Quality Assurance Project Plan

Analytical Microbiology Services

Water Research Foundation
Contract #4952

Prepared for:

The Water Research Foundation

Prepared by:

cel analytical, inc.
water, wastewater, and soil laboratory services

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Version 3.0
Version 4.0

Abstract: This Quality Assurance Project Plan (QAPP) conforms with the United States Environmental Protection Agency (EPA) publication *EPA Requirements for Quality Assurance Project Plans* dated March 2001 (QA/R-5), and. In addition, this document was created following the California Water Boards "SWAMP-EPA-QAPP Review Checklist". The QAPP details the participant laboratories' policies and approaches for planning, implementing and assessing the Quality Assurance (QA) and Quality Control (QC) procedures needed to guide the successful implementation of analytical microbiology for WRF Contract 4952, "Pathogen Monitoring in Raw Wastewater" project. The program will focus on waterborne pathogens, consisting of four virus types, 2 protozoa and 2 bacteriophages from the raw influent of publicly operated wastewater treatment works (POTWs). The program starts in October 2019 and continues through January 2021. The WRF Technical Working Group (TWG) will use the monitoring reports generated from a sampling campaign to compile scientific data during the duration of the study. The data will assist the scientists and the regulators in development of regulatory limits for contaminants and/or the log reduction values (LRVs) of contaminants by treatment systems.

The QAPP will be a working document and subject to amendment(s) that may necessitate changes in approach and/or implementation of QA/QC procedures. The QAPP and its amendment(s) are subject to WRF review and approval.

A PROJECT MANAGEMENT

Project Title: Pathogen Monitoring in Raw Wastewater WRF #4952

Contract Laboratory: Cel Analytical Inc.
82 Mary Street Suite 2
San Francisco, CA 94103
Primary Contact: Yeggie Dearborn
Laboratory Director

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Email Address: yeggie@celanalytical.com

Technical Working Group

Primary Contact: Brian Pecson, PhD, PE

Phone Number: (510) 457-2201

Email address: Brianp@trusselltech.com

Coordinating committee:

Primary Contact: Adam Olivieri, DrPH, PE

Phone Number: (510) 832-2852

Email address: awo@eoainc.com

B3. Sample Handling and Custody

A1. Approval Sheet

05.04.20

Katherine Chandler, PhD.
Cel Analytical Inc.
Quality Control Officer

Date

05.04.20

Yeggie Dearborn, Ph.D.
Cel Analytical Inc.
Lab Director

Date

Julie Minton
WRF Contracting Officer
Project Officer

Date

Walter Jakubowski
WaltJay Consulting
Independent Quality Control Project Officer

Date

A2. Table of Contents

A PROJECT MANAGEMENT 1
A1. Approval Sheet 2
A3. Distribution List 4
A4. Project/Task Organization 4
A5. Problem Definition/Background 8
A6. Project/Task Description 11
A7. Quality Objectives and Criteria 13
A8. Special Training/Certification 16
A9. Documents and Records 18

B DATA GENERATION AND ACQUISITION 20
B1. Sampling Process Design (Experimental Design) 20
B2. Sampling Methods 20
B3. Sample Handling and Custody 21
B4. Analytical Methods 23
B5. Quality Control 24
B6. Instrument/Equipment Testing, Inspection and Maintenance 30
B7. Instrument/Equipment Calibration and Frequency 30
B8. Inspection/Acceptance for Supplies and Consumables 30
B9. Non-Direct Measurements 31
B10. Data Management 31

C ASSESSMENT/OVERSIGHT 31
C1. Assessment and Response Actions 31
C2. Reports to Management 32
D DATA REVIEW AND EVALUATION 32
D1. Data Review, Verification and Validation 32
D2. Verification and Validation Methods 33
D3. Evaluating Data in Terms of User Needs 33

List of Tables

Table 1: Project Implementation Personnel 4
Table 2: Analytical Microbiology Methods 12
Table 3: Schedule of Project Under Initial Issued Task Order 12
Table 4: WWTPs and Contact information 13
Table 5. Demonstration of Capabilities: Pre-campaign Testing 14
Table 6A: QA/QC Acceptance Criteria for Full Monitoring Campaign 15
Table 6B: QA/QC Acceptance Criteria for the Pre-campaignTest 16
Table 6C: QA/QC Acceptance Criteria Applicable to Both Pre-Campaign Testing and Full Monitoring Campaign Testing 16
Table 7: Analytical Methods Shipping Temperatures and Maximum Hold Times 22
Table 8A: Quality Control Samples and Frequency of Analysis for Analytical Methods, Bacteriophage 25
Table 8B: Quality Control Samples and Frequency of Analysis for Analytical Methods, Protozoa 26
Table 8C: Quality Control Samples and Frequency of Analysis for Analytical Methods, Virus 27

List of Figures

Figure 1. Flowchart illustrating the responsibilities of the Laboratories 6
Figure 2. State Water Board Grant 1: Oversight and Communication 7
Figure 3. Wastewater sample Workflow 23

Appendices

Appendix 1. WRF 4952 Method SOPs
Appendix 2. WRF-Data Deliverable Excel Format
Appendix 3. Sampling Protocol and Schedule
Appendix 4. Laboratories' Sample Handling and Chain of Custody (COC)

A3. Distribution List

This Quality Assurance Project Plan (QAPP) documents the approach for planning, implementing and assessing the Quality Assurance (QA) and Quality Control (QC) procedures applicable to WRF #4952, "Measure Pathogens in Wastewater" by the three contract laboratories, Cel Analytical (Cel-A), BCS, and Scientific Methods.

Individuals listed on the approval sheet (subsection A1) and Project Implementation Personnel identified under Project/Task organization (subsection A4 Table 1) will receive a copy of this QAPP and its subsequent revisions and/or amendments.

This document is an extension of Cel-A Laboratory's Quality Assurance Manual (QAM) and has been prepared according to the United States Environmental Protection Agency (EPA) publication *EPA Requirements for Quality Assurance Project Plans* dated March 2001 (QA/R-5), and conforms to Part B requirements of ANSI/ASQC E4-1994.

The QAPP shall be reviewed and approved by the WRF Program Manager and by the WRF independent QA/QC Project Officer prior to its implementation.

A4. Project/Task Organization

Personnel involved in project implementation are listed in Table 1, and responsibilities of each laboratory are summarized in Figure 1. A brief description of the managers and the officers responsibilities are provided in this section.

Table 1. Project Implementation Personnel

Cel Analytical

Individual	Role in Project
Yeggie Dearborn PhD	Lab Director
Richard E. Danielson, PhD	Project Laboratory Lead
Katherine Chandler, PhD	QA Manager, QC Officer
Owen Ransom PhD	Data Management Officer
Carin Zimmerman PhD	Tissue Culture Advisor
Mohsen Foroughi,	qPCR Analyst
Jutarat Wiriyadamrikul, PhD PhD	Tissue Culture Analyst
Jamie Lam,	Indicator Analyst /Tissue
Steven Tan,	Protozoa /Indicator Analyst
Yeggie Dearborn PhD	Protozoa/qPCR Analyst

Table 1 Continued

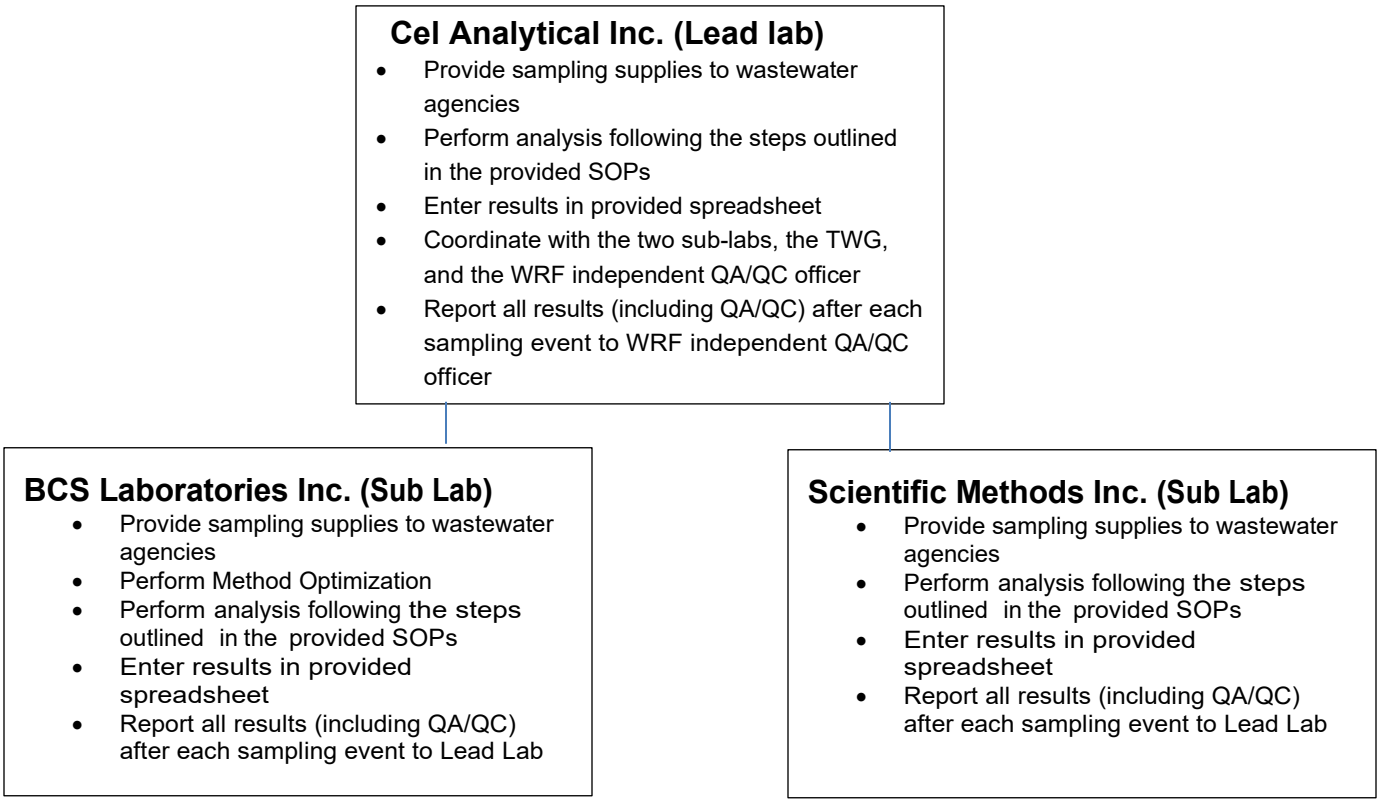
BCS Laboratory

Individual	Role in Project
George Lukasik PhD	Lab Director
Bonnie Mull MPH	QA Officer
Katherine Sayler PhD	Lead Parasitologist
Gabriela Blohm PhD	Lead Virologist
Jessica Weglarz	Analyst
Chris Benedict	Analyst

Scientific Methods

Individual	Role in Project
Fu-Chih Hsu, PhD	Project supervisor & QA officer
Rebecca Wong, PhD	Cell culture & qPCR
Rebecca Clouse, MS	Protozoa/coliphages
Alicia Jones, AA	Protozoa/coliphages/cell culture
Anne Petersen, BS	Project manager

Figure 1. Flowchart illustrating the responsibilities of the Laboratories



Coordinating Committee

The Coordinating Committee is made up independent consultants and CA State Water Board (SWB) staff to organize, oversee, review and approve information from the components of the CA SWB / WRF DPR-2 Pathogen Monitoring Project.

