2 If the field sample contains a disinfectant and the water pH is ≤9.0 (NanoCeram filters) or ≤8.0 (1MDS filters):

9.1.3.2.1 Remove the foil from the ends of an injector module (Item 6.1.4) and connect the injector module to the quick connect of the intake module. Connect the discharge module to the injector module.

9.1.3.2.2 Place 2% sodium thiosulfate (Item 7.1.3) into a chemical tank (Item 6.1.4.7). In not connected, connect the ¼-in tubing supplied with the chemical tank to the pipe adaptor elbow (Item 6.1.4.6) on the injector module. Turn on the metering pump (Item 6.1.4.8) to deliver 2% sodium thiosulfate to the sample stream.

NOTE: Before first use, adjust the metering pump to deliver 2.4 or 6 mL/min (i.e., 0.6 mL x L of disinfected water passing through the sample filtration apparatus each minute) for flow rates of 4 or 10 L/minute, respectively. Use a small graduated cylinder to measure the flow rate, and then record or mark the pump setting for each rate.

9.1.3.2.3 Set the metering pump to deliver 2.4±0.2 mL/min or 6.0±0.2 mL/min for flow rates of 4 or 10 L/min, respectively (see Table 2).

9.1.3.2.4 Turn on the water at the tap and measure the chlorine residual. If a chlorine residual is detected, re-adjust the flow rate until no residual is present. Re-mark the setting, if necessary.

9.1.3.2.5 Turn off the water at the tap and the metering pump and proceed to Step 9.1.4.

9.1.3.3 If the water does not contain a disinfectant, but has a pH >9.0 (NanoCeram filters) or >8.0 (1MDS filters):

9.1.3.3.1 Remove the foil from the ends of an injector module (Item 6.1.4) and connect the injector module to the quick connect of the intake module. Connect the discharge module to the injector module.

9.1.3.3.2 Place 0.12-M HCl (Item 7.1.2) into a chemical tank (Item 6.1.4.7). In not connected, connect the ¼-in tubing supplied with the chemical tank to the pipe adaptor elbow (Item 6.1.4.6) on the injector module. Turn on the metering pump (Item 6.1.4.8) to deliver 0.12-M HCL to the sample stream.

9.1.3.3.3 Turn on the water at the tap and measure the pH of the water exiting the discharge module. Adjust the metering pump until the pH of the water exiting the discharge module is 6.5–7.5.

9.1.3.3.4 Turn off the water at the tap and the metering pump and proceed to Step 9.1.4.

9.1.3.4 If the water is disinfected and the water pH is >9.0 (NanoCeram filters) or >8.0 (1MDS filters):

9.1.3.4.1 Remove the foil from the ends of a double injector module (Item 6.1.5) and connect the double injector module to the quick connect of the intake module. Connect the discharge module to the double injector module.

9.1.3.4.2 Follow Steps 9.1.3.2.2–9.1.3.2.4 to add sodium thiosulfate and Steps 9.1.3.3.2–9.1.3.3.3 to add HCl.

9.1.3.4.3 Turn off the water at the tap and the metering pump and proceed to Step 9.1.4.

9.1.4 Virus collection

9.1.4.1 If connected, remove the discharge module.

9.1.4.2 Remove the foil from the cartridge housing module and connect it to the end of the intake module, or if used, the injector or double injector module.

9.1.4.3 Connect the discharge module to the outlet of the cartridge housing module.

9.1.4.4 If the field sample has turbidity >20 NTU (for NanoCeram filters) or >50 NTU (for 1MDS filters), remove the foil from each end of the prefilter module (Item 6.1.6) and connect the prefilter module between the intake module (or the injector module, if used) and the cartridge housing module.

9.1.4.5 Record the unique sample number (if not added by the analytical or contract laboratory), utility or site name and address, sampler's name, water type, location at sampling site, date, time, equipment model and serial numbers, and the initial totalizer reading on a Sample Data Sheet.

9.1.4.6 If an injector or double infector module is being used, turn on the metering pump(s).

9.1.4.7 With the filter housing placed in an upright position, slowly open the water tap until it is completely open.

9.1.4.7.1 If the cartridge housing has a vent button, press it while opening the tap to expel air from the housing. When the air is totally expelled from the housing, release the button, and open the sample tap completely.

9.1.4.7.2 If the housing does not have a vent button, allow the housing to fill with water before completely opening the tap.

9.1.4.7.3 After the tap is opened completely, check the flow rate and readjust to the recommended rate from Table 2, if necessary.

9.1.4.7.4 Record the initial flow rate on the Sample Data Sheet.

9.1.4.7.5 Check and readjust the metering pump(s), if necessary.

9.1.4.8 Using the totalizer readings, pass a volume of water through the apparatus that equals the volume specified in Table 2 for the water type being sampled.

9.1.4.9 Turn off the flow of water at the sample tap at the end of the sampling period, and record the final flow rate, date, time of day, total sample volume, and totalizer reading on a Sample Data Sheet.

NOTE: Although the totalizer reading may be affected by the addition of thiosulfate, the effect is insignificant and may be ignored.

9.1.4.10 Loosen the swivel female insert on the intake module and disconnect the backflow regulator from the tap.

9.1.4.11 Disconnect the cartridge housing module and the prefilter housing module, if used, from the other modules.

9.1.4.12 Turn the filter housing(s) upside down and allow excess water to flow out.

9.1.4.13 Turn the housing(s) upright and cover the quick connects on each end of the modules with sterile aluminum foil.

9.1.4.14 Place the housing(s) into a closable plastic bag (Item 6.2.13).

9.1.4.15 Drain the water from the intake and discharge modules and, if used, from the injector module. Place the modules into one or more closable plastic bags.

9.2 Shipment of Field Samples

9.2.1 Pack the cartridge housing module(s) into an insulated shipping box (Item 6.2.9).

9.2.2 Add 6–8 small ice packs (Item 6.2.7; prefrozen at -20 °C) or double-bagged ice cubes around the cartridge housings to keep the sample cool in transit.

NOTE: The number of ice packs or bags may have to be adjusted based upon experience to ensure that the sample remains cold, but not frozen.

9.2.2.1 Add an iButton (Item 6.2.8 or other temperature recording device) to a location in the shipping box where it will not come in direct contact with the ice packs or bags.

NOTE: The temperature during shipment must be in the range of 1–10 °C.

9.2.3 Place the intake and discharge modules into the insulated shipping box.

NOTE: The discharge module may remain in a secure location at the sampling site, if field samples will be taken on a routine basis at the site.

9.2.4 Place the Sample Data Sheet, protected in a closable plastic bag, (Item 6.2.14) in with the sample.

9.2.5 Fill any void space with packing material (Item 6.2.15).

9.2.6 Close the shipping box and tape (Item 6.2.16) to prevent any leakage of water.

9.2.7 Label and address the shipping box appropriately.

9.2.8 If the shipping box cannot be directly transported to the laboratory for virus analysis by close of business on the day collected or by the next morning, ship it to the laboratory by overnight courier.

9.3 Laboratory Holding Time and Temperature

9.3.1 Immediately upon arrival at the analytical laboratory, unpack the shipping box and refrigerate the cartridge housings with filters and if used, the prefilter housings with filters.

9.3.2 Record the sample number and sampling date (from the Sample Data Sheet packed with the sample) and the date of arrival, the analytical laboratory name, identification number (if assigned), and address on a Virus Data Sheet (Item 17.2). Retain the Sample Data Sheet with all other records associated with the sample.

CAUTION: The cartridge filters must arrive from the utility or other sampling site in a refrigerated, but not frozen, condition.

9.3.2.1 Print out the transit temperature reading from the iButton or other temperature-recording device.

9.3.2.2 Record the sample number, sample date, and arrival date on the printed transit temperature readout and retain the readout with all other records associated with the sample.

NOTE: The temperature during shipment must be in the range of 1–10 °C.

9.3.2.3 Brief transient temperatures outside the acceptable range associated with the initial packing and closing of the shipping box and its opening at the analytical laboratory may be ignored.

9.3.3 Ideally, viruses should be eluted from filters within 24 h of the start of the sample collection, but all filters must be eluted within 72 h of the start of the sample collection.

10.0 FILTER ELUTION PROCEDURE

If a prefilter or more than 1 electropositive filter was used to collect a field sample, each filter must be eluted and analyzed separately using the procedures below.

10.1 Elution Equipment Setup

10.1.1 Attach the elution inlet tubing (Item 6.4.4) to the inlet and the elution outlet tubing (Item 6.4.5) to the outlet ports of the cartridge housing containing the cartridge filter (see Figure 4).

10.1.2 Place the sterile end of the tubing connected to the outlet of the cartridge housing into a sterile 2-L glass or polypropylene beaker (Item 6.4.6).

10.1.3 Connect the other end of the elution inlet tubing to the outlet port of a sterile dispensing pressure vessel (Item 6.4.3), and connect the inlet port of the pressure vessel to a positive air pressure source (Item 6.4.2).

10.2 Elution

10.2.1 First elution

10.2.1.1 Elute NanoCeram or 1MDS filters with 500 mL or 1,000 mL of buffered 1.5% beef extract, pH 9.0 (Item 7.3.1, prewarmed to

room temperature), respectively, by opening the cartridge housing and adding a sufficient amount of beef extract to cover the filter completely.

10.2.1.1.1 Pour any remaining beef extract that does not fit in the housing into the pressure vessel.

NOTE: An acceptable alternative to the use of a pressure vessel is to use a peristaltic pump and sterile tubing to push the remaining beef extract through the filter.

10.2.1.1.2 Replace the top of the pressure vessel.

10.2.1.1.3 Wipe up any spilled liquid with a disinfectant-soaked sponge (Item 6.4.15).

10.2.1.2 Allow the solution to contact the filter for 1 min.

10.2.1.3 Turn on the pressure source to force the buffered beef extract solution through the filter(s) and into the beaker.

NOTE: The solution should pass through the filter slowly to maximize the elution contact period.

NOTE: Slow passage of the solution also minimizes foaming, which may inactivate some viruses; the addition of a few drops of antifoam (Item 7.3.3) to minimize foaming in the solution collecting in the 2-L beaker is optional.

10.2.1.3.1 When air enters the line from the pressure vessel, elevate and invert the filter housing to permit complete evacuation of the solution from the filters.

10.2.1.4 Turn off the pressure at the source, and open the vent/relief valve on the pressure vessel.

10.2.2 Second elution

10.2.2.1 Repeat Steps 10.2.1.1–10.2.1.4

10.2.2.1.1 For the NanoCeram filter, repeat these sections using an additional 500 mL of buffered 1.5% beef extract and by increasing the contact time in Step 10.2.1.2 to 15 min.

10.2.2.1.2 For the 1MDS filter, repeat these sections by placing the buffered beef extract from the 2-L beaker back into the cartridge housing and pressure vessel.

10.2.2.2 Turn off the pressure at the source and open the vent/relief valve on the pressure vessel.

10.2.2.3 Combine the two 500-mL portions from the elution of the NanoCeram filters.

10.2.2.4 Record the analyst's name and identification number (if assigned), the sample batch number, the date and time of elution, and the total volume of eluate recovered on the Virus Data Sheet (see Section 9.3.2).

NOTE: If analysts work together as a team, record the names and identification numbers of all analysts. If different analysts perform different portions of this or subsequent sections of the method, each analyst should only record the steps he/she performs. If necessary, each analyst can record the steps he/she performs using separate Data Sheets.

10.2.2.5 Thoroughly mix the eluate and proceed to the organic flocculation concentration procedure (Section 11.0) immediately.

11.0 ORGANIC FLOCCULATION CONCENTRATION PROCEDURE

11.1 Organic Flocculation

11.1.1 Place a sterile stir bar into the beaker containing the buffered beef extract eluate from the cartridge filter.

11.1.2 Place the beaker onto a magnetic stirrer, and stir at a speed sufficient to develop a vortex.

NOTE: Minimize foaming (which may inactivate viruses) throughout the procedure by not stirring or mixing faster than necessary to develop a vortex.

11.1.3 Adjust the pH to 3.5±0.1.

11.1.3.1 Sterilize the electrode of a combination-type pH electrode, as described in Section 15.2.4.

11.1.3.2 Calibrate the pH meter at pH 4 and 7.

11.1.3.3 Insert the sterile pH electrode into the beef extract eluate.

11.1.3.4 Add 1.2-M HCl (Item 7.1.2) to the eluate dropwise, while moving the tip of the pipette in a circular motion away from the vortex to facilitate mixing.

CAUTION: Rapid addition of HCl will inactivate virus.

11.1.3.5 Continue adding 1.2-M HCl until the pH reaches 3.5±0.1.

11.1.4 While continuing to monitor the pH, slowly stir the eluate for 30 min at room temperature.

NOTE: A precipitate will form during the 30-min stirring period.

