Supplemental Materials for:

**TITLE:**

Method 1615. Measurement of Enterovirus and Norovirus Occurrence in Water by Culture and RT-qPCR. Part III. Virus Detection by RT-qPCR.

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**DEFINITIONS:**

1. Cq (Quantitative Cycle) [alsocalled 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
2. D (Volume of Original Water Sample Assayed) – 100 L for surface water or 500 L for groundwater
3. 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
4. DF (Dilution Factor) – The reciprocal of the dilution performed to compensate for inhibition
5. Extraction Batch and ExtractionBatchID –All test samples that are eluted from filters and concentrated by organic flocculation at a single time and a unique laboratory assigned batch identification number associated with them
6. FCSV (Final Concentrated Sample Volume) – the final filtered volume following organic flocculation
7. Field sample **–** Any environmental or drinking water sample analyzed by this method
8. GC and GCL (Genomic Copy and Genomic Copy per Liter)
9. Matrix spike **–** A field sample containing Sabin poliovirus 3 at about 1,000 MPN/mL
10. NA Batch and NABatchID –All samples processed through the nucleic acid extraction step at a single time and a unique laboratory assigned batch identification number associated with them. Each NA batch must have a negative extraction control associated with it.
11. PE (Performance Evaluation sample) **–** 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. As much as possible, laboratories should ensure that their analysts cannot distinguish PE samples from field samples.
12. PT (Performance Test sample) **–** 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.
13. QC (Quality Control) Sample Types

## LFB (Lab Fortified Blank) – A positive quality control (QC) sample prepared by analytical laboratories by adding the QC stock to 10 L of reagent grade water and passing the solution through a electropositive filter. The LFB provides a measure of overall method performance.

## LRB (Lab Reagent Blank) – A negative QC sample prepared by analytical laboratories by passing 10 L of reagent grade water through a NanoCeram filter

## LFSM (Lab Fortified Sample Matrix) – A duplicate field sample collected in series or in parallel with a regular field sample and to which laboratories add the Matrix spike. The LFSM provides a measure of matrix effects on method performance.

## QC Stock – Sabin poliovirus 3 at 500±50 MPN/mL

1. qPCR (Quantitative Polymerase Chain Reaction) **–** This is a procedure for quantitatively detecting the levels of specific deoxyribonucleic acid (DNA) in a testsample
2. Reagent water **–** This is deionized or distilled reagent grade water (dH2O) with a resistivity greater than one 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.
3. RT Batch and RTBatchID – All samples analyzed together by RT at a single time and a unique laboratory assigned batch identification number associated with them
4. RT-PCR unit – an approximate titer of a virus stock based upon measuring the presence of the virus by RT-PCR in serial dilutions of the stock either on a plus/minus basis or by running multiple replicates of each dilution and using MPN statistics; for example, a stock where virus is present in a 1:15,625 dilution, but absent in a 1:78,125 fold dilution has a titer of 15,625 RT-PCR units in the volume assayed by RT-PCR or 3,125,000 RT-PCR units per mL for a 5 µL PCR assay
5. RT-qPCR (Reverse Transcription-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)
6. S (Assay Sample Volume) – The volume of FCSV that represents 100 L for surface water or 500 L for groundwater
7. Sample Batch and SampleBatchID – All test samples received by the laboratory within one week; a week is defined as a 7-day period and a unique laboratory assigned batch identification number associated with them

## Secondary concentrate – a test sample that has been concentrated by organic flocculation following elution of virus from a positively charge filter

## Tert Batch and TertBatchID – All test samples processed together at a single time through the tertiary concentration step and a unique laboratory assigned batch identification number associated with them

1. Test sample – Any sample type that is analyzed by this method, including quality control samples, field samples, performance test samples, and performance evaluation samples
2. TSV − Total sample Volume is the total amount of water passed an electropositive filter for virus collection. This typically is 300 L for surface water and 1,500 – 1,800 L of ground or finished water.

**SUPPLEMENTAL PROTOCOLS AND EXAMPLES**

#### S1. Alternative standard curves working stocks

#### S1.1) Virus stocks

#### S1.1.1) Determine the titer of each enterovirus or norovirus stock using RT-qPCR with serial 10-fold dilutions and 10 replicates per dilution or by use of the standard curve working stock described in Protocol Section 1. If the former, obtain the MPN/mL virus titer using EPA's Most Probable Number Calculator (see [http://www.epa.gov/nerlcwww/online.html#viral\_mpn](http://www.epa.gov/nerlcwww/online.html%23viral_mpn)).