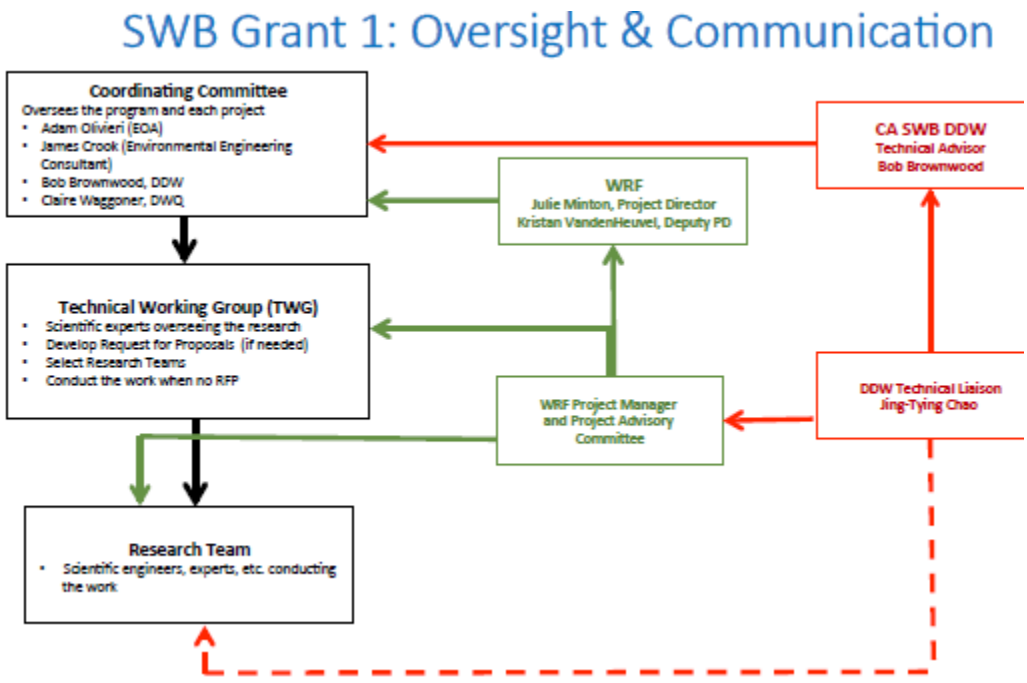
Individual	Affiliation
Adam Olivieri, DrPH, PE	EOA, Inc, Oakland, CA
James Crook, PhD, PE	Environmental Engineering, Consultant
Bob Brownwood, PE	CA SWB, Dept. Drinking Water
Claire Waggoner	CA SWB, Dept. Water Quality

Technical Working Group

The Technical Working Group (TWG) is made up of independent individuals who are familiar with the science of the methods. The TWG is responsible for review and approval of SOPs and the data produced by the laboratories.

Individual	Affiliation
Brian Pecson, PhD, PE	Trussell Tech, Oakland, CA
Menu Leddy, PhD	Consultant
Terri Slifko, PhD	Metropolitan Water District, Los Angeles, CA
Kara Nelson, PhD	University of California, Berkeley, CA

Figure 2. State Water Board Grant 1: Oversight and Communication



DDW: Department of Drinking Water DWQ: Department of Water Quality

Project Management Responsibilities

Yeggie Dearborn, PhD will serve as both the Lab Director and the supervising analyst for the purification of virus.

As the Laboratory Director she will be responsible for the following activities:

- Managing the Cel-A Project Team
- Arranging for sample shipping and receiving
- Subcontracting to the other two laboratories
- Overseeing the planning, budgeting, project schedule/tasks, and scope
- Assist WRF and project Personnel with logistic support

.

As a Supervising Analyst she will be responsible for the following:

- Participate in demonstration of capability and on-going monthly performance evaluation Conduct virus purification and concentration
- Assist in protozoa and qPCR analysis of the viruses

Richard Danielson, PhD will be the Lead Laboratory coordinator responsible for the following:

- Creating and maintaining a joint QAPP for participating laboratories
- Ensure that all laboratories are following the methods described herein (see Appendix 1)
- Train appropriate staff within the laboratories on the methods
- Ensure that samples are collected, shipped and received within the prescribed hold times
- Review all data from all labs prior to submitting to the WRF Independent Project Quality Assurance/Quality Control Officer
- Available for analysis of protozoa and virus samples
- Assembling and reporting the results from the three laboratories

Katherine Chandler, PhD will be the Quality Assurance Manager at Cel-A. Her responsibilities include the following:

- Establish the QA/QC procedures described in this QAPP
- Ensure that program deliverables meet quality standards and project requirements
- Report findings to the Laboratory Director, including requests for corrective actions
- At her discretion she may stop the analytical work or request sub-laboratories QC Officers to stop work, if there are significant deviations from required practices or if there is any evidence of potential systematic failure
- Remains independent of data generation

Owen Ransom, PhD will be the Electronic Data Quality Officer. He will be responsible for the following:

- Develop and maintain program documentations
- Data management and LIMS Data quality assurance
- Work closely with the QA/QC officers to ensure database documents are complete and analytical results are submitted in format requested by WRF

Carin Zimmerman, PhD will serve as an adviser through the life of this project

- She will provide on as-needed basis her technical expertise in cell culture-based viral assays
- She will be the temporary acting Laboratory Director in Yeggie Dearborn's absence

A5. Problem Definition/Background

The California State Water Board (SWB) sponsored an Expert Panel administered by the National Water Research Institute (NWRI). This expert Panel published a Final Report (see reference) in August 2016, which identified knowledge gaps for developing criteria for direct potable reuse (DPR). Six research projects have been identified to address these gaps. The focus of the second of these research projects, DPR-2, is to assess the concentration of relevant pathogens in raw wastewater (RWW) via a 14--month monitoring campaign. The two principal objectives of DPR-2 are:

- To collect empirical data on the concentration and variability of pathogens in raw wastewater for the purpose of verifying log removal values necessary to adequately protect public health in DPR projects.

- To develop recommendations for the collection and analysis of data on pathogens in raw wastewater. These recommendations may be used in future monitoring efforts.

During Phase 1 of DPR-2, the pathogens and/or indicator organisms that should be monitored in raw wastewater were selected. The selected organisms are *Giardia*, *Cryptosporidium*, enterovirus, adenovirus, norovirus, and male-specific coliphage. The methods for measuring the concentrations of each of these organisms were also selected during Phase 1. Raw wastewater can be a challenging matrix and, therefore, method modifications were explored during a pre-screening methods optimization study (DPR-2 WRF 4899). Based on the information learned during Phase 1, the monitoring plan described in this QAPP and the optimized SOPs (Appendix 1) were developed as part of Phase 2.

The scope of work outlined in this QAPP relates to Phase 3 (pathogen monitoring) of the DPR-2 research project. Three laboratories will receive raw wastewater samples from five wastewater agencies in California (a total of two to three samples per month per laboratory) and process the samples according to the SOPs attached in this QAPP. The sampling and analysis will start in October 2019 and will continue through January 2021. Prior to the start of the monitoring campaign, the three labs will complete pre-campaign testing to demonstrate capability of analysis (beginning in October 2019). Also, during this pre-campaign testing, one of the labs, BCS Laboratories, will optimize the methods and finalize the SOPs.

In December 2019, a novel Betacoronavirus was identified originating from the Wuhan region of China. This virus spread quickly causing a global pandemic. The virus was subsequently identified as a sudden acute respiratory syndrome (SARS) coronavirus, but different from the SARS CoV-1 virus of 2003 and the MERS-CoV of 2012. The current Betacoronavirus of concern is SARS CoV-2, which causes the disease, COVID-19 (indicating the coronavirus emergence in 2019). In order to control the spread of SARS CoV-2, extraordinary public health measures were instituted throughout the United States and the rest of the world. In regard to the WRF 4952 project, it was recognized in March 2020 the importance of monitoring for the SARS CoV-2 and adding it to the list of analytes. It was also recognized that the methods used for the originally targeted enteric viruses would not be the best methods for the SARS CoV-2, an enveloped virus. The laboratories were charge with three tasks. First, optimize a method that would be able to detect and quantify the concentration of enveloped, SARS COV-2, from raw wastewater. Second, apply the RT-qPCR assay method to the existing nucleic acid sample archive (collected using the original DPR-2 SOP established for waterborne pathogens and indicators) from the beginning of the project (November 2019 to mid-April 2020). It is hoped that this may reveal information on the temporal occurrence of SARS CoV-2 in California communities dating back to the beginning of the project. Third, apply the optimized method for concentration of SARS CoV-2 from RWW and quantification with RT-qPCR to future campaign samples (mid-April 2020 through the end of the DPR-2 campaign, January 2021).

Viruses:

Enteroviruses, adenoviruses and noroviruses are enteric viruses implicated in several epidemiological outbreaks of non-bacterial gastrointestinal illness worldwide[1]. These viruses infect humans through exposure to fecal contaminated water, ingestion of food exposed to contaminated water and readily transmitted from person-to person contact.

Enteroviruses are members of the *Picornavirus* family, a large and diverse group of small RNA viruses characterized by a single positive-strand genomic RNA. *Picornavirus* family includes Poliovirus, Coxsakievirus, echoviruses and hepatitis virus. Infection by enteroviruses can result in a wide variety of symptoms ranging from asymptomatic to mild respiratory illness (common cold), hand, foot and mouth disease, gastroenteritis, acute hemorrhagic conjunctivitis, aseptic meningitis, severe neonatal sepsis-like disease, and acute paralysis [2].