11.1.4.1 If pH falls below 3.4, add 1-M NaOH (Item 7.2.5) to bring it back to 3.5±0.1.

NOTE: Exposure to a pH below 3.4 may result in virus inactivation.

11.1.4.2 Record whether a normal amount of floc formed during this step on the Virus Data Sheet. If a normal amount of floc did not form, record whether it was lighter or heavier than normal.

11.1.5 Remove the electrode from the beaker, and pour the contents of the beaker into a centrifuge bottle (Item 6.4.9.2).

NOTE: The beef extract suspension may have to be divided into several centrifuge bottles.

11.1.5.1 To prevent the transfer of the stir bar into a centrifuge bottle, hold another stir bar or magnet against the bottom of the beaker while decanting the contents.

11.1.6 Cap the bottle and centrifuge the precipitated beef extract suspension at 2,500 x *g* for 15 min at 4 °C.

11.1.7 Carefully pour off or aspirate the supernatant, so as not to disturb the pelleted precipitate, including any loose floc on top of the pellet.

11.1.8 Discard the supernatant.

11.2 Reconcentrated Eluate

11.2.1 Place a stir bar and 30 mL of 0.15-M sodium phosphate, pH 9.0 (Item 7.3.5) into the centrifuge bottle that contains the precipitate (from Step 11.1.7).

NOTE: A smaller volume of sodium phosphate (down to 15 mL) may be used, if the analytical laboratory's PE sample sets meet the performance requirements of Section 14.0.

NOTE: When the centrifugation (Step 11.1.6) is performed in more than one bottle, dissolve the precipitates in a total of 30 mL (or in the total reduced volume from the first note) and combine into one bottle before proceeding to the next step.

11.2.2 Place the bottle onto a magnetic stirrer, and stir slowly for 10 min until the precipitate has dissolved completely.

NOTE: Significant virus loss can occur if the precipitates are not dissolved completely.

11.2.2.1 Treat precipitates that prove to be difficult to dissolve with any of the following techniques:

11.2.2.1.1 Break up the precipitate with a sterile spatula before or during the stirring procedure.

11.2.2.1.2 Use a pipette repeatedly to draw the solution up and down during the stirring.

11.2.2.1.3 Shake the precipitate at 160 rpm for 10 min on an orbital shaker, in place of stirring.

11.2.2.2 If stirring or any of the above techniques take longer than 10 min to dissolve the precipitate or if experience with the water matrix shows that precipitates are always difficult to manage, either slowly adjust the pH to 7.0–7.5 with 1.2-M HCl (Item 7.1.2) or resuspend the precipitate initially in 0.15-M sodium phosphate, pH 7.0–7.5 (Item 7.3.6).

11.2.2.2.1 Use one of the above techniques to dissolve the precipitate and then slowly re-adjust the pH to 9.0 with 1-M NaOH (Item 7.2.5).

11.2.2.2.2 Mix for 10 min at room temperature before proceeding and then remove the stir bar.

11.2.3 Centrifuge the dissolved precipitate at 4,000 x *g* for 10 min at 4 °C.

NOTE: The centrifugation speed may be increased to 10,000 x g for 10 min at 4 °C to facilitate the filtration step below.

11.2.3.1 Record the date and time concentrated and the centrifugation speed on the Virus Data.

11.2.3.2 Remove and collect the supernatant and discard the pellet.

11.2.4 Adjust the pH of the supernatant to 7.0–7.5 slowly with 1.2-M HCl (Item 7.1.2).

11.2.5 Pass the supernatant from through a sterilizing filter.

11.2.5.1 Pretreat a sterilizing filter (Item 6.4.11) or for test samples that are difficult to filter, a sterilizing filter stack (Item 6.4.12) with 10–15 mL of 1.5% beef extract, pH 7.0–7.5 (Item 7.3.2).

11.2.5.2 Load the supernatant into a 50-mL syringe and force it through the filter from Step 11.2.5.1.

11.2.5.2.1 If the sterilizing filter or filter stack begins to clog badly, empty the loaded syringe into the bottle containing the unfiltered supernatant, fill the syringe with air, and inject air into filter to force any residual sample from it.

11.2.5.2.2 Continue the filtration procedure with another filter.

11.2.5.3 Record the filtered reconcentrated eluate volume resulting from Step 11.2.5.2 (designated the *Final Concentrated Sample Volume* [FCSV]) on the Virus Data Sheet.

11.2.6 Calculation of assay volumes and preparation of subsamples

11.2.6.1 Calculate the *Assay Sample Volume (S)* for all test samples, except for QC samples using Equation 1,

$$S = \frac{D}{TSV} \times FCSV \qquad \text{Eq. 1}$$

where *D (Volume of Original Water Sample Assayed)* is the amount of reconcentrated eluate that must be assayed by the total culturable virus assay (Section 12.0) or processed for the enterovirus and norovirus molecular assay (Section 13.0) and *TSV* is the *Total Sample Volume* from the Sample Data Sheet associated with the sample.

NOTE: D is 100 L for source water or 500 L for finished or ground waters and the Assay Sample Volume (S) is the volume of the filtered reconcentrated eluate that represents 100 L of source water or 500 L of finished or ground waters.

NOTE: For example, if 1,800 L of a groundwater sample is passed through the NanoCeram filter and subsequently concentrated to 30 mL, then TSV equals 1,800 L, D equals 500 L, FCSV equals 30 mL, and S equals 8.33 mL [(500 L/1,800 L) x 30 mL].

NOTE: Go to Section 11.2.6.6 for QC samples.

11.2.6.2 Record the *S* and *D* values on the Virus Data Sheet.

11.2.6.3 Prepare 3 subsamples of the reconcentrated eluate.

11.2.6.3.1 Prepare subsamples 1 and 2 with a volume equal to 1.2 times the *Assay Sample Volume*.

11.2.6.3.2 Prepare subsample 3 with the remaining volume.

11.2.6.3.3 Label subsamples 1–3 with appropriate sampling information for identification.

11.2.6.3.4 Hold subsample 1 at 4 °C for use with the total culturable virus assay (Section 12.0) if it can be assayed within 24 h; otherwise, freeze at -70 °C.

11.2.6.3.5 Hold subsample 2 at 4 °C and analyze using the enterovirus and norovirus molecular assay (Section 13.0) within 24 h.

NOTE: Freeing and thawing leads to norovirus losses.

11.2.6.3.6 Freeze subsample 3 at -70 °C for backup and archival purposes.

11.2.6.4 Determine the *Inoculum Volume* for the total culturable virus assay (Section 12.0) by dividing the *Assay Sample Volume* (*S*; determined in Step 11.2.6.1*)* by 10.

11.2.6.4.1 Record the *Inoculum Volume* onto the Virus Data Sheet.

11.2.6.5 For ease of inoculation, a sufficient quantity of 0.15-M sodium phosphate, pH 7.0–7.5 (Item 7.3.6) may be added to the *Inoculum Volume* to give a *Final Inoculation Volume* that can be directly measured (e.g., 1.0 mL).

NOTE: Section 12.0 requires that an amount equal to the Inoculum Volume (IV) be placed onto each of 10 vessels. When inoculating many vessels, it is more practical to use large or repeater pipettes, but it can be difficult to measure some IVs using these pipettes accurately. For example, if Step 11.2.6.4 results in requiring an IV of 0.833 mL, dispersing the IV to 10 vessels can be done more reproducibly if it is brought to 1.0 mL. The calculation procedure for doing this is described in Step 11.2.6.5.1

11.2.6.5.1 Calculate the *Final Inoculation Volume* by adding a volume of Subsample 1 from Step 11.2.6.3.4 equal to 10.5 x IV to a volume of 0.15-M sodium phosphate, pH 7.0–7.5 (Item 7.3.6) equal to 10.5 x (1.0-IV). For example, using the amount (0.833 mL) from note above, 10.5 x 0.833 mL=8.75 mL of the filtered reconcentrated eluate would be added to 10.5 x (1.0-0.833 mL)=1.75 mL of sodium phosphate.

NOTE: *Final Inoculation Volumes* other than 1.0 mL can be used in the calculation by substituting the desired volume for the 1.0 in the "(1.0-IV)" component of the equation.

NOTE: The calculation uses 10.5 vessels, rather than 10, with the extra 0.5 vessel being added to account for test sample loss on the surface of the tube (e.g., Item 6.4.16) used for the preparation of the Final Inoculation Volume.

11.2.6.5.2 If the *Final Inoculation Volume* option is used, then record the volume onto the Virus Data Sheet

and substitute the term *Final Inoculation Volume* for each use of *Inoculum Volume***,** except where indicated.

11.2.6.6 For QC samples (Section 8.4), calculate assay volumes and prepare subsamples as follows:

11.2.6.6.1 Calculate the *Assay Sample Volume (S)* by multiplying the *FCSV* by 0.3.

11.2.6.6.2 Calculate the *Inoculum Volume* by dividing the *Assay Sample Volume (S)* by 10.

11.2.6.6.3 Divide the *FCSV* from QC samples into 3 subsamples and handle as described in Step 11.2.6.3.

12.0 TOTAL CULTURABLE VIRUS ASSAY

12.1 Quantal Assay

12.1.1 Preparation of cell culture test vessels

12.1.1.1 Using 10 cell culture test vessels (Item 7.4.2.4) for every test sample, code each vessel with the test sample number, subsample number, analyst initials, and date, using an indelible marker (Item 6.5.4).

12.1.1.2 Return the cell culture test vessels to a 36.5±1 °C incubator and hold at that temperature until the cell monolayer is to be inoculated.

12.1.1.3 Decant and discard the medium from the cell culture test vessels using a biosafety cabinet.

12.1.1.4 Wash the test vessels with a balanced salt solution (e.g., Item 7.4.1.1) or maintenance medium (Item 7.4.1.8.2), prepared without serum, using a wash volume of at least 0.06 mL/cm^2 of surface area.

NOTE: Add the wash solution carefully to avoid disturbing the cell monolayer.

12.1.1.4.1 Rock the wash medium over the surface of each monolayer several times and then decant and discard the wash medium.

12.1.2 Inoculation of test samples (first passage)

12.1.2.1 Rapidly thaw subsample 1 (Step 11.2.6.3.4), if frozen, in a 37 °C water bath or under warm running water at about 37 °C with shaking.

NOTE: Test samples should be removed from the warm water as soon as the last ice crystal melts.

12.1.2.2 Inoculate an amount of subsample 1 equal to the *Inoculum Volume* (Step 11.2.6.4, or *Final Inoculation Volume*, Step 11.2.6.5) onto each of the 10 cell culture test vessels.

CAUTION: Use at least a different pipetting tip or device for each set of test samples to be inoculated.

NOTE: The number of cell culture replicates was cut from 20 replicates, required by the ICR standard method, to 10 to reduce labor costs. This reduction of replicates results in wider 95% confidence limits (c. 20–40%) and reduces the maximum virus titer that can be assayed without dilutions by about 25%.

NOTE: The analysis of a second subsample is not required for this method. Subsample 2 was used in the ICR method to account for cytotoxicity. Samples with cytotoxicity should be assayed using dilutions as described in Step 12.1.2.3.

12.1.2.3 For postivie QC samples (Section 8.4) and for other test samples known or suspected of having virus concentrations greater than 0.2 MPN/L (surface waters) or 0.03 MPN/L (groundwaters), prepare 5- and 25-fold dilutions of subsample 1 for inoculation.

NOTE: Subsample 3 from Step 11.2.6.3.6 may be substituted for Subsample 1 in the steps below, if it is necessary to reanalyze test samples using dilutions.

12.1.2.3.1 Prepare a 1:5 dilution by adding a volume of subsample 1 equal to 0.1334 times the *Assay Sample Volume* (designated amount "a") to a volume of 0.15-M sodium phosphate, pH 7.0–7.5 (Item 7.3.6) equal to 0.5336 times the *Assay Sample Volume* (designated amount "b"). Mix thoroughly.

12.1.2.3.2 Prepare a 1:25 dilution by adding amount "a" of the 1:5 diluted subsample to amount "b" of 0.15-M sodium phosphate, pH 7.0–7.5.

12.1.2.3.3 Inoculate 10 cell culture test vessels each with undiluted subsample 1, subsample 1 diluted 1:5, and subsample 1 diluted 1:25, respectively, using an amount equal to the *Inoculum Volume*.

12.1.2.3.4 Freeze the remaining portions of the 1:25 dilution at -70 °C until the sample results are known.

12.1.2.3.5 Thaw and perform additional 5-fold dilutions using the dilution format above if all replicates of the undiluted to 1:25-fold dilutions develop CPE.

12.1.2.4 Inoculation of negative assay controls

12.1.2.4.1 Inoculate 3 or more cell culture test vessels with a volume of 0.15-M sodium phosphate, pH 7.0–7.5 (Item 7.3.6) equal to the *Inoculum Volume*, as a negative control.

12.1.2.4.2 If any negative control develops CPE, all sub*sequent assays* should be halted until the cause of the positive result is determined.