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##### S1.1.2) Dilute each enterovirus and norovirus stock to 2.5 x108 MPN/mL. Disperse into 250 μL aliquots and freeze the stocks at or below -70°C. Although not necessarily equivalent, the term GC/mL can be substituted for MPN/mL if the efficiency of the standard curve derived from each virus stock is in the acceptable range (see protocol section 5.6).

#### S1.2) Plasmids

#### S1.2.1) Prepare RNA from plasmids containing the targets for each assay and determine transcript copy numbers using standard methods [e.g., see reference [1](#_ENREF_1)].

##### S1.2.2) Dilute the transcribed RNA to 2.5 x 108 transcripts/mL. Disperse into 250 μL aliquots and freeze at or below -70°C. Substitute the term GC/mL for transcripts/mL.

S2) Example for calculation of S: if 300 L (i.e., TSV) of a surface water sample is passed through the cartridge filter and subsequently concentrated to 30 ml (i.e., FCSV) by organic flocculation, then TSV equals 300 L, D equals 100 L, FCSV equals 30 ml, and

 = = 10.0 ml.

S3) Examples for calculation of standard curve acceptance criteria values.

S3.1) Example of calculation of the overall standard deviation (StdDev) using equation 2 and data from Table S1:

= = 0.173

S3.2) Example of Slope calculation using Equation 3 and data from Table S1:

= = -3.314

S3.3) Example of the calculation of the R2 value using Equation 4 and data from Table S1:

= = .9977

S3.4) Example of calculation of the % Efficiency using Equation 5 and the slope calculated in section S3.2:

 = 100 × (10-1/-3.214 -1) = 104.7%

S4) Example of calculation of GCL where the mean GC value of a 1:25 dilution of a surface water sample is 3.2:

**Figure Legends:**

**Figure S1. Schematic for RT Plate (1)**

(1) The schematic assumes that five samples are received by an analytical laboratory on a Tuesday and another five on a Wednesday, and that one NA batch was used for the ten samples. Although one NA batch is used, the ten samples require the running of two RT batches, one for the Tuesday samples and another for the Wednesday samples. This figure shows a suggested format for the first RT batch. A second RT batch (not shown) is needed to run the samples that arrived on Wednesday. The second RT batch must include additional NTC controls, but not the NA batch negative extraction control. Note that the first plate contains sufficient Armored RNA EPA-1615 samples for the qPCR plates (see Figure S2), so it is not necessary to run Armored RNA samples on the second RT plate.

(2) Abbreviations used: Col. = column; Sam = test sample; Rep = replicate; NTC = no template control; Ext = NA Batch negative extraction control; LRB = Lab Reagent Blank; LFB = Lab Fortified Blank; AR = Armored RNA EPA-1615; Superscripts attached to abbreviations are the sample dilution factor

**Figure S2. Schematic for qPCR Plates (1)**

(1) The schematic assumes the analysis of the samples from both RT batches described in Figure S1 using a single PCR batch. Separate optical reaction plates should be used for each qPCR assay unless very few samples are being analyzed because the standard curves would take up the majority of a single plate. Do not include the standard curve on the hepatitis G assay plate.

(2) See the legend to Figure S1 for abbreviations.

(3) Include all controls from RT batch 1 and 2 with each qPCR assay, but only the test samples dilutions that are not inhibited based upon the hepatitis G inhibition assay for each qPCR assay.

(4) Do not include the Armored RNA EPA-1615 standard curve samples with the hepatitis G qPCR assay.

**Table S1. Data for Standard Curve Performance Criteria Examples**

(1) Values are based upon 6 standards and 12 total Cq values

(2) Values in columns labeled A are derived by subtracting the mean for the duplicates at each standard level from each individual Cq values at that level.

(3) Values in columns labeled B are derived by subtracting the mean of the Cq values for all standard levels from each individual Cq value.

**Table S2. Molecular Virus Protocol Data Sheet**

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.

**Table S3. Molecular Virus Quality Control Data Sheet**

(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

(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.

**Table S4. Molecular Virus Results Data Sheet**

(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 Protocol Equation 6. For field samples with a mean value of zero, report the Genomic Copies per L as less than or equal to the detection limit.

**References**

1 Parshionikar, S. U., Cashdollar, J. & Fout, G. S. Development of homologous viral internal controls for use in RT-PCR assays of waterborne enteric viruses. *J. Virol. Methods* **121**, 39-48 (2004).

Figure S1.