Noroviruses are a genetically diverse group of single-stranded RNA, non-enveloped viruses in the *Caliciviridae* family.[3] According to the Centers for Disease Control and Prevention (CDC), noroviruses are the most common cause of viral gastroenteritis in the U.S., and worldwide [1, 4]. The disease is characterized by nausea, vomiting, severe diarrhea, and abdominal pain, and in some cases, loss of taste. While severe illness is rare, a small number of people die from severe dehydration, mostly the very young, elderly, and persons with weakened immune systems.

Adenoviruses are non-enveloped 90 – 100 nm, double-stranded DNA, spherical viruses in the family Adenoviridae. There are 57 accepted types that have been associated with infection in humans. The majority are responsible for respiratory tract diseases and conjunctivitis, while three types, 40, 41 and 52, are associated with gastrointestinal disease. Adenovirus infections and disease are very common and these viruses occur in great concentrations in wastewater.

SARS Cov-2 is a novel enveloped, positive- stranded RNA, Betacoronavirus. As of this writing, the exact origin of this specific strain of Coronavirus is under investigation. Early analysis of epidemiological and sequencing data has indicated that it originated from the Wuhan region of China. Its origin may or may not have been from an animal reservoir (bats). It has round or elliptic and often pleomorphic form, and a diameter of approximately 60–140 nm. Like other

CoVs, it is sensitive to ultraviolet rays and heat. Furthermore, these viruses can be effectively inactivated by lipid solvents including ether (75%), ethanol, chlorine-containing disinfectant, peroxyacetic acid and chloroform except for chlorhexidine (Cascella, et al., 2020).

OC-43 (Betacoronavirus 1, strain OC-43, ATCC VR-1558) is an enveloped RNA Coronavirus and will be used in this project as a surrogate for SARS CoV-2 virus in matrix spikes to evaluate method performance. Until a vaccine or anti-viral drug treatments are identified, the propagation and use of the SARS CoV2 in matrix spike studies is deemed to present a biohazard that would require BSL-3 containment. Therefore, the OC-43 was chosen due to its BSL-2 status for laboratory use.

Bacteriophage (a.k.a. phage) are viruses which infect bacterial hosts. These viruses can be infective to several genera of bacteria or be species specific. The phage of those indicator bacteria of human fecal pollution (i.e., coliforms) have themselves been employed as indicators of fecal pollution. Further, due to the plentiful nature and relative ease of analysis for phage, they are often used as surrogates for human infective viruses in demonstrating the effectiveness of wastewater treatment in overall virus reduction. For this project, a male-specific phage, MS2, and a somatic phage, PhiX174, will be used as the matrix spike for assessing virus recovery. The concentration of native male-specific phage will also be assessed. Two *Escherichia coli* bacterial hosts will be used to quantify the concentration of phage. One *E. coli* host will be for those phages which specifically target the bacterial F+ pilus (a.k.a, male-specific or MS phage). The other *E. coli* will be a host which is susceptible to non-F+ pilus phage (a.k.a somatic phage).

Protozoa
Human parasitic protozoa are common and pervasive in all communities in the United States and have been implicated in many waterborne and foodborne outbreaks. The two protozoa of concern in this study are *Giardia* sp. and *Cryptosporidium* sp.. *Giardia* is a protozoan flagellate which requires an animal host to complete its life cycle and is responsible for the animal diarrheal disease giardiasis. *Giardia* develop into a highly resistant cyst-form which can survive outside the host. *Cryptosporidium* is a coccidian protozoan parasite responsible for the animal diarrheal disease cryptosporidiosis. *Cryptosporidium* requires an animal host to complete its life cycle. *Cryptosporidium* develop into a highly resistant oocyst-form which can survive outside the host.

Rationale for Initiating the Project

The California State Water Resources Control Board (SWB) has recommended research be conducted to address knowledge gaps for developing criteria for Direct Potable Reuse (DPR). One of these knowledge gaps is the concentration and variability of relevant pathogens in raw wastewater.

The primary objectives of WRF 4952 (“Measure Pathogens in Wastewater” include:

- Collection of the occurrence data for norovirus, adenovirus, enterovirus, SARS CoV-2, bacteriophage, *Cryptosporidium* and *Giardia* from 5 public wastewater water treatment plants (WWTPs)
- Modification and optimization of methods for collection and analysis of pathogen data in raw wastewater

The analytical data will assist CA Water Boards in addressing the following key question(s)/issues:

- Verify log removal values necessary to adequately protect public health in DPR projects
- Methods to use for analyzing pathogen concentration in raw wastewater for future monitoring efforts.

Regulatory Criteria

This project will contribute to the development of DPR regulations in California in support of the findings of the SB 918 Expert Panel "*A Proposed Framework for Regulating Direct Potable Reuse in California.*"

A6. Project/Task Description

Project Overview

Analytical microbiology contracts supporting the WRF 4952 monitoring program will focus solely on a survey of protozoan and viral contaminants in domestic raw wastewater (RWW). The program starts in October 2019 and continues through January 2021 (or other modified date) and is organized into multiple samples per month from 5 wastewater treatment plants within the State of California.

There will be three qualified laboratories in support of the monitoring campaign. A "lead" laboratory has been selected from the three to act as coordinator with the other two labs.

Prior to the monitoring campaign, one of the laboratories will evaluate and optimize the methods to finalize the standard operating procedures (SOPs). All three of the laboratories will conduct pre-campaign testing to demonstrate capability of analysis and to determine method acceptance criteria.

There will be strict quality control review of the data. Each laboratory will submit their results to the lead lab where a designated "Lead" will compile and check to see if the analyses met with established quality control criteria. The lead lab will then submit the results to a WRF independent quality assurance/quality control (QA/QC) officer who will determine if acceptance criteria has been met before allowing the data set(s) to be delivered to the TWG.

Constituents to be Monitored and Measurement Techniques

The microbes and the proposed measurement techniques are summarized in Table 2. Analysis for each microbe will be in accordance with the SOPs specified for each method and as required by the Details of the measurement techniques provided in Section B4 "Analytical Methods" of this QAPP.

Table 2. Analytical Microbiology Methods

Analytical Method	Microbe
EPA Method 1615v1.1 (modified, 08/2019)	Viruses: 1) Enterovirus cell culture and RT-qPCR 2) Norovirus RT-qPCR 3) Adenovirus cell culture and RT-qPCR 4) Bacteriophage RT-qPCR
EPA Method 1693 (modified, 08/2019)	*Cryptosporidium* and *Giardia*
WRF_COV_SARS2	SARS CoV-2 RT-qPCR OC-43 RT-qPCR
EPA Method 1602 (modified, 08/2019)	Bacteriophages

Notes/Definitions:

- EPA Method 1615v1.1 is the newly revised version (EPA600/R-10/181, version 1.1, revised January 2012) modified by WRF Project #4988
- qPCR= Quantitative Polymerase Chain Reaction
- EPA Method 1693 modified by WRF Project #4988
- EPA Method 1602 modified by WRF Project #4988

A6.1 and A6.2 Project Summary and Work Schedule

The project's deliverables under the statement of work (SOW) are summarized in Table 3 below. The QAPP will be amended to replace estimated period of performance dates and issuance of subsequent modifications following the pre-campaign laboratory performance evaluation indicated in the SOW.

Table 3: Project Schedule

Schedule for Pathogen Monitoring	
Task	**Date(s)**
Pre-campaign testing (Method Optimization & Laboratory Evaluation)	October 2019 - December 2019
Sampling starts	December 2019
Analyze 24 samples/WWTP from 5 WWTP (dividing among 3 labs)	December 2019 - January 2021
Finish analyses and report results from the final sampling	January 2021

A6.3 Geographic Focus

The geographic areas under this WRF study are located in the State of California.

WRF has selected 5 WWTPs located in California for this study. The facilities will be identified by NPDES IDs. The agency and the contact information are provided in Table 4.

Table 4: WWTPs and contact information

Agency	Contact Information
Sanitation District of Los Angeles County (LACSD)	Mike Liu 562-908-4288 mliu@lacsd.org
City of Los Angeles Sanitation (LASAN)	Nasir Emami 310-648-5646 nasir.emami@lacity.org
City of San Diego	Joseph Quicho 858-292-6479 jquicho@sandiego.gov
San Francisco Public Utilities Commission (SFPUC)	George Engel 415-385-3245
Orange County Sanitation District	Samuel Choi 714-593-7497 SChoi@OCSD.COM

A6.4 Resource and Time Constraints

The project requires that all microbiological tests without exception to be conducted by the laboratories receiving the original sample and responsibilities of each analyst and the managers clearly delineated. This constraint is primarily due to short holding times of the samples and the special technical skills required to conduct the analytical testing for the project. Change in staff related to performance issues, and/or leave due to sickness will be communicated to the WRF Contracting Officers (COs)/Task Officers. Additionally, the Project Lead will assist with sampling logistics to minimize time constraints.

A7. Quality Objectives and Criteria

This section identifies the statistical methods that will be used to establish trends and quality control limits necessary to improve the quality of the data that will be used in the decision process.

Demonstration of Capability (DoC). Each laboratory will participate in a DoC in the pre-campaign testing. A 30 L sample will be sent to the lead laboratory for sample spiking and distribution to the sub labs. Each analysis will require 1 L of RWW, and, each sample will be analyzed in triplicate as indicated in Table 5.

Table 5. Demonstration of Capabilities: Pre-campaign Testing

Sub-sample	Organism Type	Enumeration Method	Specific Target	Number of Analyses per Lab
Sub-sample 1	Protozoa	Microscopy	Native (*Crypto*. and *Giardia*)	3[2]
			Blind Spike (ColorSeed)	3[2]
Sub-sample 2	Virus	Culture	Blind Spike (Poliovirus)[1]	3[2]
			Matrix Spike (MS2 and PhiX174)	3[2]
		Molecular	Blind Spike (Poliovirus)[1]	3[2]
			Matrix Spike (MS2 and PhiX174)	3[2]
Sub-sample 3	Virus	Culture	Native (Enterovirus)	3[2]
			Matrix Spike (MS2 and PhiX174)	3[2]
		Molecular	Native (Enterovirus)	3[2]
			Matrix Spike (MS2 and PhiX174)	3[2]

[1]Poliovirus is enumerated using the same culture and molecular methods that are used for enterovirus
[2]Each sub-sample is divided into triplicates, and each triplicate is processed and analyzed independently

Method specific performance measures or Data Quality Indicators-(DQIs) are outlined below in Tables 6 a, b and c.

Note: Target Reporting Limits in Table 6 are theoretical estimations and subject to modification based on data set outcomes in the monitoring campaign.