12.1.2.5 Inoculation of positive assay controls

NOTE: Run a positive control with every test sample; this control will provide a measure for continued sensitivity of the cell cultures to virus infection.

12.1.2.5.1 Inoculate 3 or more cell culture test vessels with the positive assay control (Item 7.4.3).

12.1.2.5.2 If any positive control fails to develop CPE, all subsequent assays should be halted until the cause of the negative result is determined.

12.1.2.6 Record the date of inoculation on the Virus Data Sheet in the cell for the 1st passage of Subsample 1.

12.1.2.7 Rock the inoculated cell culture test vessels gently to achieve uniform distribution of inoculum over the surface of the cell monolayers.

12.1.2.7.1 Place the cell culture test vessels on a mechanical rocking platform (Item 6.5.8) set at 1–5 oscillations/min at room temperature.

12.1.2.7.2 If a rocking platform is not available, the vessels may be placed on a level laboratory surface, but the vessels should be rocked every 15–20 min during the adsorption period to prevent cell death in the middle of the vessels from dehydration.

12.1.2.8 Continue incubating the inoculated cell cultures for 80–120 min at room temperature to permit viruses to adsorb onto and infect cells.

12.1.2.9 Add maintenance medium (Section 7.4.1.8.2) and incubate at 36.5±1 °C.

CAUTION: Never touch the pipetting device to the inside rim of the cell culture test vessels during

medium addition. This step represents the most likely place where cross contamination of cultures can occur. Cross contamination will result in invalid MPN values and can cause false positive results. Laboratories must ensure that analysts take great precaution in performing this step.

CAUTION: Warm the maintenance medium to 36.5±1 °C before placing it onto the cell monolayers.

CAUTION: Add the medium to the side of the cell culture vessel opposite the cell monolayer.

12.1.2.10 If CPE has not started to develop, the cultures may be re-fed with fresh maintenance medium after 4–7 d.

12.1.3 CPE development

12.1.3.1 Examine each culture microscopically for the appearance of CPE daily for the first 3 d and then every couple of days for a total of 14 d.

12.1.3.2 Freeze cultures at -70 °C when more than 75% of the monolayer has developed CPE.

12.1.3.3 Freeze all remaining cultures, including controls, at -70 °C after 14 d.

12.1.4 Second passage

12.1.4.1 Perform a second passage for confirmation.

NOTE: Confirmation passages may be performed in small vessels or multiwell trays, however, it may be necessary to distribute the inoculum into several vessels or wells to ensure that the inoculum volume is ≤0.04 mL/cm^2 of surface area.

12.1.4.2 Thaw all the cultures, including the negative and positive assay controls, to confirm the results of the previous passage.

12.1.4.3 Refreeze at least 2 mL of the medium from each vessel at -70 °C for optional analysis by molecular methods (Section 13.0).

12.1.4.4 Filter at least 10% of the medium from each vessel that was positive for CPE through separate 0.2-µm sterilizing filters (Item 6.5.9).

12.1.4.4.1 If the medium is difficult to filter, it can be centrifuged at 1,500–18,000 x *g* for 10 min at 4 °C prior to filtration.

12.1.4.5 Prepare fresh cell culture test vessels as described in Step 12.1.1.

12.1.4.6 Inoculate the fresh cultures with the thawed medium from all negative cell culture test vessels (Step 12.1.4.2) and the filtered medium from Step 12.1.4.4, using an inoculation volume that represents 10% of the medium from the first passage.

12.1.4.7 Repeat Steps 12.1.2.7–12.1.3.1.

12.1.4.7.1 Record the date of inoculation on the Virus Data Sheet in the cell for the 2nd passage of Subsample 1.

12.1.4.7.2 Freeze any cell culture test vessels that were negative on the first passage and positive on the second passage at -70 °C when more than 75% of the monolayer has developed CPE.

12.1.5 Score cultures that developed CPE in both the first and second passages as confirmed positives.

12.1.6 Third passage

12.1.6.1 Perform a third passage, as described in Section 12.1.4, with the negative assay controls and any cell cultures that were negative during the first passage and positive in the second passage.

NOTE: Other vessels that were either negative or positive in both the first and second passages do not need to be carried through the third passage.

12.1.6.2 Score cultures that develop CPE in both the second and third passages as confirmed positives.

12.2 Virus Quantitation

12.2.1 Record the total number of confirmed and not confirmed positive and negative cultures for each test sample on a Total Culturable Virus Data Sheet (Section 17.3).

12.2.2 Transfer the number of cultures inoculated and the number of confirmed positive cultures for each test sample from the Total Culturable Virus Data Sheet to the Quantitation of Total Culturable Virus Data Sheet (Section 17.4).

12.2.3 Calculate the MPN/mL value (M_{mL}) and the upper (CL_{umL}) and lower (CL_{lmL}) 95% confidence limits/mL, using the number of confirmed positive cultures from Step 12.2.2 and EPA's Most Probable Number Calculator (Item 6.5.11).

12.2.4 Record the MPN/mL and upper and lower 95% confidence limits/mL values obtained on the Quantitation of Total Culturable Virus Data Sheet.

12.2.5 Calculate the MPN/L value (M_L) of the original test sample using Equation 2,

$$M_L = \frac{M_{mL}\ S}{D} \qquad \text{Eq. 2}$$

where M_{ml} is the MPN/mL value in Step 12.2.4, *S* is the *Assay Sample Volume*, and *D* is the *Volume of Original Water Sample Assayed*; the values for *S* and *D* can be found on the Virus Data Sheet.

NOTE: For example, if the test sample described in the second note to Step 11.2.6.1, (with an *Inoculum Volume* equal to 0.833 mL) had 4 positive replicates, the MPN/mL value would be 0.61 with 95% Confidence Limits of 0.12–1.31. The MPN/L value then equals 0.0102 [(0.61 MPN/mL x 8.33 mL)/500 L].

12.2.5.1 For M_{mL} values of 0, calculate the test sample detection limit rather than the M_L value, by dividing 1 by *D*. Report as equal to or less than the calculated detection limit.

12.2.6 Record the MPN/L (M_L) value on the Virus Data Sheet.

12.2.6.1 For test samples where more than 1 cartridge filter or a prefilter was used, record the total MPN/L value and Confidence Limits/L values (calculated in Steps 12.2.7 and 12.2.8) for all filters on the Virus Data Sheet, recording individual totals for each filter under "Other Comments."

12.2.7 Calculate the lower 95% confidence limit/L value (CL_L) for each test sample using Equation 3,

$$CL_L = \frac{CL_{lmL}S}{D} \qquad \text{Eq. 3}$$

where CL_{lmL} is the lower 95% confidence limit/mL from the Quantitation of Total Culturable Virus Data Sheet, *S* is the *Assay Sample Volume*, and *D* is the *Volume of Original Water Sample Assayed*; the values for *S* and *D* can be found on the Virus Data Sheet.

NOTE: Continuing with the example in the note to Step 12.2.5, the CL_L of this test sample equals 0.002 [(0.12 CL_{lmL} x 8.33 mL)/500 L].

12.2.7.1 Record the lower 95% confidence limits/L values on the Virus Data Sheet.

12.2.8 Calculate the upper 95% confidence limit/L value (CL_U) using Equation 4,

$$CL_U = \frac{CL_{umL}S}{D} \qquad \text{Eq. 4}$$

where CL_{umL} is the upper 95% confidence limit/mL from the Quantitation of Total Culturable Virus Data Sheet, *S* is the *Assay Sample Volume*, and *D* is the *Volume of Original Water Sample Assayed*; the values for *S* and *D* can be found on the Virus Data Sheet.

NOTE: Continuing with the example from the note to Step 12.2.5, the CL_U of this test sample equals 0.0218 [(1.31 CL_{umL} x 8.33 mL)/500 L].

12.2.8.1 Record the upper 95% confidence limits/L values on the Virus Data Sheet.

12.2.9 Calculate the total MPN value and the total 95% confidence limit values for each QC samples by multiplying the values/mL by *S* and dividing by 0.3.

13.0 ENTEROVIRUS AND NOROVIRUS MOLECULAR ASSAY

The molecular assay uses RT-qPCR to provide a quantitative estimate of enterovirus and norovirus genomic copies per liter (GC_L) in environmental and drinking waters. Only microliter (µL) volumes can be analyzed by RT-qPCR, so the procedure includes additional concentration (Section 13.2) of any viruses present in the test sample beyond that required for culture. The RNA from each test sample is reversed transcribed using triplicate assays and random primers (Item 7.5.11) to prime the transcription (Section 13.4). The cDNA from each reverse transcription reaction is split into five separate assays and analyzed by qPCR (Section 13.5; Figure 5).

Surface and ground waters may contain substances that interfere with RT-qPCR, so the assay uses RNA extraction (section 13.3) to reduce inhibition. The assay also uses a hepatitis G control to identify test samples that are inhibitory to RT-qPCR (section 13.6).

The assay uses primers and probes from the scientific literature (Table 4) that are designed to detect many enteroviruses and noroviruses. Standard curves (sections 13.7) or stored standard curves with calibrators (section 13.8) are used for quantitation. These standards are prepared from an Armored RNA® reagent that contains the target sequence for the primer/probe sets. Armored RNA was chosen for standard curves and calibrators because it is difficult to obtain high-titered norovirus stocks.

13.1 Preliminary Procedures

13.1.1 Prepare 100-µM stock solutions of each oligonucleotide primer and probe (Item 7.5.1), if not supplied as 100-µM solutions.

NOTE: Preparation of primers and probes must be performed in a clean room or other location to minimize the possibility of false positive reactions. A clean room or location is one in which molecular and microbiological procedures are not performed.

13.1.1.1 Centrifuge the vial containing the primer or probe in a microcentrifuge (Item 6.6.4) for 30 sec.

13.1.1.2 Dissolve each primer or probe in a microliter volume of PCR-grade water (Item 7.5.2) that equals the number of nanomoles (nmol) shipped (as identified on the specification sheet from the manufacturer) times 10 (e.g., if a primer contains 51.0 nmol, resuspend in 510 µL). Vortex (Item 6.6.6) to mix.

13.1.1.2.1 Measure the absorbance (e.g., Item 6.6.1 using 260 10 mm path function) of a 100-fold dilution of the primer or probe at 260 nm.

NOTE: This step is recommended but optional for primers or probes supplied as 100-μM solutions by the manufacturer.

13.1.1.2.2 Calculate the total extinction coefficient for each primer and probe as described in Table 5.

NOTE: Total extinction coefficients supplied by the manufacturer may be used, if the units are converted to μM^{-1} cm^{-1} (e.g., units on Applied Biosystems specification sheets are in M^{-1} cm^{-1} and can be converted by dividing their total extinction coefficients by 10^6).

13.1.1.2.3 Calculate the theoretical absorbance. The theoretical absorbance for a 100 μM solution diluted 100-fold and measured at 260 nm in a 10 mm light path equals the total extinction coefficient for the primer [e.g., the theoretical absorbance for the EntP probe (Table 4) with a total extinction coefficient of 0.3178 (see example in Table 5) is 0.3178].

NOTE: If a dilution other than 100-fold is used to obtain the observed absorbance, multiply the theoretical absorbance by a factor equal to 100 μM divided by the dilution factor of the dilution used (e.g., if a 1000-fold dilution is used with the enterovirus TaqMan probe above, the theoretical absorbance is 0.3178 x 100/1000 = 0.0318).

NOTE: If a light path other than 10 mm is used to obtain the observed absorbance, multiply the theoretical absorbance by a factor equal to the light path used in mm divided by 10 mm (e.g., if a 100-fold dilution and 3 mm light path is used with the enterovirus TaqMan probe above, the theoretical absorbance is 0.3178 x 3/10 = 0.0953).

13.1.1.2.4 Compare the theoretical absorbance with the 260 nm reading from Step 13.1.1.2.1.

13.1.1.2.5 If the observed reading differs by more than ±10% from the theoretical absorbance value (e.g., <0.3078->0.3278 for the example in Step 13.1.1.2.3), check to ensure that the correct volume was used to dilute the oligonucleotide primer or probe, that the 100-fold dilution was performed correctly, and that the theoretical absorbance value was calculated properly. If these values are correct, repeat Step 13.1.1.2.1.

13.1.1.2.6 If after repeating the 260 nm reading, the value is still more than 10% from the theoretical value, calculate the actual concentration by dividing the absorbance reading by total extinction coefficient and multiplying the result by 100.

NOTE: If a dilution other than 100-fold was used for the 260 nm reading, multiply the concentration by the dilution factor used instead of 100. If a light path other than 10 mm was used, multiply the resulting concentration by 10 and divide by the actual light path mm value.

13.1.2 Prepare 10-µM primer and probe working solutions by diluting the stock solutions 1:10 (or by a dilution that compensates for the actual concentration calculated in Step 13.1.1.2.6) in PCR-grade water.