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Col./ Row** (2) | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** |
| A | Sam11 Rep1 | Sam11 Rep2 | Sam11 Rep3 | Sam15 Rep1 | Sam15 Rep2 | Sam15 Rep3 | Sam125 Rep1 | Sam125 Rep2 | Sam125 Rep3 | NTC1 | Ext1 Rep1 |  |
| B | Sam21 Rep1 | Sam21 Rep2 | Sam21 Rep3 | Sam25 Rep1 | Sam25 Rep2 | Sam25 Rep3 | Sam225 Rep1 | Sam225 Rep2 | Sam225 Rep3 | LRB Rep1 | LRB Rep2 | LRB Rep3 |
| C | NTC2 | Ext1 Rep2 | Sam31 Rep1 | Sam31 Rep2 | Sam31 Rep3 | Sam35 Rep1 | Sam35 Rep2 | Sam35 Rep3 | Sam325 Rep1 | Sam325 Rep2 | Sam325 Rep3 |  |
| D | Sam41 Rep1 | Sam41 Rep2 | Sam41 Rep3 | Sam45 Rep1 | Sam45 Rep2 | Sam45 Rep3 | Sam425 Rep1 | Sam425 Rep2 | Sam425 Rep3 | NTC3 | Ext1 Rep3 |  |
| E | Sam51 Rep1 | Sam51 Rep2 | Sam51 Rep3 | Sam55 Rep1 | Sam55 Rep2 | Sam55 Rep3 | Sam525 Rep1 | Sam525 Rep2 | Sam525 Rep3 |  |  |  |
| F | NTC4 | LFB Rep1 | LFB Rep2 | LFB Rep3 |  |  |  |  |  |  |  |  |
| G |  |  |  |  |  |  |  |  |  |  |  |  |
| H | AR1 Rep1 | AR1 Rep2 | AR10 Rep1 | AR10 Rep2 | AR100 Rep1 | AR100 Rep2 | AR1,000 Rep1 | AR1,000 Rep2 | AR10,000 Rep1 | AR10,000 Rep2 | AR100,000 Rep1 | AR100,000 Rep2 |

Figure S2

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Col./ Row** (2) | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** |
| A | Sam1 (3) Rep1 | Sam1 Rep2 | Sam1 Rep3 | Sam2 Rep1 | Sam2 Rep2 | Sam2 Rep3 | NTC1 | Ext1 Rep1 | LRB Rep1 | LRB Rep2 | LRB Rep3 |  |
| B | Sam3 Rep1 | Sam3 Rep2 | Sam3 Rep3 | NTC2 | Ext1 Rep2 | Sam4 Rep1 | Sam4 Rep2 | Sam4 Rep3 | NTC3 | Ext1 Rep3 |  |  |
| C | Sam51 Rep1 | Sam51 Rep2 | Sam51 Rep3 | NTC4 | LFB Rep1 | LFB Rep2 | LFB Rep3 | Sam6 Rep1 | Sam6 Rep2 | Sam6 Rep3 | NTC5 |  |
| D | Sam7 Rep1 | Sam7 Rep2 | Sam7 Rep3 | Sam8 Rep1 | Sam8 Rep2 | Sam8 Rep3 | NTC6 | Sam9 Rep1 | Sam9 Rep2 | Sam9 Rep3 |  |  |
| E | NTC7 | Sam10 Rep1 | Sam10 Rep2 | Sam10 Rep3 | NTC8 |  |  |  |  |  |  |  |
| F | AR1 Rep1 (4) | AR1 Rep2 | AR10 Rep1 | AR10 Rep2 | AR100 Rep1 | AR100 Rep2 | AR1,000 Rep1 | AR1,000 Rep2 | AR10,000 Rep1 | AR10,000 Rep2 | AR100,000 Rep1 | AR100,000 Rep2 |
| G |  |  |  |  |  |  |  |  |  |  |  |  |
| H |  |  |  |  |  |  |  |  |  |  |  |  |