Data Quality Indicators are defined as follows with associated statistical calculation described under Section D3 of this QAPP.

Precision is defined as the measure of agreement among repeated measurements of the same property under identical or substantially similar conditions. In this study precision is expressed as coefficient of variation. The coefficient of variation should be less than 120% for replicates. The QC Officer will track the precision of each method by evaluating historical data for any indication of variability caused by the analyst's performance, change in reagents/supplies or the need for equipment maintenance.

Accuracy: While precision measures the closeness of duplicate sample analysis, it does not ensure the accuracy of the data generated at the laboratory. Accuracy of data reflects the degree to which the analytical measurement represents the actual concentration of the tested sample or its "true value".

Microbial samples pose a special problem of reproducibility due to natural variations in the number of organisms found from sample to sample. Apart from blank samples (laboratory blank or field blank), additional control samples will be utilized to determine accuracy to establish method performance (refer to Tables 6 and 8) for summary of method specific QC requirements).

Sensitivity is monitored through instrument calibration and determination of method detection limits based on number of replicate samples, and sample dilutions if any.

Completeness of data is established by the percentage of laboratory results backed up by accepted quality assurance data. Completeness of data however, also relies on the sampling

process, transportation and treatment of samples prior to receipt by the laboratory in addition to the analytical procedures.

Bias and Representativeness are assessed through the sample design process and method performance. Some methods inherently are subject to a number of biases that will reduce the accuracy and precision, partly due to limitation of a given method but also due to the nature of microbes and their occurrence in the environmental samples. Ongoing performance evaluation of QC samples such a matrix spike (MS) and matrix spike duplicate (MSD), PE sample of a known quantity but unknown to analyst(s), and inter-laboratory comparison can minimize bias and assist in establishing acceptance limits for a given method. Representativeness is ensured through proper sample handling, and sample storage conditions as well as analysis of samples within specified holding time(s).

Table 6 A. QA/QC Acceptance Criteria for Full Monitoring Campaign

Assay	Acceptable Recovery Range
Matrix Spike	
MS2 Culture	5 – 200%
PhiX174 Culture	5 – 200%
MS2 Molecular	5 – 200%
PhiX174 Molecular	5 – 200%
OC43 Molecular	To be determined
Giardia ColorSeed	10 – 100%
Crypto. ColorSeed	10 – 100%
QC/OPR[1]	
Poliovirus Culture	5 – 200%
Poliovirus Molecular	5 – 200%
OC43 Molecular	To be determined
Giardia ColorSeed	18 – 100%
Crypto. ColorSeed	14 – 100%

[1]QC (quality control) and OPR (ongoing precision and recovery) samples are positive controls (organisms spiked at a concentration known to the analyst in reagent water) for EPA Method 1615 and EPA Method 1693, respectively. The QC and OPR sample sets include a negative control (reagent water) in addition to the positive control. For this study, QC and OPR sample sets will be processed and analyzed once per month during the duration of the study.

Table 6 B. QA/QC Acceptance Criteria for the Pre-Campaign Testing

	Acceptable Range
Percent accuracy of average poliovirus culture measurement (corrected for recovery)[1]	-50 – 50%
Percent accuracy of average poliovirus molecular measurement (corrected for recovery)[1]	-50 – 50%
Coefficient of variation of poliovirus culture measurements (corrected for recovery)[2]	≤ 120%
Coefficient of variation of poliovirus molecular measurements (corrected for recovery)[2]	≤ 120%
Culture MS2 matrix spike recovery	5 – 200%
Culture PhiX174 matrix spike recovery	5 – 200%
Molecular MS2 matrix spike recovery	5 – 200%
Molecular PhiX174 matrix spike recovery	5 – 200%
Giardia ColorSeed matrix spike recovery	10 – 100%
Crypto. ColorSeed matrix spike recovery	29 – 100%

[1]Percent accuracy is calculated using the equation: $\frac{Observed-Actual}{Actual} \times 100\%$

[2]Coefficient of variation is calculated using the equation: $\frac{Standard\ deviation}{Average\ of\ the\ three\ measurements} \times 100\%$

Table 6 C. QA/QC Acceptance Criteria Applicable to Both Pre-Campaign Testing and Full Monitoring Campaign Testing

Criteria that always results in processing/analyzing a repeat sample at no cost to WRF
If a result includes qualifiers other than "non-detect" and "matrix interference" (provided that the appropriate inhibition controls and dilutions were performed), the lab must process and analyze a repeat sample at no cost to WRF. Examples of **unacceptable qualifiers** include: • Lost analysis • Analyzed past hold time
If a sample was **not analyzed by the methods specified** (e.g., enumeration by PFU instead of MPN), the lab must process and analyze a repeat sample at no cost to WRF.
If a sample was not analyzed with **all of the quality controls** specified in the SOPs (e.g., positive controls, inhibition controls, negative controls, dilutions), the lab must process/analyze a repeat sample at no cost to WRF. For example, if the lab reports "matrix interference" for a molecular assay but no inhibition control was included and no dilutions were performed, the lab must process/analyze a repeat sample at no cost to WRF.

A8. Special Training/Certification

CEL Analytical, Inc.

Cel-A stands committed to establishing compatibility levels of routine analytical results by participating in inter-laboratory Proficiency Testing (PT). Laboratory staff will analyze reference material(s) obtained through authorized vendors where their performance is evaluated through inter-laboratory comparisons. The performance audit results are then reviewed by California Department of Public Health Bureau of Laboratory Accreditation (ELAP) authorities and accreditation status is granted upon successful analysis of referenced material. Accreditation status is renewed through the same process every 2 years.

To comply with the requirements of this contract, and in addition to proficiency testing conducted for routine analytical tests, Cel A laboratory personnel will receive training that includes the review of proper laboratory procedures and sample-handling techniques, including receiving, handling/storage, and chain-of-custody procedures, prior to conducting any sample analysis, and only those staff with proficiency will be permitted to conduct laboratory analysis. All personnel are required to read and acknowledge the requirements specified under the PQAPP. Laboratory program managers and QA managers will provide training for all laboratory personnel identified in this QAPP and retain administrative files documenting all training.

BCS Laboratories, Inc. (4609 NW 6th Street, Ste. A; Gainesville, FL 32609)
BCS Laboratories is an ISO17025:2017 accredited facility and is also accredited by the State of Florida Department of Health (Lab ID E82924) as per The National Environmental Laboratory Accreditation Conference (NELAC / NELAP) and The NELAC Institute (TNI). BCS Laboratories is a member of the US EPA Compendium of Environmental Testing Laboratories/Environmental Response Laboratory Network (ERLN; Lab FL01147). Additionally, BCS is a United States Department of Agriculture (USDA) approved facility for the analysis of pathogens in soil (ID 2949). BCS Laboratories holds permit P526 for the receipt and analysis of foreign soil and biosolids samples for pathogens (Animal Health and Plant Inspection Service Permit P526P-13-01699). BCS is a member of the Centers for Disease Control and Prevention (CDC) Environmental Legionella Isolation Techniques Evaluation (ELITE) Program. We also hold NELAP accreditations in Louisiana, Pennsylvania, Puerto Rico, South Carolina, Texas, and Virginia.

Scientific Methods, Inc. (12441 Beckley St, Granger, IN 46530)
Principal microbiologists (Dr. Hsu and Dr. Wong) have more than 20-years of experience detecting protozoa in environmental samples (water, soils, biosolids, etc.) using ICR method, Method 1622 and 1623. All analysts in SMI have broad experience in different concentration techniques such as filtration (EnviroChek™, Filta-Max™) and continuous flow centrifugation (CFC). Scientific Methods was certified by EPA for Method 1623 in the first round of LT2 monitoring as well as the current round, and is currently certified by NHELAP using the CFC which is an approved method by USEPA for concentration of *Cryptosporidium* and *Giardia.*

Dr. Hsu is the key person who has assisted the USEPA for the development of Method 1601 and 1602, and has been a leading expert in the area of coliphage testing for more than 22 years. He was a consultant during a Method 1601, 1602 round-robin study in 2001. Dr. Wong was selected to participate in the round-robin study as well. Scientific Methods was a reference laboratory during Method 1642 and 1643 development/validation, and provided comments and technical assistance.

Dr. Wong has more than 27 years of experience in cell culture techniques and has conducted research funded by the USEPA for concentrating human enteric viruses in water using positively charged particles, and for novel methodologies to detect and identify environmental pathogens including viruses, bacteria and protozoans in water samples. She was trained for Real-Time PCR at a workshop in the Mayo Clinic in 2008.

To comply with the requirement of this sub-contract, all personnel will receive training according to this QAPP.

A9. Documents and Records

Report format/Information

The format for all data reporting packages will be consistent with the requirements and procedures used for data validation and data assessment described in this QAPP.

Document/Record Control

Cel-A records and maintains documentation of all laboratory activities ranging from original observations, calculations and derived data to calibration records and copies of the test reports. Records are maintained primarily in electronic format, and some records in paper format, in a manner as to allow historical reconstruction of all laboratory activities. Records include:

- A Cel-A proprietary database, containing information for all processed samples: results, pertinent QC information, and all necessary information pertaining to the collection and processing of the sample, including personnel and analysts who handled or analyzed the sample.
- A LIMS which will track reception of the sample, tying it to the associated customer data, assignment of the sample for analysis, processing and QC of the sample including what data was used for analysis, storage of the sample, and location of the associated physical and electronic results of sample analysis within the Cel-A database.
- Hand-recorded data will be taken with indelible ink, and changes to such data records will be made by drawing a single line through the error with an initial and date by the responsible person.
- The Lab Director will have ultimate responsibility for any and all changes to records and documents. Similar controls will be put in place for electronic records.

Cel-A QA Officer shall retain all updated versions of the QAPP and be responsible for distribution of the current version of the QAPP. The WRF independent QA/QC officer will approve all updates to this plan prior to its distribution. Cel-A's Program Manager shall retain copies of all management reports, memoranda, and all correspondence between the WRF and all project personnel identified in A4.

BCS Laboratories of North Florida, Inc. has specific procedures set forth to approve, store, control, revise, and distribute documents and records generated both internally and externally. Documents and records are legible and stored to be readily retrievable, and in suitable environments to prevent damage, deterioration or loss. Documents set forth procedures to be followed (i.e. SOP's) while records allow for the historical reconstruction of laboratory activities related to sample-handling and analysis and show adherence to the Laboratory's documents. The laboratory maintains a document/record system appropriate to its needs, records all laboratory activities, and complies with applicable standards or regulations as required.
It is ensured that the most up-to-date version of each appropriate SOP is available at each workstation and that obsolete SOP's are promptly removed from service, marked as such and retained as a record.