13.1.3 Aliquot primer and probe stocks and working solutions and store at -20 ºC.

13.1.4 Record the sample number (from the Sample Data Sheet that was packed with the test sample), the analytical laboratory name and identification number (if assigned), the analytical laboratory address, and the analyst name and identification number (if assigned) on a Molecular Virus Protocol Data Sheet (Item 17.5), a Molecular Virus Quality Control Data Sheet (Item 17.6), and a Molecular Virus Results Data Sheet (Item 17.7).

NOTE: If analysts work together as a team, record the names and identification numbers of all analysts. If different analysts perform portions of the molecular protocol steps, each analyst should only record the steps he/she performs. If necessary, separate data sheets for each analyst may be used.

13.2 Tertiary Concentration

13.2.1 Preliminary procedures

13.2.1.1 For each test sample to be analyzed, label a Vivaspin 20 unit (Item 6.6.2) with the sample number, analyst's initials, and date.

13.2.1.2 Fill the Vivaspin 20 unit with PBS, 0.2% BSA (Item 7.5.5), and soak at least 2 h at room temperature or overnight at 4 ºC.

13.2.1.3 Record the subsample number and the sample batch number on the Molecular Virus Protocol Data Sheet, the Molecular Virus Quality Control Data Sheet, and the Molecular Virus Results Data Sheet.

13.2.2 Discard the PBS, 0.2% BSA from the Vivaspin 20 unit, and add an amount of the appropriate subsample 2 (Section 11.2.6.3.5) equal to the *Assay Sample Volume (S)* noted on the test sample's Virus Data Sheet.

13.2.2.1 Record the date and time of tertiary concentration and the initials of the analyst performing the concentration on the Molecular Virus Protocol Data Sheet.

13.2.2.2 Record the concentrator catalog and lot numbers and the *Assay Sample Volume* on the Molecular Virus Protocol Data Sheet.

13.2.2.3 Centrifuge at 3,000 x *g* and 4 °C with swinging buckets (Items 6.4.9 and 6.6.3) until the subsample has been concentrated down to about 50 µL.

13.2.2.4 Add 1 mL of sterile 0.15-M sodium phosphate, pH 7–7.5 (Item 7.3.6), and repeat Step 13.2.2.3.

13.2.2.5 Repeat Step 13.2.2.4 one additional time.

13.2.3 Transfer the concentrate to a 1.5-mL microcentrifuge tube (Item 6.6.5).

13.2.4 Measure the volume, and add 0.15-M sodium phosphate, pH 7–7.5 to bring the total volume to 0.4 mL.

13.2.4.1 Record this final tertiary concentrated sample volume on the Molecular Virus Protocol Data Sheet.

13.2.4.2 Immediately proceed to Section 13.3 or hold at 4 °C for no more than 24 h.

CAUTION: Freezing and thawing leads to norovirus losses.

13.3 Nucleic Acid Isolation

13.3.1 Preliminary procedures

13.3.1.1 Record the date and time the nucleic acid extraction is performed and the initials of the analyst performing the extraction on the Molecular Virus Protocol Data Sheet.

13.3.1.2 Record the catalog and lot number of the nucleic acid extraction kit (Item 7.5.6) used on the Molecular Virus Protocol Data Sheet.

NOTE: Although a DNA extraction kit is used, the modifications to the manufacturer's protocol

described below must be used for efficient extraction of RNA.

13.3.1.3 Prepare a stock solution of carrier RNA (from Item 7.5.7)

13.3.1.3.1 Add 310 µL of Buffer AVE (Item 7.5.8) to the vial with the carrier RNA to obtain a final concentration of 1 µg/µL and mix to dissolve. Aliquot the dissolved carrier RNA and store at -20 °C.

NOTE: Prepare a sufficient number of aliquots so that each aliquot does not have to be frozen and thawed more than three times.

13.3.1.4 Prepare a working solution of carrier RNA

13.3.1.4.1 Add dissolved carrier RNA (Step 13.3.1.3.1) to Buffer AVL (Item 7.5.7) to give a concentration of 0.027 µg/µL.

NOTE: A concentration of 0.027 µg/µL can be prepared by adding 5.6 µL of the dissolved carrier RNA to 200 µL of Buffer AVL per test sample (i.e., 5.6 µL carrier RNA x number of test samples + 200 µL Buffer AVL x number of test samples).

CAUTION: Do not use the Buffer AL supplied with Item 7.5.6.

13.3.2 RNA Extraction

13.3.2.1 For each test sample and control to be processed, label a 1.5-mL microcentrifuge tube with test sample identification, add 200 µL of the a final tertiary concentrated sample from Step 13.2.4, a standard curve from Step 13.7.4, a calibrator from Step 13.8.2.1.2, or culture positive lysate from Step 12.1.4.3 (for confirmation of culture positive results), and vortex briefly to mix.

13.3.2.1.1 Record the amount of final tertiary concentrated sample used on the Molecular Virus Protocol Data Sheet.

13.3.2.1.2 Freeze any remaining tertiary concentrate at -70 °C.

13.3.2.1.3 Run a negative RNA extraction control each time RNA extractions are performed. Prepare the negative RNA extraction control by adding 200

μL of AE buffer (from item 7.5.6) to a labeled 1.5 mL microcentrifuge tube.

13.3.2.2 Add 200 μL of Buffer AVL with carrier RNA from Step 13.3.1.4 to the microcentrifuge tube and vortex for 15 sec.

13.3.2.3 Incubate at 56 °C for 10 min.

13.3.2.4 Centrifuge at >5,000 x *g* for about 5 sec in a microcentrifuge.

13.3.2.5 Add 200 μL of ethanol (Item 7.5.9), vortex for 15 sec, and then centrifuge at >5,000 x *g* for about 5 sec.

13.3.2.6 Add the mixture to a QIAamp Mini Spin column (Item 7.5.6), taking precautions to avoid wetting the rim of the tube.

13.3.2.7 Close the cap, and centrifuge at 6,000 x *g* for 1 min.

13.3.2.8 Check to determine if the sample has completely passed through the column.

13.3.2.8.1 If it has not, centrifuge again for 1 min at 10,000–20,000 x *g*, or for longer times, until the sample has completely passed through the column.

13.3.2.9 Place the Mini Spin column into a clean 2-mL collection tube (Item 7.5.6), and discard the collection tube containing the filtrate.

13.3.2.10 Add 500 μL of Buffer AW1 (Item 7.5.6) without touching the tube rim.

13.3.2.11 Centrifuge at 6,000 x *g* for 1 min, and again, transfer the column to a clean collection tube and discard the tube containing the filtrate.

13.3.2.12 Add 500 μL of Buffer AW2 (Item 7.5.6) without touching the tube rim.

13.3.2.13 Centrifuge at 20,000 x *g* for 3 min, and again, transfer the column to a clean collection tube and discard the tube containing the filtrate.

13.3.2.14 Centrifuge at 20,000 x *g* for 1 min.

13.3.2.15 Add 40 units of RNase Inhibitor (Item 7.5.10) to a clean 1.5-mL microcentrifuge tube (Item 6.6.5), and transfer the column from the collection tube to the microcentrifuge tube. Discard the collection tube.

NOTE: Alternatively, RNase Inhibitor can be added to an amount of Buffer AE (Item 7.5.6) sufficient for the number of samples to be eluted, at a concentration of 400 units/mL (i.e., in place of adding it to the microcentrifuge tubes).

13.3.2.16 Add 50 µL of Buffer AE to the column.

13.3.2.17 Incubate at room temperature for 1 min, and then centrifuge for 1 min at 6,000 x *g*.

13.3.2.18 Repeat Steps 13.3.2.16–13.3.2.17.

13.3.2.19 Remove and discard the column.

13.3.2.20 Proceed immediately to Section 13.4, or prepare aliquots and store the RNA at -70 °C until it can be assayed.

13.3.2.20.1 Record the RNA extract final volume on the Molecular Virus Protocol Data Sheet.

13.4 Reverse Transcription (RT)

13.4.1 Preliminary procedures (to be performed in a clean room)

13.4.1.1 Label PCR plates or tubes (Item 6.6.15) with appropriate test sample numbers.

13.4.1.2 Prepare RT Master Mix 1 and 2 using the guide in Table 6.

NOTE: The amounts shown for the volume per master mix can be scaled up or down according to the number of samples that need to be analyzed.

13.4.1.2.1 Record the date and time prepared and the initials of the preparer on the Molecular Virus Protocol Data Sheet.

13.4.1.3 Vortex the master mixes after the addition of all ingredients.

13.4.1.4 Centrifuge at ≥500 x *g* for 10 sec in a microcentrifuge.

13.4.2 Use a multichannel pipette (Items 6.6.9 and 6.6.11) to aliquot 16.5 µL of RT Master Mix 1 (Step 13.4.1.2) to the labeled PCR tubes or plate wells.

13.4.3 Run the RNA from every test sample in triplicate by adding 6.7 µL of the appropriate sample to each of the tubes or plate wells labeled for that sample (see Figure 5 for a schematic of the RT-qPCR process).

NOTE: It is not necessary to prepare 1:5 and 1:25 dilutions of the QC samples as done for the culture assay (in Step 12.1.2.3).

13.4.3.1 Record the RNA extract volume used, the date and time that the reverse transcription assays are performed, and the initials of the person running the assays on the Molecular Virus Protocol Data Sheet.

13.4.4 Add 6.7 µL of PCR grade water (Item 7.5.2) to one or more tubes or plate wells (Item 6.6.15) as no template controls (NTC).

13.4.4.1 Include at least one NTC for the replicates associated with every fourth test sample run on a plate.

NOTE: NTC controls must be distributed throughout the plate.

13.4.4.2 If any NTC control is positive, the cause of the false positive value should be investigated. After fixing the cause of the problem, all test samples must be rerun.

13.4.5 Close the tubes or seal the plates, and heat at 99 °C for 4 min, followed by quenching on ice, or a hold temperature of 4 °C.

13.4.6 Add 16.8 µL of RT Master Mix 2 (Step 13.4.1.2) to each tube or well.

13.4.7 Centrifuge at ≥500 x *g* for 10 sec at 4 °C in a centrifuge (Item 6.6.4 for tubes; Item 6.6.13 for plates).

13.4.8 Place the tubes or plates in a thermal cycler and run at 25 °C for 15 min, 42 °C for 60 min, and 99 °C for 5 min, followed by a 4 °C hold cycle.

NOTE: Thermal cyclers from a number of different manufacturers can be used for this and the following real-time quantitative PCR step (Section 13.5). Analysts must follow the manufacturers' instructions for set-up, runs, and analysis for the instrument used.

13.4.8.1 Record the make and model of the thermal cycler used on the Molecular Virus Protocol Data Sheet.

13.4.9 Centrifuge at ≥500 x *g* for 10 sec at 4 °C in a centrifuge (Item 6.6.4 for tubes; Item 6.6.13 for plates).

13.4.10 Proceed immediately to Section 13.5, or store reverse transcribed samples at -70 °C until they can be processed.

NOTE: Samples can be held at 4 °C for up to 4 h prior to qPCR

13.5 Real-Time Quantitative PCR (qPCR)

13.5.1 Preliminary procedures

13.5.1.1 Label PCR plates or tubes (Item 6.6.15) with appropriate test sample numbers.

NOTE: Each test sample will require 15 plate wells or tubes (i.e., 3 RT replicates x 5 qPCR assays; see Figure 5).

13.5.1.2 Prepare PCR master mixes using the guides in Table 7 for enterovirus, Table 8 and Table 9 for norovirus genogroup I, Table 10 for norovirus genogroup II, and Table 11 for hepatitis G.

NOTE: The amounts shown for the volume per master mix can be scaled up or down according to the number of test samples that need to be analyzed.

13.5.1.2.1 Vortex the master mix after the addition of all ingredients.

13.5.1.2.2 Centrifuge at ≥500 x *g* for 10 sec at 4 °C in a centrifuge (Item 6.6.4 for tubes; Item 6.6.13 for plates).

13.5.1.2.3 Record the date and time prepared and the initials of the preparer on the Molecular Virus Protocol Data Sheet.

13.5.2 Dispense 14 µL of the appropriate mix to the labeled plates or tubes.

13.5.3 Add 6 µL of the appropriate test sample from Step 13.4.10 to each tube or plate (Figure 5).

13.5.3.1 Record the volume used on the Molecular Virus Protocol Data Sheet.

13.5.4 Place tubes or plates in a thermal cycler and run with a setting of 1 cycle at 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 sec, and 60 °C for 1 min.

13.5.4.1 Record the run number on the Molecular Virus Protocol Data Sheet, Molecular Virus Quality Control Data Sheet, and the Molecular Virus Results Data Sheet.

13.5.4.2 Record the date and time that the qPCR assays are performed and the initials of the person running the assays on the Molecular Virus Protocol Data Sheet.