Table S1

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | Standard GC (1) | Log GC | Cq | |  | | --- | |  | | | |  | | --- | | LogGC− | | | |  | | --- | | A (2) | | | |  | | --- | | B (3) | | | |  | | --- | | A | | | |  | | --- | | B | | | )2   |  | | --- | | (LogGC− | | | x   |  | | --- | |  | | |
|
|  | 502500 | 5.7 | 20.9 | 21.05 | 2.5 | -0.13 | -8.54 | 0.02 | 72.94 | 6.25 | -21.35 |
|  | 502500 | 5.7 | 21.2 | 21.05 | 2.5 | 0.13 | -8.28 | 0.02 | 68.48 | 6.25 | -20.69 |
|  | 50250 | 4.7 | 24.5 | 24.48 | 1.5 | 0 | -4.99 | 0 | 24.86 | 2.25 | -7.48 |
|  | 50250 | 4.7 | 24.5 | 24.48 | 1.5 | 0 | -4.99 | 0 | 24.85 | 2.25 | -7.48 |
|  | 5025 | 3.7 | 27.5 | 27.49 | 0.5 | -0.02 | -2 | 0 | 3.99 | 0.25 | -1 |
|  | 5025 | 3.7 | 27.5 | 27.49 | 0.5 | 0.02 | -1.95 | 0 | 3.81 | 0.25 | -0.98 |
|  | 502.5 | 2.7 | 31 | 31.15 | -0.5 | -0.12 | 1.57 | 0.01 | 2.45 | 0.25 | -0.78 |
|  | 502.5 | 2.7 | 31.3 | 31.15 | -0.5 | 0.12 | 1.8 | 0.01 | 3.25 | 0.25 | -0.9 |
|  | 50.25 | 1.7 | 35.1 | 34.99 | -1.5 | 0.1 | 5.63 | 0.01 | 31.65 | 2.25 | -8.44 |
|  | 50.25 | 1.7 | 34.9 | 34.99 | -1.5 | -0.1 | 5.42 | 0.01 | 29.38 | 2.25 | -8.13 |
|  | 5.025 | 0.7 | 37.4 | 37.62 | -2.5 | -0.22 | 7.94 | 0.05 | 63.04 | 6.25 | -19.85 |
|  | 5.025 | 0.7 | 37.8 | 37.62 | -2.5 | 0.22 | 8.38 | 0.05 | 70.25 | 6.25 | -20.95 |
| Sum |  |  |  |  |  |  |  | 0.18 | 398.96 | 35 | -118.03 |
| Mean |  | 3.2 | 29.5 |  |  |  |  |  |  |  |  |

Table S2

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| SampleKitID: | | | | | |
| Analytical Laboratory Name/Identification No.: | | | | | |
| Analytical Laboratory Address: | | | | | |
| City: State: Zip: | | | | | |
| Analyst Name/Identification No.: | | | | | |
| SampleBatchID: | | | | | |
| ExtractionBatchID: | | | | | |
| Tertiary Concentration | Date: | | | Time: | Initials: (1) |
| TertBatchID: | | | | | |
| Concentrator Cat. No/Lot No.: | | | | | |
| Assay Sample Volume: (2) mL | | Final Tertiary Concentrated Sample Volume: μL | | | |
| RNA Extraction | Date: | | | Time: | Initials: |
| NABatchID: | | | | | |
| 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 | | | | | |
| RTBatchID | | | | | |
| 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 | | | | | |
| AnalysisBatchID: | | | | | |
| 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) | | | | | |

Table S3

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| SampleKitID: | | | | | | |
| Analytical Laboratory Name/Identification Number: | | | | | | |
|
| Analytical Laboratory Address: | | | | | | |
| City: State: Zip: | | | | | | |
| Analyst Name /Identification Number | | | | | | |
| SampleBatchID: | | | | | | |
| Subsample Number: | | | | | | |
| ExtractionBatchID: | | | | | | |
| TertBatchID: | | | | | | |
| NABatchID: | | | | | | |
| RTBatchID: | | | | | | |
| AnalysisBatchID: | | | | | | |
| All No Template Controls Negative? Yes No (1) | | | | | | |
| All Negative RNA Extraction Controls Negative? Yes No | | | | | | |
| Standard Curves Used | | | | | | |
| Enterovirus | Lot # (2) | | Eff. (3) | R2 | SD (4) | |
| Norovirus GIA | Lot # | | Eff. | R2 | SD | |
| Norovirus GIB | Lot # | | Eff. | R2 | SD | |
| Norovirus GII | Lot # | | Eff. | R2 | SD | |
| Sample Type | | Lot # | | Mean (5) | | SD (5) |
| Inhibition Control | |  | |  | |  |

Table S4

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| SampleKitID: | | | | | |
| Analytical Laboratory Name/Identification Number: | | | | | |
| Analytical Laboratory Address: | | | | | |
| City: State: Zip: | | | | | |
| Analyst Name(s) | | | | | |
| SampleBatchID: | | | | | |
| ExtractionBatchID: | | | | | |
| TertBatchID: | | | | | |
| NABatchID: | | | | | |
| RTBatchID: | | | | | |
| AnalysisBatchID: | | | | | |
| *Enterovirus* | If required, dilution used in calibration of test sample concentration: | | | | |
| Replicate (1) | | 1 | 2 | 3 | Mean (SD) |
| Genomic Copies | |  |  |  |  |
| Genomic Copies per L (GCL): (2) | | | | | |
| Inhibition Control 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 (GCL): (2) | | | | | |
| Inhibition Control 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 (GCL): (2) | | | | | |
| Inhibition Control 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 (GCL): (2) | | | | | |
| Inhibition Control Cq Value: | | | | | |