At Scientific Methods, Inc., data are generated from the moment the sample and paperwork enters the Laboratory building. Our receiving personnel document temperature and sample condition, which is recorded on the Chain of Custody (COC) as well as our sample log-in register. COC documents and any other documents related to a particular sample are kept in holding bins in close proximity to where the sample is currently. As analytical data is generated in the laboratory, bench sheets are populated, and then added to the other

documents related to the sample. Raw data and calculations are reviewed by a senior analyst and the QA Manager and entered into our laboratory's database. Data and reports are generated in a program such as Microsoft Word and/or Excel, depending on the project. The final result as well as the report itself are reviewed and require a signature from two approved staff members.

The final results will be reported to WRF in the electronic data deliverable (EDD) Excel Format provided by the Technical Working Group (TWG). See Appendix 2.

Additional Records/Documents

Records and documents that will be produced in conjunction with this project include:

Standard Operating Procedures
For this contract, laboratory personnel will conduct all analyses and sample handling with strict adherence to method SOPs outlined in Table 2 (A6, above).

Documentation of Data Collection Activities
Each laboratory will be responsible to maintain records for samples submitted to the laboratories. Similarly, each laboratory shall document and track the aspects of the field sample collection process, including the generation of field sheets at each sampling site and Chain of Custody (COC) forms (Appendix 4) for the samples collected by the WWTPs. COC forms will accompany RWW samples to the laboratory for analysis. Laboratories will record and document the temperature at which the sample was received and report any sampling or shipping related problems to Cel A and the WRF independent QA/QC officer. Sub-contractor labs are required to submit a copy of the COC to the lead lab within 48 hr of sample receipt.

Laboratories Records
Laboratory Directors will be responsible for documenting and tracking the aspects of sample receipt and storage, analyses, and reporting. Hard copy records or electronic files when available from vendors pertaining to supplies, certificate of qualities for reagents, media and microbes, tissue culture media, cell lines including passage numbers will be kept and tracked.

Upon completion of laboratory analysis, laboratory data review, and data validation, the laboratory will issue reports in electronic format(s) specified for Demonstration of Capability, pre-campaign and campaign monitoring describing the results of analysis for each sample submitted. Prior to issuance of the laboratory report, the lead laboratory's Project Lead and QA Manager will review and approve the report prior to submittal to the WRF independent QA/QC officer.

Storage of Project Information

Cel-A maintains all documentation regarding analysis, along with all equipment maintenance and repair records, for a minimum of five (5) years. Electronic records will be maintained on LIMS. Hard copies of select records will be filed under the corresponding laboratory ID or records kept next to the equipment(s) such as incubator temperature monitoring logs, autoclave, water baths, Class II hoods along with other equipment specified in Cel-A Quality Assurance Plan v. 11. Refer to the following sections for examples of these SOPs. Backup of all information relating to this project will occur as follows:

- All handwritten notes pertaining to each sample will be electronically scanned and filed with a file name relating directly to the sample/samples which they reference.

- All electronic files will be backed up to the local server nightly.
- All files will be copied and uploaded to an off-site server at least weekly.

Each completed Data Input Form is stored individually within the database. The database is run from a networked computer, with both onsite and remote redundant and automatic backup, ensuring that data are saved and backed up immediately after each addition to the database. Any changes or addenda to this procedure will be included in this section of the QAPP.

At BCS all electronically generated data are stored on network drives, and backed up daily. All raw data in the form of bench sheets, plots, and laboratory notes are scanned and stored electronically in batch analysis work sheet files. All backup data are maintained in multiple formats. All hard copy records including but not limited to bench sheets, instrument maintenance logs, analysis run logs, and media prep logs are archived in secure access-controlled storage. The dedicated storage area is environmentally protected. All access to the archived documents is documented and monitored. Electronic copies of all final reports are stored in batch files. The files are maintained in redundant digital format. These files are maintained for a period of ten years unless the client specifies a longer term. All computers and software used by BCS Laboratories utilizes passwords and the Laboratory Director has Administrator authority granting or limiting access by BCS staff.

Scientific Methods stores all records electronically and in hard copy with a minimum of 5 years from generation of the last entry in the records. Backup copies of electronic records will be generated regularly and maintained in secure storage on and off site. Off-site storage will allow for reproduction of the data in the event of a failure of the computer system or natural disaster.

B DATA GENERATION AND ACQUISITION

B1. Sampling Process Design (Experimental Design)

A key initial task in this project is the pre-campaign evaluation/optimization and laboratory proficiency tests to generate data for demonstration of capability (pre-campaign testing Table 5 in Section A7, above) and, describe the project's performance and quality control criteria (see Table 6 in Section A7, above). These samples will be prepared by the lead laboratory. This section of the QAPP will be amended upon completion of the project-specific performance testing to include information for subsequent tasks.

B2. Sampling Methods

Grab samples will be collected at the individual sites by WWTP staff personnel. The laboratories will not be directly involved in sample collection, but will be responsible for supplying sampling containers and shipping materials. A sampling protocol has been provided to the WWTPs (Appendix 3).

B3. Sample Handling and Custody

Sample kits will be shipped overnight with delivery expected from Monday through Thursday. The laboratories will provide adequate logistic support to the WWTPs to ensure the integrity of the samples upon arrival to the laboratory.

Sample Custody

The integrity of samples will be verified using the following sample acceptance criteria routinely employed at the laboratory and specifications identified in the contract:

- Accurate and extensive documentation (i.e. sample identification, location, date/ time of collection, collector's name, preservation type, sample type, and remarks, analysis requested);
- Correct labeling of samples (i.e. durability of labels, use of permanent ink) containing their unique identification;
- Integrity of sample containers/ shipping package;
- Observance of required holding times/ proper temperature during transit (< 10 ºC and ≥ 4 ºC) as shown in Table 6;
- Sufficient sample volume.

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Each individual laboratory will have ultimate responsibility for ensuring samples are properly handled and transferred. In case of damage and/or contamination, the Laboratory Director or laboratory QA Officer will notify WRF Project Managers identified above in an immediate and timely manner and (no later than 24hr from the time of receipt) to request re-sampling. Sample handling procedure and example COCs from the labs are listed in Appendix 4.

Sample Tracking

Upon receipt, each sample is given its own unique laboratory identification code, as well as, sub-samples or dilutions to be tested to prevent any mix-ups or errors with respect to the sample's identity. Identification codes are recorded in the laboratory information management system (LIMS) and on the chain of custody form upon receipt at the laboratory and on sample container(s) in the form of a durable label. All subsequent processing (i.e. preparation, concentration, assays, amplification reactions, etc.) are documented by the laboratory using identifiable codes. Each lab will be responsible for tracking sample ID, batch ID, subsamples or dilutions, analysis requested, hold times and report due date.

Sample Storage

All samples received by the laboratory are kept in a designated and secure storage area, away from standards, reagents, food, and other potential contaminating sources. All samples will have ample refrigerator space, and will be handled by minimal personnel. Once the samples are received, the laboratory QC Officer or designee will sign the sample receipt and ensure that samples are stored at the appropriate temperature. It is the responsibility of the laboratory QC Officer or designee to assure that all microbiological samples are correctly preserved (if needed) and stored at 1°C to 10 °C (see Table 7). Storage times and temperatures are documented, and later taken into account in the interpretation of results.

Table 7. Analytical Methods Shipping Temperatures and Maximum Hold Times

Analytical Method	Microbe	Shipping Temperature	Hold Time
EPA Method 1615v1.1	Viruses: 1) Enterovirus 2) Adenovirus 2) Norovirus	1-10°C	< 72 hours
WRF_COV_SARS2	1) SARS CoV-2 2) OC 43	1-10ºC	< 72 hours
EPA Method 1602	Bacteriophages	1-10°C	< 72 hours
EPA 1693	*Cryptosporidium* & *Giardia*	1-10ºC	< 72 hours

Notes/Definitions:

- EPA Method 1615v1.1 modified.
- qPCR= Quantitative Polymerase Chain Reaction
- °C= degree centigrade

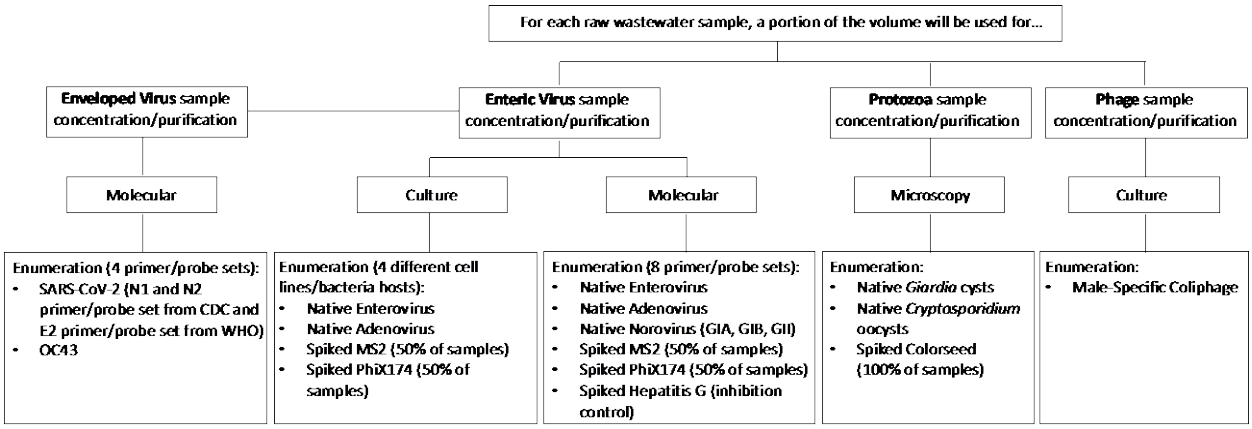
Sample Archiving

After analysis is complete, archived virus samples will be properly labeled and stored at -70°C for the duration of the project or shipped to WRF upon request for a period not to exceed 6 months after completion of the project. Protozoan samples will not be archived.

B4. Analytical Methods

A brief summary of analytical methods is provided in this section. This project will follow with strict adherence to test methods identified in Table 2 Section A6, above. Methods with no change in content are provided as a link below and/or copies in Appendix 1. A brief overview of the workflow is presented in Figure 2 below.

Fig. 3. Wastewater Sample Workflow.