13.5.4.3 Record the make and model of the thermal cycler used on the Molecular Virus Protocol Data Sheet.

13.5.5 Analyze the results of each run (according to the instructions of the manufacturer of the thermal cycler used) to calculate the *Genomic Copy* numbers of unknown test samples based upon the standard curve samples described in Sections 13.7 or 13.8.

13.5.5.1 Record the *GC* values of each replicate on the Molecular Virus Results Data, along with the mean and standard deviation of the 3 replicates for each test sample.

NOTE: Include non-detects (zeros) in the calculation of the mean.

13.5.5.2 Record the results of the negative RNA extraction control and the no template controls on the Molecular Virus Quality Control Data Sheet.

13.5.6 Calculate the *Genomic Copies per L (GC_L)* for each test sample using Equation 5 and the mean *GC* value from Step 13.5.5,

$$GC_L = \frac{GC \times 199 \times DF}{D} \qquad \text{Eq. 5}$$

where *DF* equals the reciprocal of any dilution performed to compensate for inhibition (see Section 13.6; e.g., 5 and 25 for 1:5 and 1:25 dilutions, respectively, or 1 for undiluted samples) and *D* equals the *Volume of Original Water Sample Assayed* (see Step 11.2.6.1).

NOTE: 199 is the total dilution factor for the volume reductions that occur in Sections 13.2–13.5.

NOTE: For example, if the PCR assay from the test sample described in the second note to Step 11.2.6.1 detects 15 genomic copies in a 1:5 dilution, then the number of *Genomic Copies per L* is 29.85 [(15 x 199 x 5)/500 L].

13.5.6.1 Record the GC_L value on the Molecular Virus Results Data Sheet.

13.5.6.2 For test samples with a mean value of 0, report the *Genomic Copies per L* as less than or equal to the detection limit (i.e., $\leq$1/D).

13.5.7 Calculate the *Genomic Copies* of QC samples by multiplying the mean *GC* value by 199 and dividing by 0.3.

13.6 Inhibition Control

NOTE: A control for inhibition must be performed to reduce false negative results caused by matrix interference (Section 4.2). This method uses hepatitis G Armored RNA as an inhibition control. The inclusion of hepatitis G Armored RNA in all assays has two major advantages compared to the typical approach of seeding a portion of each field sample with a specific enterovirus or norovirus strain (18.18). First, it reduces the number of assays that need to be run by one-half, thereby reducing labor and assay costs. Second, it reduces cross-contamination that can occur between seeded and unseeded field samples when the typical approach is used.

NOTE: Laboratories may choose from three options when running the inhibition control. All three options are evaluated using Step 13.6.2.1 and may require dilutions as defined in Step 13.6.2.1-13.6.2.2. Option 1: the hepatitis G RT-qPCR assay is run on all test samples before all the other RT-qPCR assays. The enterovirus and norovirus assays are then run either without dilution for test samples giving no inhibition and with dilution for test samples showing inhibition. Option 2: all test samples are diluted as described in Step 13.6.2.1 and then the RT assay (Section 13.4) is performed on all diluted and undiluted test samples, followed by running the hepatitis G qPCR assay. The enterovirus and norovirus qPCR assays are then performed using the cDNA from the undiluted test sample, if not inhibited, or from the lowest dilution that does not show inhibition.

Option 3: RT-qPCR assays are run on all test samples, followed by rerunning any sample showing inhibition using the Steps 13.6.2.1-13.6.2.2.

13.6.1 Preliminary procedure

13.6.1.1 Process a volume of FCSV from at least 2 negative QC controls (Step 8.4.1), hereafter designated "negative FCSV," equal to the *Assay Sample Volume (S)* using Sections 13.2 to 13.3.

13.6.1.1.1 Using 6.7 µL of the RNA from one of the negative FCSV samples from Step 13.6.1.1 for each replicate, run 5 hepatitis G RT and qPCR assays. Run 5 additional replicate hepatitis G RT and qPCR assays using the other negative FCSV.

13.6.1.1.2 Calculate the mean *Cq* value and standard deviation for the 10 replicate hepatitis G assays.

13.6.1.1.3 Assign a lot number to the mean value of the 10 replicates, starting with "1," and record the lot number on the Molecular Virus Quality Control Data Sheet. Increment the lot number for subsequent repeats of Step 13.6.1.1.

13.6.1.1.4 Record the mean *Cq* and standard deviation values on the Molecular Virus Quality Control Data Sheet.

NOTE: The mean value should be 25–32 Cq units. The standard deviation of the mean should be <0.3 units.

13.6.1.1.5 If the mean value is not between 25 and 32 Cq units, readjust the amount of Hepatitis G Armored RNA added to RT Master Mix 1 (see Table 6) and repeat Step 13.6.1 until the value is within the acceptable range. Once an acceptable value is found, substitute the amount of Hepatitis G Armored RNA indicated in Table 6 with the amount that produced an acceptable level and compensate by adjusting the amount of PCR grade water added.

13.6.2 Compare the Hepatitis G Cq values obtained with all test samples against the mean Cq value calculated in Step 13.6.1.1.2.

13.6.2.1 If the value in the unknown test samples is more than 1 Cq value higher than that calculated in Step 13.6.1.1.2, dilute the unknown test sample 1:5 and 1:25 in PCR grade water (Item 7.5.2).

13.6.2.2 Re-run the unknown test sample, along with the 1:5 and 1:25 dilutions.

13.6.2.3 Calculate the test sample concentration using the highest dilution for which the Hepatitis G Cq values are within 1 unit of the value calculated in Step 13.6.1.1.2.

13.6.2.4 If the inhibition control fails again and the test sample Cq value is lower than 38, re-run the sample at higher 5-fold dilutions.

13.6.2.5 If any test sample run at the higher dilution fails the inhibition control again, or if any unknown test samples are below the detection limit (e.g., Cq values of 45 or higher), list the test sample as a potential false negative sample on the Molecular Virus Results Data Sheet.

13.7 Standard curves

NOTE: Standard curves must be run with every test sample (e.g., every field and quality control) as described in this section or in Section 13.8. Standard curves should be prepared using Armored RNA (Item 7.5.19), but unless specified otherwise by EPA, may be prepared using Sabin poliovirus 3 and norovirus GI and GII stocks or transcribed RNA from plasmids containing the appropriate viral sequence.

13.7.1 Preparation of working stocks for standard curves

13.7.1.1 To use Armored RNA containing the enterovirus, norovirus GI, and norovirus GII sequences (Item 7.5.19) for standard curves, dilute the Armored RNA in negative FCSV (see Step 13.6.1.1) to give a concentration of 2.5 $x10^8$ particles/mL based upon the concentration of the Armored RNA lot supplied.

NOTE: 1 Armored RNA particle/mL equals 1 Genomic Copy/mL.

13.7.1.2 To use virus stocks, determine the titer of each stock using RT-qPCR.

13.7.1.2.1 Perform RT-qPCR assays on each stock using serial 10-fold dilutions and 10 replicates per dilution. Obtain the MPN/mL virus titer using EPA's Most Probable Number Calculator (Item 6.5.11). Change the calculator's "Number of Dilutions" to 3, the "Number of Tubes per dilution" to 10, and the "Dilution Type" to Standard 10-Fold Serial. For each stock, input the number of positive replicates from the highest dilution giving at least one positive replicate and from the next two lower dilutions.

13.7.1.2.2 Dilute each viral stock to 2.5 $x10^8$ MPN/mL.

NOTE: If the efficiency of the standard curve derived from each virus stock is in the acceptable range (see notes to Steps 13.7.5–13.7.6), substitute the term Genomic Copy/mL for MPN/mL.

13.7.1.3 To used transcribed RNA, prepare RNA from plasmids containing the targets for each assay and titer each using standard methods [e.g., see Reference (18.34)].

13.7.1.3.1 Dilute the transcribed RNA to 2.5 x 10^8 transcripts/mL.

NOTE: Substitute the term Genomic Copy/mL for transcripts/mL.

13.7.2 Divide the standard curve working stocks into 250 µL aliquots and freeze at or below -70 °C.

13.7.3 Prepare 5 ten-fold serial dilutions of each Armored RNA working stock (or of each alternative virus or transcribed RNA working stock).

13.7.3.1 Add 25 µL of the working stock containing 2.5 x 10^8 Genomic Copies/mL (Step 13.7.1) to 225 µL of negative FCSV. Vortex for 5–15 sec.

13.7.3.2 Add 25 µL of the dilution in Step 13.7.3.1 to 225 µL of negative FCSV. Vortex again and continue the dilution process to prepare a total of 5 ten-fold dilutions.

NOTE: The final concentrations of the 5 dilutions are $2.5x10^7$, $2.5x10^6$, $2.5 x10^5$, $2.5x10^4$, and $2.5x10^3$ Genomic Copy/mL.

13.7.4 For each Armored RNA standard (or alternative), run 200 µL of the working stock and each of the 5 ten-fold dilutions separately through Steps 13.3–13.5.5, using the volumes described in the steps and only the specific primers/probe for the Armored RNA standard.

13.7.4.1 Identify the samples as standards in the thermal cycler (Item 6.6.16) software.

13.7.4.2 For each dilution, enter the genomic copy values shown in Table 12 into the standards section of the software.

13.7.5 Calculate the standard curve slope and R^2 values for each standard curve by plotting Cq values against the log of the concentration for each point or, if available, by using the slope and R^2 values determined by the qPCR instrument.

NOTE: An acceptable standard curve will have an R^2 value >0.97 and a standard deviation of <0.25. Standard deviations ≥0.25 represent errors in preparing dilutions or in pipetting.

13.7.6 Calculate the percent amplification efficiency using Equation 6:

$$\%Efficiency = 100 \times (10^{-1/slope} - 1) \quad \text{Eq. 6}$$

13.7.6.1 An acceptable standard curve will have an amplification efficiency of 80–110%.

NOTE: The ideal efficiency occurs when the slope equals -3.32; in this case, the % Efficiency equals 100 [100 x ($10^{-1/-3.32}$ – 1) = 100 x (2.0-1)].

NOTE: Efficiencies less than 90% may indicate technical problems. Laboratories should strive to have standard curves in the 90–110% range.

13.7.7 Record the amplification efficiencies on the Molecular Virus Protocol Data Sheet.

13.7.8 Standard curves that meet the criteria specified in Steps 13.7.5–13.7.6 must be used to calculate genomic copies of unknown test samples in Step 13.5.5.

13.8 Preparation of stored standard curves and calibrators

13.8.1 Stored standard curves

13.8.1.1 If all the enterovirus and norovirus standard curves can be stored in the quantitative PCR thermal cycler (Item 6.6.16), stored standard curves may be used as an alternative to running standard curves with every test sample analyzed (Section 13.7).

CAUTION: Calibrators (Step 13.8.2) must be run with all unknown test samples when using stored standard curves, but they may be run even if standard curves are run with every sample as an additional quality check.

13.8.1.1.1 Prepare stored standard curves by running each standard as described in Section 13.7 three times.

13.8.1.1.2 Calculate the mean for each dilution for each standard and store the mean values in the thermocycler.

CAUTION: The stored standard curve must meet the acceptance criteria found in Steps 13.7.5 and 13.7.6.

13.8.1.1.3 Record the amplification efficiencies of each stored standard curve on the Molecular Virus Protocol Data Sheet.

13.8.1.2 Generate and record new stored standard curve sets, as described in Sections 13.8.1.1.1 every eighth analysis batch

(Section 3.1) or every 2 months, whichever comes first; or anytime a calibrator fails twice in a row to meet acceptance criteria.

13.8.2 Calibrators

13.8.2.1 Prepare calibrators for each virus standard by choosing the dilution from the standard curve that gives the Cq value closest to, but not greater than 32.

13.8.2.1.1 Prepare the dilution corresponding to the chosen value in negative FCSV and extract the RNA as described in Section 13.3.

NOTE: Prepare a sufficient number of dilutions to last for the entire study, taking into consideration that each 200-µL extraction will yield sufficient material for about 14 runs.

13.8.2.1.2 Aliquot into single run batches and store at -70 °C.

13.8.2.2 Run a set of at least 10 calibrators from each Armored RNA standard.

13.8.2.2.1 Calculate the mean *Cq* value and standard deviations.

13.8.2.2.2 Record the mean and standard deviation values as the *Target Value* on the Molecular Virus Results Data Sheet.

NOTE: The standard deviation must be <0.25 units.

13.8.2.3 Run all calibrators with every set of unknown test samples.

13.8.2.4 Accept a test sample if the value of the calibrator for each corresponding PCR assay falls within 1.0 Cq unit of the calibrators' mean values.

13.8.2.4.1 Record each calibrator's Cq value on the Molecular Virus Results Data Sheet.

13.8.2.5 Reject and rerun test samples from a PCR assay where the calibrator for that assay falls outside the acceptance criteria.