Method Summaries

EPA Method 1615v1.1. Measurement *of Enterovirus, Adenovirus and Norovirus Occurrences in Water By Culture and RT-qPCR (Modified).* Approximately one liter portions of wastewater will be analyzed for enterovirus, adenovirus, norovirus and spiked bacteriophage using this modified 1615 SOP. The virus sample will be concentrated/purified using Polyethylene Glycol (PEG) flocculation and chloroform extraction. The concentrated sample will be processed using culturable and molecular methods. The portion of the sample for culture enumeration will be divided into four parts. Two of the sub-samples will be used to enumerate enterovirus (using BGM cells) and adenovirus (A549) via mammalian cell culture (total culturable virus--TCV), and two of the sub-samples will be used to enumerate the matrix spikes (MS2 and PhiX174) via culture method (1602 described below). For the portion of the sample used for molecular enumeration, eight different primer/probe sets will be used: five of the primer/probe sets will be used to enumerate enterovirus, adenovirus and norovirus (GIA, GIB, and GII); two primer/probe sets will be used to enumerate the matrix spikes (MS2 & PhiX174); and one of the primer/probe sets will be used to enumerate the inhibition control (Hepatitis G). TCV 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. The viral nucleic acid is extracted from the concentrate and tested for enterovirus and norovirus RNA using RT-qPCR and viral deoxyribonucleic acid (DNA) is tested for adenovirus using qPCR. Virus concentrations for the molecular assay are calculated in terms of genomic copies of viral RNA/DNA per liter. In order to limit the variability inherent in multi-laboratory testing of viruses, all laboratories will be using the same cell lines and molecular supplies.

Enveloped Virus Method – This method was developed in-house with collaboration between BCS and Cel Analytical. Approximately one liter of raw sewage (same sample that is used for analysis of enterovirus, adenovirus, norovirus) is spiked with surrogate virus (OC43) and allowed to mix for 30 min. A buffered (pH 7.2 – 7.5) beef extract solution is added and stirred for 30 min at room temperature. 100 mL of this suspension is aliquoted for concentration and

analysis of enveloped virus; the remaining 1 L is processed as described above for analysis of enterovirus, adenovirus, and norovirus. 15 mL of the aliquot is loaded onto a Amicon ultrafiltration-Vivaspin 20. The Amicon filter is centrifuged at 4500-5000 x g for 30 minutes or until the concentrate is reduced to less than 1 mL. The column will be reloaded as many times as possible until filter begins to show signs of clogging. Typically, 45-60 mL can be concentrated 1 mL of RNA lysis buffer is added directly to the sample in the Amicon and resuspended. This suspension can be stored at 4ºC for up to 24 hours or at -70ºC for long term storage. For the virus assay, four primer sets will be used, three for the SARS CoV-2 (N1, N2, and E3) and one for OC43. 100 µL of the viral nucleic acid is extracted from the concentrate and a portion of this extract is tested for SARS CoV-2 and OC43 RNA using RT-qPCR. Virus concentrations for the molecular assay are calculated in terms of genomic copies of viral RNA/DNA per liter. In order to limit the variability inherent in multi-laboratory testing of viruses, all laboratories will be using the same molecular supplies.

EPA 1602/1601: Male-specific (F+) and Somatic Coliphage in Water--describes the double layer agar (DAL) and single agar layer (SAL) procedures. In the SAL procedure, a volume of the diluted sample is assayed by adding $MgCl_2$ (magnesium chloride), selected antibiotics, log-phase host bacteria (*E. coli* Famp for F+ coliphage and *E. coli* CN-13 for somatic coliphage), to molten tryptic soy agar (TSA). The sample is thoroughly mixed and the total volume is poured into plates. The Double Layer Agar (DAL) method (1601) can also be used to quantitate samples. Briefly, the sample (or dilutions of samples) is mixed with the molten agar with additives and poured onto a bottom layer of solidified TSA *(Note: Optimization testing will determine if labs use the SAL or DAL method).* After an overnight incubation, circular lysis zones (plaques) are counted and summed for all plates from a single sample. The quantity of coliphage in a sample is expressed as plaque forming units (PFU) /ml.

EPA 1693: Method 1693: *Cryptosporidium* and *Giardia* in Disinfected Wastewater by Concentration/IMS/IFA. Method 1693 describes a procedure for the detection of *Cryptosporidium* (CAS Registry number 137259-50-8) and *Giardia* (CAS Registry number 137259-49-5) in disinfected wastewater by concentration, immunomagnetic separation (IMS), and immunofluorescence assay (IFA) microscopy. Following detection using fluorescein isothiocyanate (FITC) for the IFA, *Cryptosporidium* and *Giardia* are examined and characterized using 4',6-diamidino-2-phenylindole (DAPI) staining and differential interference contrast (DIC) microscopy.

For matrix spikes, observation under Texas Red fluorescence is used to distinguish commercially supplied protozoa (the spike organisms) from the naturally occurring background. This method identifies the genera, *Cryptosporidium* or *Giardia*, but not the species. The method cannot determine the host species of origin, nor can it determine the viability or infectivity of detected oocysts and cysts. There are some modifications to this method to account for the raw wastewater matrix (see Appendix 1).

Quality Controls associated with the methods specified are described under Section B5.

B5. Quality Control

All analytical methods include reference to the organisms used as positive and negative controls, method blanks and spike samples for precision and recovery. For PCR and cell culture-based assays, additional controls are required by the method to validate test results, assess the analyst performance and minimize false negatives or false positives caused by interfering substances or other contaminant(s) that may be present in RWW samples.

This project will undertake the following specific quality control steps to measure/estimate the effect of data errors. QC samples required for each method and frequency of analysis are outlined in Table 6 and summarized below.

Laboratory Fortified Blanks (LFB) for Virus Analysis consists of a reagent water sample that has been fortified with a known concentration of organism of interest. LFB will be processed, analyzed and reported as a QC sample for all methods as identified in Table 8 below. Analysis of pure culture or spiked sample recovery should conform to a known result, within an acceptable margin of error.

Laboratory Reagent Blanks (LRB) also known as method blanks provide bias information on possible contaminants for the entire laboratory analytical system. The laboratory will process laboratory blanks through the laboratory sample handling, preparation and analytical processes. These blanks will be made from sterile purified water (for viruses) or reagent grade water (for protozoa) that are known to have no detectable levels of the target organism. They will be processed with each batch of sample to document background contamination of the analytical measurement system. LRB must be less than the reporting limit or contain no organisms.

Laboratory Fortified Sample Matrix (LFSM) Pre-Campaign Only. One mL of 10,000 MPN/mL matrix spike of poliovirus Sabin 3 will be added to 1L of RWW that will be sent to the lab. Each lab will add 10^7 pfu of each bacteriophage to 1 L of RWW. The labs will also receive 1L of RWW spiked with ColorSeed (approx..100 each of *Cryptosporidium* and *Giardia*). If the recovery is not within the expected range, but the control sample recovery is, this data must be identified as suspect due to matrix effects and the lab(s) may be asked to process and analyze a repeat sample at no cost to WRF.

Laboratory Fortified Sample Matrix (LFSM) Monitoring Campaign One mL of 10^7 pfu/mL bacteriophage phiX174 and MS2 will be added to 1L of RWW received by the lab in every other sampling event for a given WWTP. Each lab will add one vial of ColorSeed (approx..100 each of *Cryptosporidium* and *Giardia*) to 1 L RWW. If the recovery is not within the expected range, while control sample recovery is, this data must be identified as suspect due to matrix effects and the lab(s) may be asked to process and analyze a repeat sample at no cost to WRF.

Table 8A: Quality Control Samples and Frequency of Analysis for Analytical Methods, Bacteriophage

QC Sample	Description	Method	Frequency/Timing	Purpose	Acceptance Criteria
Negative Control (method blank)	1 culture test vessel with buffer	PFU culture assay (with *E. coli* F_{amp})	Every *E. coli* F_{amp} culture assay	To verify that external factors aren't preventing the growth of *E. coli*	If *E. coli* fails to grow, troubleshoot and repeat assay
Positive Control	At least one culture test with MS2 in buffer (appropriate number of dilutions to observe plaques)	PFU culture assay (with *E. coli* F_{amp})	Every *E. coli* F_{amp} culture assay	To verify the continued sensitivity of the *E. coli* to phage infection	If no plaques are observed, troubleshoot and repeat assay

There is no demonstration of capability or ongoing performance and recovery testing for this method.

Table 8B: Quality Control Samples and Frequency of Analysis for Analytical Methods, Protozoa.

QC Sample	Description	Method	Frequency/Timing	Purpose	Acceptance Criteria
Initial demonstration of capability (IDC) for 1623/1693	Initial precision and recovery (IPR) set: one sample with ColorSeed (or equivalent) spiked to reagent water, one method blank (reagent water), and a MS/MSD in field sample	Either 1623 or 1693	Once (sometime before pre-campaign testing)	To establish initial control over the analytical system and demonstrate acceptable method performance	Table 3, 4, and 5 of 1693
Demonstration of capability for this study	Three field sub-samples with blind matrix spikes (ColorSeed[1])	Modified 1693 with direct centrifugation	Once (during pre-campaign testing)	To demonstrate acceptable method performance using the modified method	See Table 6 above
Ongoing precision and recovery (OPR)	One positive control (ColorSeed[1] in reagent water) and one method blank (reagent water)	Modified 1693 with direct centrifugation	Once initially and then once per month for the duration of the study	To demonstrate ongoing control of the analytical system and verify continuing method performance	See Table 6 above
Matrix spike (MS)	ColorSeed[1] in field sample	Modified 1693 with direct centrifugation	Every sample (during full monitoring campaign)	To determine the effect of the matrix on (oo)cyst recoveries	See Table 6 above
Positive staining control	Positive antigen that comes with EasyStain ™ stain kit[1]	Prepare according to the manufacturer recommendations	Process each time samples are stained; examine each microscope session	To demonstrate ongoing control of the staining process and performance of reagents and microscope	Control should display fluorescence for IFA and DAPI stain
Negative staining control	PBS	Prepare according to the manufacturer recommendations	Process each time samples are stained; examine each microscope session	To demonstrate the absence of contamination through staining process	Control should not contain any fluorescent (oo)cysts

1. ColorSeed ™ and EasyStain™ from BioPoint Pty, Ltd (formerly BFT, Ltd)

Table 8C: Quality Control Samples and Frequency of Analysis for Analytical Methods, Virus

QC Sample	Description	Method	Frequency/Timing	Purpose	Acceptance Criteria
Demonstration of capability for this study	Three field sub-samples with blind spikes (poliovirus) and three field sub-samples without blind spikes; both shall include matrix spikes (MS2 and PhiX174)	Modified 1615 (PEG/chloroform extraction, culture, and molecular) (Do not need to run adenovirus or norovirus assays)	Once (during pre-campaign testing)	To demonstrate acceptable method performance using the modified method	See Table 6 above
QC sample set-Enteric viruses	One positive control (poliovirus in reagent water) and one negative control	Modified 1615 (PEG/chloroform extraction, culture, and molecular) (For positive control, do not need to run adenovirus or norovirus assays)	Once initially and then once per month for the duration of the study	To demonstrate ongoing control of the analytical system and verify continuing method performance	See Table 6 above
Matrix spike Enteric viruses	MS2 and PhiX174 in field sample	Modified 1615 (PEG/chloroform extraction, culture, and molecular) Verify the concentration of the spiking suspension in terms of both PFU/L and GC/L every time a matrix spike is performed	Every other field sample from each WWTP during full monitoring campaign; every field sample during pre-campaign testing	To determine the effect of the matrix on virus recoveries	See Table 6 above
QC sample set – Enveloped Viruses	One positive control (OC43 in reagent water) and one negative control	Direct incubation in beef extract Amicon filter Concentration, RNA extraction, molecular assay	Once initially and then once per month for the duration of the study	To demonstrate ongoing control of the analytical system and verify continuing method performance	To Be Determined
Matrix Spike Enveloped Viruses	OC43 in field sample	Direct incubation in beef extract Amicon filter Concentration, RNA extraction, molecular assay Verify the concentration of the spiking suspension (GC/L)	Every field sample from each WWTP during campaign testing	To determine the effect of the matrix on enveloped virus recovery	To Be Determined

QC Sample	Description	Method	Frequency/Timing	Purpose	Acceptance Criteria
		every time a matrix spike is performed			
Positive Control: Enterovirus Culture Assay	3 culture test vessels with poliovirus in buffer	MPN culture assay with BGMK cells	Every BGMK culture assay	To verify the continued sensitivity of the cell cultures to virus infection	EPA 1615 Section 12.1.2.5.2
Positive Control: Adenovirus Culture Assay	3 culture test vessels with adenovirus in buffer	MPN culture assay with A549 cells	Every A549 culture assay	To verify the continued sensitivity of the cell cultures to virus infection	EPA 1615 Section 12.1.2.5.2
Negative Control: Enterovirus Culture Assay	3 culture test vessels with buffer	MPN culture assay with BGMK cells	Every BGMK culture assay	To verify that external factors aren't harming the cells	EPA 1615 Section 12.1.2.4.2
Negative Control: Adenovirus Culture Assay	3 culture test vessels with buffer	MPN culture assay with A549 cells	Every A549 culture assay	To verify that external factors aren't harming the cells	EPA 1615 Section 12.1.2.4.2
Negative Control: MS2 Culture Assay	1 culture test vessel with buffer	PFU culture assay (with *E. coli* F_{amp})	Every *E. coli* F_{amp} culture assay	To verify that external factors aren't preventing the growth of *E. coli*	If *E. coli* fails to grow, troubleshoot and repeat assay
Negative Control: PhiX174 Culture Assay	1 culture test vessel with buffer	PFU culture assay (with *E. coli* CN-13)	Every *E. coli* CN-13 culture assay	To verify that external factors aren't preventing the growth of *E. coli*	If *E. coli* fails to grow, troubleshoot and repeat assay
Negative Control: Molecular Assay	1 sample with PCR grade water	(rt)-qPCR	One per every fourth sample on a plate	To verify the lack of contamination	EPA 1615 Section 13.4.4.2
Inhibition Control (Positive Control): Molecular Assay – Enteric Viruses	1 test sample with hepatitis G armored RNA (compare to hepatitis G in reagent water)	rt-qPCR	Every molecular assay (one per field sample)	To identify any matrix inhibition	EPA 1615 Section 13.6.2
Inhibition Control (Positive Control): Molecular Assay, Enveloped Viruses	TaqMan™ Internal Controls run with OC43 assay	rt-qPCR	Every molecular assay (one per field sample)	To identify any matrix inhibition	To Be Determined

Additional Quality Control Measures for Nucleic Acid Amplification (qPCR/RTPCR)

To minimize cross contamination, sample processing and nucleic acid amplification will be performed under sterile conditions following one-way work flow protocols to prevent nucleic acid contamination of reagents followed by daily cleaning of laboratory work areas. Additionally, qPCR by virtue of being a closed system has minimized contamination problems often encountered with conventional PCR that requires analysis of amplified samples by gel electrophoresis. However, controls remain an essential part of assays requiring DNA amplification or RNA amplification for gene expression or viral load determination.

Examples include but are not limited to:

- ***NTC***- no template control at least in duplicate to evaluate possible cross-contamination applicable to both qPCR and RT-qPCR. If NTC is positive, investigate how a positive reaction could have happened, apply appropriate corrections and repeat the assay.
- ***RT-negative***: RNA preparations may contain residual genomic DNA, which may be detected in real-time RT-PCR. DNA contamination can be detected by performing a no RT control reaction in which no reverse transcriptase is added.
- ***DNA (Adenovirus, phix174), RNA (MS2), and Armored RNA standard curves*** at different dilutions to control inter-assay variability.
- ***Inhibition Assay***: Hepatitis G RNA. The inhibition assay is critical in determining if the sample is contributing compounds or elements which are interfering with the assay. The outcome of the inhibition assay will determine which dilution of the sample is to be run. For SARs_CoV2, a TaqMan™ Exogenous Internal Positive Control Reagents will be spiked into samples to distinguish true target negatives from PCR inhibition.

Quality Control Statistics

The quality control statistical calculations such as calculation for determining accuracy, precision and bias, are described in more detail under Section D3.

Corrective Actions

When problems associated with equipment(s), sample management, analytical results and data entry or reporting occur it is the responsibility of the laboratory QA Officer to immediately notify the Lead Laboratory and WRF independent QA/QC Officer and implement corrective action measures. The effectiveness of the corrective action must be established by follow up procedures and an internal audit and verified by the WRF independent QA/QC Officer.

Data Entry QA Procedures

In order to ensure accuracy of data, the Laboratory QA/QC Officer will enact and uphold policies for data entry. Results are entered by a technician/analyst and validated before being forwarded for final approval and reporting. Sub-labs will provide their data in their report format and as WRF electronic data deliverable (EDD) spreadsheet (Appendix 2) to the lead laboratory coordinator prior to submission to the WRF independent QA/QC Officer.

QA/QC Acceptance Criteria

The WRF independent QA/QC Officer will check that results meet the specified QA/QC criteria (Tables 6 & 7, above). If the results do not meet the specified QA/QC criteria, the independent QA/QC Officer will notify the lead lab. The lead lab will be responsible for

corresponding with the appropriate sub-lab (as applicable) to repeat the analysis on a new sample.

Pre-Campaign Testing

During the two months prior to the start of the monitoring campaign, the three laboratories will complete the pre-campaign testing. There are two goals of this pre-campaign testing:

- **Method Optimization to Finalize the SOPs**. The SOPs have been developed based on the pre-screening methods optimization study; however, there may be additional modifications/optimizations necessary prior to finalizing these SOPs. BCS laboratories was selected by the WRF Technical Working Group (TWG) for this task.
- **Demonstration of Capability.** Verify that all three labs can perform the analysis and obtain results that pass the QA/QC acceptance criteria, and verify that the performance among the three labs is similar.

B6. Instrument/Equipment Testing, Inspection and Maintenance

The QC Officers of each laboratory set policies for routine checks and repairs of laboratory equipment depending on the manufacturer's recommendations and prior to sample analysis when applicable. Details of the inspection and maintenance schedule are either marked by stickers on the equipment or maintained in the equipment log book to record all checks and repairs.

Corrective actions reports will be created for equipment out of spec. Preventative actions will be documented to limit future occurrence.

B7. Instrument/Equipment Calibration and Frequency

Equipment performance is monitored and logged to ensure that all equipment meets the appropriate levels of accuracy and precision. A calibration log is kept for equipment to record all checks and repairs. QA/QC logbooks are kept in the laboratory for autoclaves, microscopes, pipettors, biological hoods, vacuum pumps, the laboratory water purification system, incubators, refrigerators, freezers and the PCR machine(s). Balances, thermometers and the pH meter are routinely calibrated to meet the NIST and EPA standards.

B8. Inspection/Acceptance for Supplies and Consumables

Per specification of each laboratory's Internal Quality Assurance Plan (iQAP), prepared media and reagents will be purchased from manufacturer/suppliers with the certificate of quality assurance and quality control for the batch received. Once received at the lab, the information is documented. Lot number(s), date opened and the expiration dates are clearly marked on the media and reagents. New lots of dehydrated or commercially prepared, as well as in-house prepared media/reagents, are checked before use with a positive and a negative control culture as appropriate.

BGMK cell line was kindly provided by US EPA, Office of Groundwater, Cincinnati, OH. A549 cells were purchased from American Type Culture Collection (ATCC). The cells are seeded as monolayers in T-25 flasks and ready for use to eliminate handling and maintenance in-house. Host bacteria and phages for Method 1602/1601 are purchased from ATCC. A log book will be used to track the strains, phage titers and generations.

Blank media, buffer dilution and rinse water are routinely included in the test runs to verify sterility. Media sterility is checked by incubating a representative batch (identified by batch reference number), at the appropriate temperature for 24 to 48 hr and observed for growth. Sterile flasks, tissue culture dishes, pipettes and micropipette tips from each new batch are randomly tested to ensure sterility.

B9. Non-Direct Measurements

Non-direct measurements, also referred to as secondary data, are data previously collected under an effort outside this project. There will be no data obtained for this project that are derived from non-direct measurement sources, with the exception of geographical location maps for WWTPs under study.

B10. Data Management

All laboratories will implement their data management in accordance to their internal quality assurance plan (iQAP) and specifications identified for this QAPP to reflect any changes before the implementation process.

Any amendment to data management will be detailed in this section of the QAPP affecting any of the following elements:

- Sample Management from receipt to storage described
- Standard recordkeeping and tracking practices, and document control system
- Data handling equipment/procedures that will be used to process, compile, analyze, and transmit data reliably and accurately
- Individuals responsible for changes to elements of the data management
- Process for LIMS data archival and retrieval

Reports from the lead and sub-contract laboratories will be submitted to the WRF independent QA/QC Officer for final review.