13.8.2.5.1 Repeat the run once upon failure.

13.8.2.5.2 If the assay fails again, generate new stored standard curves or take steps to determine the cause of the failure.

14.0 METHOD PERFORMANCE

14.1 Culturable Assay

14.1.1 This method is subject to a number of biases that reduce its precision and accuracy.

14.1.1.1 The isoelectric point of the virus particle affects its ability to bind to and be eluted from electropositive filters. The isoelectric point can vary significantly across virus species and even within members of the same species.

14.1.1.2 Other capsid and matrix related characteristics and substances could affect virus recovery at various stages of the method.

14.1.1.3 The passage number of the BGM cell line and the media used to passage and maintain cells is known to affect the ability of viruses to replicate in cells.

14.1.2 The best performance data for the method comes from the PE samples that were analyzed during the ICR.

NOTE: The performance characteristics given below are based upon Sabin poliovirus type 3 and may not be reflective of other viruses that are detected by this method.

14.1.2.1 In total, 12 laboratories with 25 ICR-approved analysts analyzed 828 PE samples, consisting of low (<300 MPN per filter), medium (300–1,500 MPN per filter) and high (>1,500 MPN per filter) virus levels. The mean interlaboratory recovery was 56% with a coefficient of variation (CV) of 92%, a false negative rate of 1.3%, and a false positive rate of 1.1%. The highest mean recovery values (71%) were obtained from PE samples containing low virus levels. Table 13 shows the mean recovery and CV value ranges for individual analysts and for intralaboratory variation.

14.1.2.2 Although Method 1615 uses a different electropositive filter than the ICR study, both filters have been shown to give similar recoveries in a single study (18.25) and a four laboratory validation study (unpublished data).

14.1.3 The detection limit of the culture method is about 0.05 MPN/L for surface water and 0.01 MPN/L for groundwater.

14.1.4 The acceptance criteria for PE are set for the culturable assay at a mean recovery of 20–150%, with a CV $\leq$ 120%.

14.1.5 The acceptance criterion for QC and matrix spikes for the culturable procedure is a recovery of 5–200%.

14.2 Molecular Procedure

14.2.1 The molecular procedure is subject to the same bias as the culturable procedure in terms of virus adsorption and recovery from the electropositive filters and secondary concentration procedures. Additional bias can occur during tertiary concentration, RNA extraction, and RT-qPCR.

14.2.2 The method was tested using 7 groundwater test samples from 5 different wells with a range of physicochemical characteristics. In addition to bias from matrix effects, these tests may have had additional bias, because they were performed as matrix spikes as described in Section 8.6. The 7 groundwater test samples gave a mean recovery of 26% with a recovery range of 5–60% and a CV of 73%. These same test samples were also tested for norovirus recovery using murine norovirus and murine norovirus-specific primers and probe (not shown) with the Method 1615 protocols. Mean recovery of murine norovirus was 35%, with a recovery range of 7–63% and a CV of 69%.

14.2.3 The detection limit of the molecular method is based upon the overall detection limit of the RT-qPCR assay and the volume of the field sample assayed.

14.2.3.1 The detection limit for the poliovirus assay is about 2 *Genomic Copies per L* and 0.4 *Genomic Copies per L* for surface water and groundwater, respectively.

14.2.3.2 The detection limit can be increased by running more than 3 RT-qPCR replicates from each test sample.

14.2.4 The acceptance criteria for PE samples are set for the molecular procedure at a mean recovery of 15–175%, with a CV $\leq$ 130%.

14.2.5 The acceptance criterion for QC and matrix spikes for the molecular procedure is a recovery of 5–200%.

14.3 Performance Record

14.3.1 The laboratory shall maintain a record of the performance of QC and PE samples for both the culture and molecular portions of this method as described in Sections 8.4.3, 8.5.3, 8.6.3, and 8.7. This record can be useful for tracking and correcting decreases in performance before they become result in generation of unacceptable data.

14.3.2 EPA may maintain the performance record for EPA based studies.

15.0 STERILIZATION AND DISINFECTION

15.1 General Guidelines

15.1.1 Use aseptic techniques for handling test waters, eluates, and cell cultures.

15.1.2 Sterilize apparatus and containers that will be exposed to test waters and all solutions that will be added to test waters, unless otherwise indicated.

15.1.3 Thoroughly clean all items before final sterilization using laboratory SOPs.

15.1.4 Sterilize all contaminated materials before discarding.

15.1.5 Disinfect all spills and splatters.

15.2 Sterilization Techniques

15.2.1 Solutions

15.2.1.1 Sterilize all solutions, except those used for cleansing, standard buffers, HCl, NaOH, and disinfectants, by autoclaving them (Item 6.7.1) at 121 °C, 15 psi for at least 15 min.

NOTE: The HCl, NaOH, and disinfectants used are self-sterilizing.

15.2.1.2 When autoclaving buffered beef extract, use a vessel large enough to accommodate foaming.

15.2.2 Autoclavable vessels, glassware, plasticware, and equipment

15.2.2.1 Sterilize stainless steel vessels (dispensing pressure vessel) in an autoclave at 121 °C, 15 psi for at least 30 min.

NOTE: Add sufficient dH_2O to all vessels to be autoclaved, equal to about 1–2% of the vessel's rated volume. Water speeds the sterilization process by enhancing the transfer of heat.

NOTE: Place large vessels on their sides in the autoclave, if possible, to facilitate the displacement of air in the vessels by flowing steam.

NOTE: If vessel is equipped with a vent-relief valve, open during autoclaving and close immediately when vessel is removed from the autoclave.

15.2.2.2 Autoclavable glassware and plasticware

15.2.2.2.1 Cover the openings into autoclavable glassware, plasticware, and equipment loosely with aluminum foil (Item 6.7.3) before autoclaving and autoclave at 121 °C, 15 psi for at least 30 min.

NOTE: Glassware may also be sterilized in a dry heat oven (Item 6.7.2) at a temperature of 170 °C for at least 1 h.

15.2.2.2.2 Pre-sterilize 1MDS filters (Item 6.1.2.4), prefilters (Item 6.1.6.2), sterilizing filter stacks (Item

6.4.12), and aluminum foil (Item 6.2.10) by wrapping them in Kraft paper (Item 6.7.4) and autoclaving at 121 °C, 15 psi for 30 min.

CAUTION: Do not autoclave the NanoCeram filters specified in Item 6.1.2.4. These filters are sterilized by the manufacturer and have housings that cannot be autoclaved.

NOTE: Ten (10)-in cartridge prefilters (Item 6.1.6.2), but not NanoCeram or 1MDS filters, may be presterilized with sodium hypochlorite (see Section 15.2.4), as an alternative to autoclaving.

15.2.3 Instruments, such as scissors and forceps

15.2.3.1 Sterilize instruments, such as scissors and forceps, by immersing them in 95% ethanol (Item 7.6.1) and flaming them between uses.

15.2.4 Non-autoclavable equipment, plasticware (filter housings), tubing, and vessels

NOTE: Filter apparatus modules should be disinfected after use by sterilization and then cleaned according to laboratory SOPs before final sterilization.

15.2.4.1 Sterilize items that cannot be autoclaved by recirculating or immersing the items in 0.525% sodium hypochlorite (Item 7.6.2) for 30 min; pH electrodes should be sterilized with 0.525% sodium hypochlorite for at least 5 min.

15.2.4.2 Drain the hypochlorite from the objects being sterilized and rinse in sterile water.

15.2.4.3 Dechlorinate by recirculating or immersing the items in a solution containing 50 mL of 1-M sodium thiosulfate (Item 7.6.3) per liter of sterile dH_2O.

CAUTION: Ensure that the sodium hypochlorite (Step 15.2.4.1) and sodium thiosulfate (Step 15.2.4.3) solutions come in full contact with all surfaces when performing this procedure.

15.2.4.4 Cover the apparatus module ends and the injector port(s) with sterile aluminum foil.

15.2.4.5 Place the injector module and tubing into a sterile bag or wrapping in such a way that they may be removed without contaminating them.

15.2.5 Contaminated materials

15.2.5.1 Autoclave (Item 6.7.1) contaminated materials for at least 30 min at 121 °C, 15 psi.

NOTE: Be sure that steam can enter contaminated materials freely.

15.2.5.2 Disinfect spills and other contamination on surfaces with either a solution of 0.5% iodine (Item 7.6.4) or 0.525% sodium hypochlorite (Item 7.6.2) to ensure thorough disinfection.

NOTE: Many commercial disinfectants do not adequately kill enteric viruses.

NOTE: The iodine solution has the advantage of drying more rapidly on surfaces than sodium hypochlorite, but may stain some surfaces.

16.0 **TABLES AND FIGURES**

Table 1. Viruses Detected by EPA Method 1615

Virus genus or species	Detected by TCVA [(1)]	Detected by qPCR
Human enterovirus A	Some serotypes	Yes
Human enterovirus B	Most serotypes	Yes
Human enterovirus C	Some serotypes	Yes
Human enterovirus D	Some serotypes	Yes
Norovirus genogroup I and II	No	Many genotypes
Mammalian orthoreovirus	Yes	No

(1) TCVA – Total Culturable Virus Assay (Section 12.0)

Table 2. Specified and Recommended Field Sample Volumes

Water type	Flow rate [1] (L/min)	Sampling duration (h)	Sample volume (L) [2,3]
Sewage effluent	10	0.2	120 [4]
Surface	10	0.6	360 [5]
Finished/groundwater	10 [6]	3.0	1,800 [7]
Finished/groundwater	4 [8]	16±2	≤4,320 [7,9]

(1) Poliovirus retention is independent of flow rates between 4–20 L/min for NanoCeram filters (18.25), but a constant flow rate, such as described here, should be used for any single study. EPA may specify alternative flow rates for specific studies.
(2) Consistent field sample volumes should be used for any single study. EPA may specify alternative sample volumes for specific studies.
(3) Turbidity and other factors may affect the volume collected during any sampling event. The sampling duration must be increased to meet the specified or recommended volume during these situations. As an alternative, 2 cartridge filter modules may be used to obtain the specified volume.
(4) This is a recommended value for final sewage effluents. There is no recommended volume for raw sewage.
(5) The minimum specified volume is 300 L for surface waters.
(6) For disinfected waters, add 2% thiosulfate at a flow rate of 6.0±0.2 mL/min.
(7) The minimum specified volume is 1,500 L for treated tap or untreated groundwater.
(8) For disinfected waters, add 2% thiosulfate at a flow rate of 2.4±0.2 mL/min.
(9) For convenience, field samples may be collected by starting the sampling at the end of a workday and stopping it in the morning of the next day.

Table 3. MPN Program Settings

Item	Setting
Data entry mode	Keyboard
Dilution type	Standard 5-fold serial
Approximation type	Cornish & Fisher limits
Confidence level	95%
Number of dilutions	1 (or, is used, the number of dilutions)
Number of tubes per dilution	10
Inoculum volume (mL)	*Inoculum Volume* (Step 11.2.6.4)

Table 4. Primers and TaqMan® Probes for Virus Detection by RT-qPCR

Virus Group [1]	Primer/Probe Name/Sequence [2,3,4]	Reference
Enterovirus		(18.17)
	EntF: CCTCCGGCCCCTGAATG	
	EntR: ACCGGATGGCCAATCCAA	
	EntP: 6FAM-CGGAACCGACTACTTTGGGTGTCCGT-TAMRA	(18.33)
Norovirus GIA		(18.24)
	NorGIAF: GCCATGTTCCGITGGATG	
	NorGIAR: TCCTTAGACGCCATCATCAT	
	NorGIAP: 6FAM-TGTGGACAGGAGATCGCAATCTC-TAMRA	
Norovirus GIB		(18.12)
	NorGIBF: CGCTGGATGCGNTTCCAT	
	NorGIBR: CCTTAGACGCCATCATCATTTAC	
	NorGIBP: 6FAM-TGGACAGGAGAYCGCRATCT-TAMRA	
Norovirus GII		(18.12)
	NorGIIF: ATGTTCAGRTGGATGAGRTTCTCWGA	
	NorGIIR: TCGACGCCATCTTCATTCACA	
	NorGIIP: 6FAM-AGCACGTGGGAGGGGATCG-TAMRA	
Hepatitis G		(18.39)
	HepF: CGGCCAAAAGGTGGTGGATG	
	HepR: CGACGAGCCTGACGTCGGG	
	HepP: 6FAM-AGGTCCCTCTGGCGCTTGTGGCGAG-TAMRA	

(1) EPA may specify additional or alternative primer and probe sets for specific applications.
(2) Primers and probes are designated by the first three letters of the virus name followed by F, R, or P for forward, reverse, and probe. GIA, GIB, or GII are also added to the norovirus designations.
(3) All primer and probe sequences are 5' to 3'.
(4) Degenerate bases in primers and probes are as follows: N equals a mixture of all four nucleotides; R equals A + G; Y equals T + C; W equals A + T; and I equals inosine.

Table 5. Extinction Coefficients for Primers and Probes [1]

Chromophore	*Chromophore Extinction Coefficient* [2]
A	0.0152
T	0.0084
G	0.01201
C	0.00702
6FAM	0.020958
TAMRA	0.03198

(1) Calculate the total extinction coefficient of an oligonucleotide primer or probe by 1) multiplying the total number of each chromophore by its corresponding chromophore extinction coefficient and 2) summing the resulting values. Using the EntP probe from Table 4 as an example, the total extinction coefficient is 1 x 0.020958 + 4 x 0.0152 + 7 x 0.0084 + 8 x 0.01201 + 7 x 0.00702 + 0.03198 = 0.3178.

(2) Units for the extinction coefficients are $\mu M^{-1}\ cm^{-1}$

Table 6. RT Master Mix 1 and 2

Ingredient	Volume per reaction (µL) [1]	Final concentration	Volume per Master Mix (µL) [2]
	RT Master Mix 1		
Random primer (Item 7.5.11)	0.8	10 ng/µL (c. 5.6 µM)	84.0
Hepatitis G Armored RNA [3] (Item 7.5.12)	1.0		105.0
PCR grade water (Item 7.5.2)	14.7		1543.5
Total	16.5		1732.5
	RT Master Mix 2		
10X PCR Buffer II (Item 7.5.13)	4.0	10 mM tris, pH 8.3, 50 mM KCL	420.0
25-mM $MgCl_2$ (Item 7.5.13)	4.8	3 mM	504.0
10-mM dNTPs (Item 7.5.14)	3.2	0.8 mM	336.0
100-mM DTT (Item 7.5.15)	4.0	10 mM	420.0
RNase Inhibitor (Item 7.5.10)	0.5	0.5 units/µL	52.5
SuperScript II RT (Item 7.5.16)	0.3	1.6 units/µL	31.5
Total	16.8		1764.0

(1) The volumes given are for 40-µL RT assays.

(2) Reagent amounts sufficient for a 96-well PCR plate are given. The volumes shown were calculated by multiplying the volume per reaction amount by the number of assays to be performed, plus an additional 9 assays to account for losses during transfer of the master mix to plates (Item 6.6.15) using items 6.6.9 and 6.6.12. The amount of additional assays to add can be reduced if experience shows that lower amounts are adequate.

(3) Hepatitis G Armored RNA is supplied as an untitered stock. The amount to use must be determined for each lot, as described in Step 13.6.1.

Table 7. PCR Master Mix for Enterovirus Assay

Ingredient	Volume per reaction (µL) [1]	Final concentration	Volume per Master Mix (µL) [2]
2X LightCycler 480 Probes Master Mix (Item 7.5.17) [3]	10.0	Proprietary	1050.0
ROX reference dye (Item 7.5.18) [4]	0.4	0.5 mM	42.0
PCR grade water (Item 7.5.2)	1.0		105.0
10 µM EntF (Table 4)	0.6	300 nM	63.0
10 µM EntR (Table 4)	1.8	900 nM	189.0
10 µM EntP (Table 4)	0.2	100 nM	21.0
Total	14.0		1470.0

(1) The volumes given are for using 6 µL of cDNA from Step 13.5.3 in a qPCR assay using a total qPCR volume of 20 µL.
(2) Reagent amounts sufficient for a 96-well PCR plate are given. The volumes shown were calculated by multiplying the volume per reaction amount by the number of assays to be performed, plus an additional 9 assays to account for losses during transfer of the master mix to tubes or plates. The amount of additional assays to add can be reduced if experience shows that lower amounts are adequate.
(3) 10X PCR Buffer II (2 µL/reaction), 25-mM $MgCl_2$ (5 µL/reaction), and AmpliTaq Gold (0.2 µL/reaction) can be substituted for the LightCycler 480 Probe Master Mix.
(4) This reagent is necessary for use with Applied Biosystems and similar instruments. It should be substituted with PCR grade water for use with the LightCycler and similar instruments.

Table 8. PCR Master Mix for Norovirus GIA Assay

Ingredient	Volume per reaction (µL) [1]	Final concentration	Volume per Master Mix (µL) [2]
2X LightCycler 480 Probes Master Mix (Item 7.5.17) [3]	10.0	Proprietary	1050.0
ROX reference dye (Item 7.5.18) [4]	0.4	0.5 mM	42.0
PCR grade water (Item 7.5.2)	1.4		147.0
10 µM NorGIAF (Table 4)	1.0	500 nM	105.0
10 µM NorGIAR (Table 4)	1.0	500 nM	105.0
10 µM NorGIAP (Table 4)	0.2	100 nM	21.0
Total	14.0		1470.0

See Table 7 for footnotes (1)–(4).

Table 9. PCR Master Mix for Norovirus GIB Assay

Ingredient	Volume per Reaction (µL) [(1)]	Final Concentration	Volume per Master Mix (µL) [(2)]
2X LightCycler 480 Probes Master Mix (Item 7.5.17) [(3)]	10.0	Proprietary	1050.0
ROX reference dye (Item 7.5.18) [(4)]	0.4	0.5 mM	42.0
PCR grade water (Item 7.5.2)	0.3		31.5
10 µM NorGIBF (Table 4)	1.0	500 nM	105.0
10 µM NorGIBR (Table 4)	1.8	900 nM	189.0
10 µM NorGIBP (Table 4)	0.5	250 nM	52.5
Total	14.0		1470

See Table 7 for footnotes (1)–(4).

Table 10. PCR Master Mix for Norovirus GII Assay

Ingredient	Volume per Reaction (µL) [(1)]	Final Concentration	Volume per Master Mix (µL) [(2)]
2X LightCycler 480 Probes Master Mix (Item 7.5.17) [(3)]	10.0	Proprietary	1050.0
ROX reference dye (Item 7.5.18) [(4)]	0.4	0.5 mM	42.0
PCR grade water (Item 7.5.2)	0.3		31.5
10 µM NorGIIF (Table 4)	1.0	500 nM	105.0
10 µM NorGIIR (Table 4)	1.8	900 nM	189.0
10 µM NorGIIP (Table 4)	0.5	250 nM	52.5
Total	14.0		1358

See Table 7 for footnotes (1)–(4).

Table 11. PCR Master Mix for Hepatitis G Assay

Ingredient	Volume per Reaction (µL) [(1)]	Final Concentration	Volume per Master Mix (µL) [(2)]
2X LightCycler 480 Probes Master Mix (Item 7.5.17) [(3)]	10.0	Proprietary	1050.0
ROX reference dye (Item 7.5.18) [(4)]	0.4	0.5 mM	42.0
PCR grade water (Item 7.5.2)	1.4		147.0
10 µM HepF (Table 4)	1.0	500 nM	105.0
10 µM HepR (Table 4)	1.0	500 nM	105.0
10 µM HepP (Table 4)	0.2	100 nM	21.0
Total	14.0		1470.0

See Table 7 for footnotes (1)–(4).

Table 12. Standard Curve Genomic Copies

Standard Curve Concentration	**Genomic Copies per RT-qPCR Assay [(1)]**
2.5×10^8	502,500
2.5×10^7	50, 250
2.5×10^6	5,025
2.5×10^5	502.5
2.5×10^4	50.25
2.5×10^3	5.025

(1) Place the indicated genomic copy values in the standards section for the real time thermal cycler used

Table 13. Mean Recovery and Coefficient of Variation Range

Variation type	Mean recovery range (%)	CV range [(1)]
Interlaboratory	56	92
Individual analysts	33–98	34–157
Intralaboratory	36–85	58–131

(1) CV – coefficient of variation

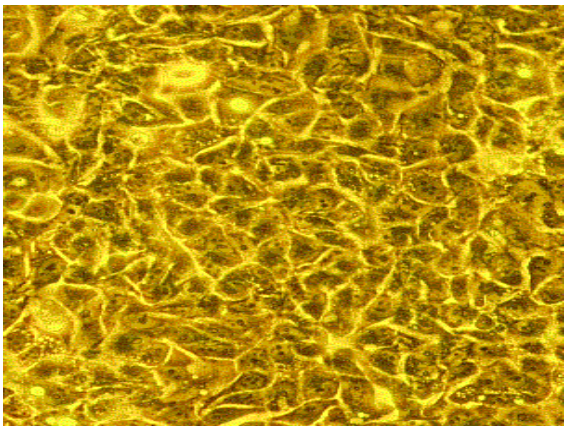

Figure 1. **Uninfected BGM cells**

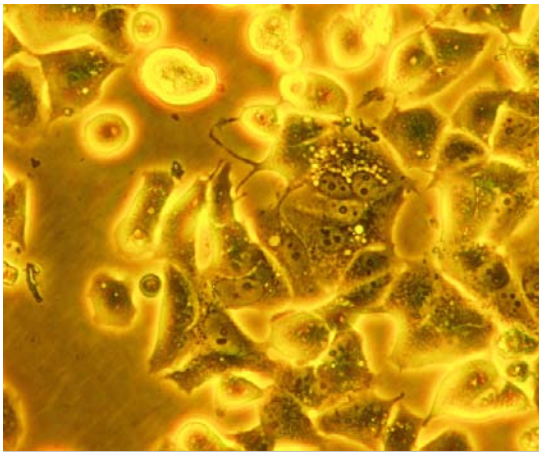

Figure 2. BGM cells showing early cytopathic effect from poliovirus

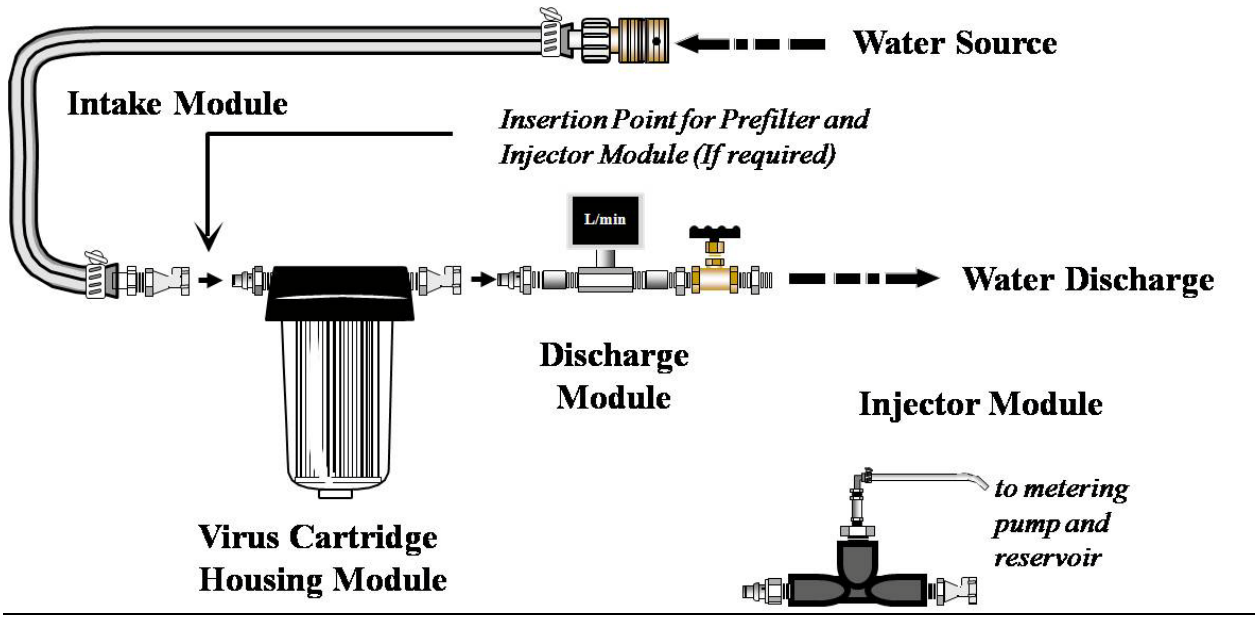

Figure 3. Sample filtration apparatus

Figure 4. Elution of an electropositive filter with beef extract

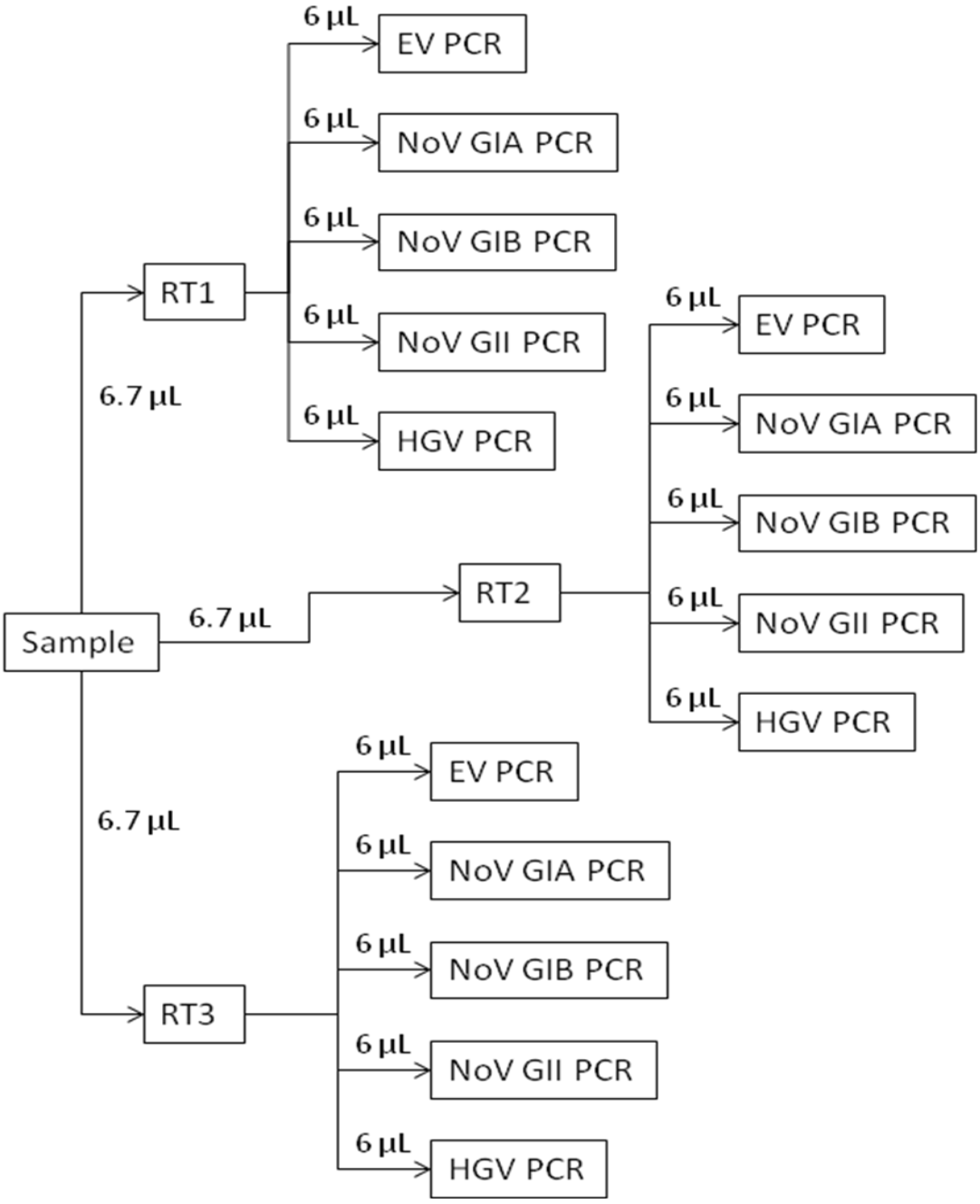

Figure 5. RT-qPCR schematic

Each test sample is reverse transcribed in triplicate (RT1, RT2, and RT3) using 6.7 µL of extracted sample RNA for each RT assay, in a 40-µL assay volume. Five (5) qPCR assays (EV PCR, NoV GIA PCR, NoV GIB PCR, NoV GII PCR, and HGV PCR) are run from each of the triplicate RT reactions using 6 µL of cDNA for each qPCR assay.

17.0 DATA SHEETS

17.1 Sample Data Sheet

SAMPLE DATA SHEET				
Sample Number				
Utility/Site Name				
Site Address				
City, State				
Sampler's Name (1)				
Water Type	☐ Surface Waters	☐ Treated Surface or Groundwaters	☐ Untreated Groundwater	☐ Other (specify in comments section
Location at Sampling Site	☐ Treatment Plant/Pumping Station	☐ Distribution System	☐ Other (specify in comments section)	☐ Matrix Spike

	Start of Sampling Event	End of Sampling Event
Date		
Time		
Totalizer Reading (L)		
Flow Rate (L/min)		
Total Sample Volume (L)		
Water Parameter Readings		
Water Temperature		
pH		
Turbidity (NTU)		
Free Chlorine (mg/L)		
Quality Controls		
Flow meter model and serial number:		
Totalizer model and serial number:		
Date of last flow meter/totalizer calibration:		
Metering pump model and serial number		
Temperature meter model and serial number:		
pH meter model and serial number		
Turbidity meter model and serial number		
Chlorine test meter model and serial number		
Metering pump flow rate QC check performed	☐ Yes	
Comments:		

(1) If any other individuals assist the sampler, include their name in the comments section and add the initials of the person who performed measurements after the recorded value.

17.2 Virus Data Sheet

VIRUS DATA SHEET			
Sample Number:	Sample Date:		
Sample Arrival Date:	Hold Time/Temperature Met (Y/N) (1)		
Analytical Laboratory Name and ID:			
Analytical Laboratory Address: City: State: Zip:			
Analyst Name (Please print or type):			
Sample Batch Number:			
Date Eluted:	Time:		
Eluate Volume Recovered:			L
Date Concentrated:	Time:		
Centrifugation Speed (Step 11.2.3):			x *g*
Final Concentrated Sample Volume (FCSV):			mL
Volume Of Original Water Sample Assayed (D) (2):			L
Assay Sample Volume (S):			mL
Inoculum Volume:			mL
Final Inoculation Volume (If Used):			mL
Date of Inoculation:	1st Passage	2nd Passage	3rd Passage (If necessary)
Subsample 1:			
MPN/L (3):	95% Confidence Limits/L Lower:	Upper:	
Comments: Did a heavy floc form during the organic flocculation step? Yes___ No___ Was the floc difficult to dissolve? Yes___ No___ Other comments:			
Analyst Signature:			

(1) If not met, record the failure under "Other comments;" consult QA guidance on how to proceed.
(2) e.g., 100 L of surface water or 500 L of finished or ground waters
(3) Value calculated from the Quantitation of Total Culturable Virus Data Sheet as described in the Virus Quantitation section.

17.3 Total Culturable Virus Data Sheet

TOTAL CULTURABLE VIRUS DATA SHEET					
Sample Number:					
Incubator Model and Serial Number:					
Passage	Sample Type	Confirmed (1) (indicated by √)	Total Number of Replicates		
			Inoculated	Without CPE	With CPE
1st	Neg. Cont.				
	Pos. Cont.				
	Undiluted				
	1:5 Dil.				
	1:25 Dil.				
	1:125 Dil.				
2nd (2)	Neg. Cont.				
	Pos. Cont.				
	Undiluted				
	1:5 Dil.				
	1:25 Dil.				
	1:125 Dil.				
3rd (3)	Neg. Cont.				
	Pos. Cont.				
	Undiluted				
	1:5 Dil.				
	1:25 Dil.				
	1:125 Dil.				

(1) Place a check (√) next to the negative controls and dilutions that were confirmed.
(2) A portion of medium from each 1st passage vessel, including negative controls, must be passaged again for confirmation. The terms "Undiluted," "1:5 Dilution" and "1:25 Dilution" under the 2nd and 3rd Passage headings refer to the original test sample dilutions for the 1st passage.
(3) Test samples that were negative on the first passage and positive on the 2nd passage must be passaged a third time for confirmation. If a third passage is required, negative controls must be passaged again.

17.4 Quantitation of Total Culturable Virus Data Sheet

QUANTITATION OF TOTAL CULTURABLE VIRUS DATA SHEET					
Sample Number:					
Sample	Number Replicates Inoculated	Number with CPE (1)	MPN/mL (2)	95% Confidence Limits/mL	
				Lower	Upper
Undiluted					
1:5 Dilution					
1:25 Dilution					
1:125 Dilution					

(1) The number of flasks with confirmed CPE from the second passage (or third passage, if necessary).
(2) The MPN/mL and 95% Confidence Limit values must be obtained using the computer program supplied by EPA.

17.5 Molecular Virus Protocol Data Sheet

MOLECULAR VIRUS PROTOCOL DATA SHEET			
Sample Number:			
Analytical Laboratory Name/Identification No.:			
Analytical Laboratory Address: City: State: Zip:			
Analyst Name/Identification No.:			
Subsample Number:			
Sample Batch Number:			
Tertiary Concentration	Date:	Time:	Initials: (1)
Concentrator Cat. No/Lot No.:			
Assay Sample Volume: (2) mL	Final Tertiary Concentrated Sample Volume: µL		
RNA Extraction	Date:	Time:	Initials:
RNA Extraction Kit Cat. No./Lot No.:			
Amount of Final Tertiary Concentrated Sample Used For RNA Extraction: µL			
RNA Extract Final Volume: µL			
Reverse Transcription (RT) Step			
RT Master Mix 1 Prepared	Date:	Time:	Initials:
RT Master Mix 2 Prepared	Date:	Time:	Initials:
RNA Extract Volume Used For RT: µL			
RT Samples Run:	Date:	Time:	Initials:
Thermal Cycler Used: (3)			
qPCR Step			
Enterovirus Master Mix Prepared:	Date:	Time:	Initials:
Norovirus GIA Master Mix Prepared:	Date:	Time:	Initials:
Norovirus GIB Master Mix Prepared:	Date:	Time:	Initials:
Norovirus GII Master Mix Prepared:	Date:	Time:	Initials:
Hepatitis G Master Mix Prepared:	Date:	Time:	Initials:
Volume Of RT Used For PCR: µL			
Run Number: (4)			
PCR Samples Run	Date:	Time:	Initials:
Thermal Cycler Used: (3)			
(1) Record the initials of the analyst at the time this procedure is performed. (2) A volume equal to the Assay Sample Volume must be concentrated. (3) Record the thermal cycler make and model. (4) A serial record identification of test samples that have to be re-run.			

17.6 Molecular Virus Quality Control Data Sheet

MOLECULAR VIRUS QUALITY CONTROL DATA SHEET				
Sample Number:				
Analytical Laboratory Name/Identification Number:				
Analytical Laboratory Address: City:		State:	Zip:	
Analyst Name /Identification Number				
Subsample Number:				
Sample Batch Number:				
Run Number				
All No Template Controls Negative?		Yes	No (1)	
All Negative RNA Extraction Controls Negative?		Yes	No	
Standard Curves Used				
Enterovirus	Lot # (2)	Eff. (3)	R^2	SD (4)
Norovirus GIA	Lot #	Eff.	R^2	SD
Norovirus GIB	Lot #	Eff.	R^2	SD
Norovirus GII	Lot #	Eff.	R^2	SD

Sample Type	Lot #	Mean (5)	SD (5)
Inhibition Control	Lot #		
Enterovirus Calibrator	Lot #		
Norovirus GI Calibrator	Lot #		
Norovirus GII Calibrator	Lot #		

(1) If any no template controls are positive or the inhibition control or calibrator falls outside specification limits, the test samples must be re-run with each run being recorded on a separate data sheet.
(2) Assign a new lot number to each new standard curve, inhibition control, and calibrator.
(3) Percent efficiency (Step 13.7.6)
(4) Record the largest standard deviation among the different concentrations of the standard curve lot.
(5) Record the mean and the standard deviation values for the sample type.

17.7 Molecular Virus Results Data Sheet

MOLECULAR VIRUS RESULTS DATA SHEET				
Sample Number:				
Analytical Laboratory Name/Identification Number:				
Analytical Laboratory Address: City: State: Zip:				
Analyst Name /Identification Number				
Subsample Number:				
Sample Batch Number:				
Run Number				
Enterovirus	If required, dilution used in calibration of test sample concentration:			
Replicate [(1)]	1	2	3	Mean (SD)
Genomic Copies (				
Genomic Copies per L (GC_L): [(2)]				
Inhibition Control Cq Value:		*Enterovirus* Calibrator Cq Value:		
Norovirus GIA	If required, dilution used in calibration of test sample concentration:			
Replicate [a]	1	2	3	Mean (SD)
Genomic Copies				
Genomic Copies per L (GC_L): [(2)]				
Inhibition Control Cq Value:		*Norovirus* GIA Calibrator Cq Value:		
Norovirus GIB	If required, dilution used in calibration of test sample concentration:			
Replicate [a]	1	2	3	Mean (SD)
Genomic Copies				
Genomic Copies per L (GC_L): [(2)]				
Inhibition Control Cq Value:		Norovirus GIB Calibrator Cq Value:		
Norovirus GII	If required, dilution used in calibration of test sample concentration:			
Replicate [a]	1	2	3	Mean (SD)
Genomic Copies				
Genomic Copies per L (GC_L): [(2)]				
Inhibition Control Cq Value:		*Norovirus* GII Calibrator Cq Value:		

(1) If more than three replicates are used, record the data from the additional replicates onto another Molecular Virus Results Data Sheet.

(2) Calculate the Genomic Copies per L using Equation 5. For field samples with a mean value of zero, report the Genomic Copies per L as less than or equal to the detection limit.

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