C ASSESSMENT/OVERSIGHT

C1. Assessment and Response Actions

The laboratory QA Officer will conduct a Readiness Review immediately prior to major analytical testing tasks: identifying availability and integrity of supplies and consumables, inspection of equipment, analysts' readiness, data verification and data entry. The laboratory QA Officer will report findings to the Laboratory Director, who will take corrective action (if any is necessary) before the data collection task begins.

Further, the Laboratory Director and laboratory QA Officer will report any emerging/unanticipated problems to the Lead Laboratory, WRF independent QA/QC Officer and Project Managers as soon as discovered including the corrective actions taken, if necessary.

C2. Reports to WRF

Reports and results from the two sub-laboratories will be submitted to the lead laboratory for evaluation. Results for the analysis of *Giardia*, *Cryptosporidium*, bacteriophage (culture and PCR); and, Norovirus, Enterovirus, SARS CoV-2, OC43, and Adenovirus (by PCR) are to be reported to WRF independent QA/QC Officer who then submits to Project Management within 30 days of sample receipt. Analysis results for viruses by tissue culture (Enterovirus and Adenovirus) will be reported to WRF Project Management within 60 days of sample receipt. In order to limit the variability inherent in multi-laboratory testing of viruses by tissue culture, all laboratories will be using the same cell line and media sources whenever possible. Therefore, in order to expedite the start of the various campaigns, the tissue culture results may be delayed for the first two months of samples to allow the labs to propagate the cell lines in preparation of assay.

D DATA REVIEW AND EVALUATION

D1. Data Review, Verification and Validation

Each responsible party listed in Section A4 shall adhere to all the procedures required by the QAPP and ensure that all personnel involved in this project do likewise.

This QAPP shall be reviewed at least once every 6 months of the project to ensure that the project will achieve all intended purposes. All the responsible persons listed in Section A4 shall participate in the review of the QAPP. The Lab Lead and the WRF independent QA/QC Officer are responsible for determining that data are of adequate quality to support this project. As appropriate, the project will be modified as directed by the WRF Project Management. The WRF Project Management shall be responsible for the implementation of changes to the project and shall document the effective date of all changes made.

It is expected that from time to time ongoing and perhaps unexpected changes will need to be made to the project. The WRF Project Management shall authorize all changes or deviations in the operation of the project. Any significant changes will be considered an amendment to the QAPP. All verification and validation methods will be noted in the analysis reports.

All reports include quality control data, initials and/or signature of the analyst performing the analysis and the signature of the reviewer. An EDD in the WRF format will be provided.

Data Reduction (DR)/Analyst Bench Sheets

Each analyst records raw laboratory data consisting of project name, unique laboratory ID number, method procedures, batch ID, dilutions, calculations, and QC measures as appropriate to the method. Some laboratories are using electronic LIMS for recording test outcomes as well as bench sheets.

The analyst can also record information in his/her laboratory note book in permanent ink. Any changes in data shall be marked with a line through in a manner that the initial account is evident. All changes are initialed and dated. The analyst reduces the raw data and the QC results into the laboratory reportable format which then becomes part of the laboratory's electronic documentation system.

Data Validation

The analyst is responsible for data accuracy and completeness, for calibration and performance verification of instruments, and for analyzing the correct type and quantity of quality control samples, which must meet the laboratory's pre-determined control limits.

The quality control results must be checked against method limits. Should these limits not be met, analytical results are flagged, documented, and justified by the analyst. The laboratory QA Officer will review all available data and provide corrective action if deemed necessary.

All results will be checked by a second individual. If using an electronic LIMS, employ automated checking to ensure data falls within predefined method limits. If there are any violations in procedural or measured values, the sample is tagged for re-analysis, if feasible, by the laboratory QA Officer before the sample can be processed for reporting.

Data Reporting

The final report of the data is generated in the TWG EDD after all the internal review and validations are completed and signed by the analyst, laboratory QA Officer and/or the Laboratory Director.

Data Recording

All laboratories record and maintain documentation of all laboratory activities ranging from original observations, calculations and derived data to calibration records and copies of the test report. Records are maintained in a manner as to allow historical reconstruction of all laboratory activities.

D2. Verification and Validation Methods

To confirm that QA/QC steps have been handled in accordance with the QAPP, a readiness review will be conducted by the laboratory QC Officer before key data collection/analysis steps, and data handling reports. Statistical tests described in Section D3 will be used to determine the validity of the sample data. This QAPP will be amended to determine the best approach in verification and validation methods specific to this project or the task at hand.

D3. Evaluating Data in Terms of User Needs

This Section presents the approach in data evaluation.

Meeting and Reporting Needs of the Project

Information from analytical data reports (including corrective actions, and audits), laboratory data reviews (including errors involving data entry, transcriptions, omissions, and calculations and laboratory audit reports), reviews of data versus Data Quality Indicators, reviews against QA/QC requirements, data verification reports, data validation reports, independent data checking reports, and error handling reports will be used to determine whether or not the project's objectives have been met.

Quality Control Statistics

Total Culturable Virus data by Most Probable Number:

MPN/ml value (M_{ml}), upper (CL_{uml}) and lower (CL_{lml}) 95% confidence limits/ml are calculated from cultures with confirmed CPE and the EPA Most Probable Number Calculator program http://www.epa.gov/nerlwww/mpn.html

MPN/L value (M_L) or the original sample is calculated as follows:

$$\mathbf{M_L = (M_{ml}\, S) / D}$$

Where, M_{ml}= MPN/ml
S= Assay Sample Volume
D= Volume of the Water Sample Assayed.
Note: for M_{ml} values of 0, test sample detection limit rather than M_L value is calculated by dividing 1/D

95% Lower and Upper Confidence limits are calculated as follows:

$$\mathbf{CL_L = CL_{lml} S / D}$$

Where, CL_{lml} is the lower 95% confidence limit/mL
S= the Assay Sample Volume
D=original water sample assayed

$$\mathbf{CL_L = CL_{uml} S / D}$$

Where, CL_{uml} is the upper 95% confidence limit/mL
S= the Assay Sample Volume
D=original Water sample Assayed

For QC samples the calculation is modified as follows: Total MPN value and total 95% confidence limit values are calculated by multiplying MPN/mL values by S and dividing by D.

Molecular Virus data calculation-Genomic Copies per Liter (GC_L)

Genome copy number of unknown test samples are calculated based on the standard curve samples used in the test run as show in Equation 1 below.

Equation 1:

$$GC_{original\ sample\ per\ L} = \frac{GC_{assay} \times V_{nucleic\ acid\ extract} \times FCSV \times DF}{V_{assay} \times V_{pellet\ for\ molecular} \times TSV}$$

Where,

GCoriginal sample per L = Calculate genome copies per liter in the original sample
GCassay = Genome copies measured from the qPCR assay
Vnucleic acid extract = Volume of the concentrate after nucleic acid extraction (should be 0.2 mL)
FCSV = Final concentrated sample volume (before chloroform extraction) (should be 2.0 mL)

DF = dilution factor = the reciprocal of any dilution performed to compensate for inhibition (e.g., 5 and 25 for 1:5 and 1:25 dilutions, respectively)
Vassay = volume of the nucleic acid extraction used for each (rt)qPCR assay (should be 0.006 mL)
Vpellet for molecular = volume of the re-dissolved pellet/concentration used for nucleic acid extraction (should be 1 mL)
TSV = total sample volume (should be 1 L)

Approach to Managing Unusable Data

If there are any data quality problems, the Laboratory Lead and WRF independent QA/QC Officer will identify whether the problem is a result of project design issues, sampling issues, analytical methodology issues, or QA/QC issues. If the source of the problem can be traced to one or more of these basic activities then the person or people in charge of the areas where the issue lies will be contacted and efforts will be made to immediately resolve the problem. If the issue is too broad or severe to be easily corrected then the appropriate people involved will be assembled to discuss and try to resolve the issue(s) as a group. Ultimately the Laboratory Director is responsible to notify the WRF Project Managers that the data is unusable and is not billable with an explanation provided in writing.

Hyperlinks to EPA Method 1615 and 1602 are given below:

Method 1615v1.1 is available in pdf form at the USEPA website
https://naldc.nal.usda.gov/download/55313/PDF
Fout, G.S., Martinson, B.C., Moyer, M.W.N., and Dahling, D.R., 2003, A multiplex reverse transcription-PCR method for detection of human enteric viruses in groundwater: Applied and Environmental Microbiology, v. 69, no. 6, p. 3158-3164.

Method 1602 is available in pdf form at the USEPA website
https://www.epa.gov/sites/production/files/2015-12/documents/method_1602_2001.pdf

Method 1693 is available in pdf form at the US EPA website
https://www.epa.gov/sites/production/files/2015-08/documents/method_1693_2014.pdf

References

Olivieri, A.W., J. Crook, M.A. Anderson, R.J. Bull, J.E. Drewes, C.N. Haas, W. Jakubowski, P.L. McCarty, K.L. Nelson, J.B. Rose, D.L. Sedlak, and T.J. Wade (2016). Expert Panel Final Report: Evaluation of the Feasibility of Developing Uniform Water Recycling Criteria for Direct Potable Reuse. Prepared August 2016 by the National Water Research Institute for the State Water Resources Control Board, Sacramento, CA.

California Water Boards “SWAMP-EPA-QAPP Review Checklist”.
https://www.waterboards.ca.gov/water_issues/programs/swamp/quality_assurance.html

Features, Evaluation and Treatment Coronavirus (COVID-19). Marco Cascella; Michael Rajnik; Arturo Cuomo; Scott C. Dulebohn; Raffaela Di Napoli. StatPearls.
https://www.ncbi.nlm.nih.gov/books/NBK554776/; April 6, 2020.

Vincent R. Hill • Bonnie Mull •Narayanan Jothikumar • Karen Ferdinand •Jan Vinje´Food Environ Virol (2010) 2:218–224: Detection of GI and GII Noroviruses in Ground Water Using Ultrafiltration and TaqMan Real-time RT-PCR

Turgeon, N. et al. 2014; Appl Environ Microbiol 80(14):4242-4250: Comparison of Five Bacteriophages as Models for Viral Aerosol Studies

Ko et al., J. Virol Methods, 2005, 127: 148-153: Rapid detection of infectious adenoviruses by mRNA real-time RT-PCR

Dare, R.K. et al. J Infect. Diseases, 2007, 196: 1321-8: Human coronavirus infections in rural Thailand: a comprehensive study using real-time reverse-transcription polymerase chain reaction assays.

WHO Protocol: Diagnostic detection of 2019-nCoV by real-time RT-PCR on Jan 17, 2020

End Section

Appendix 1

WRF 4952 -Method SOPs

Standard Operating Procedure for Processing and Enumerating Native Male-Specific Coliphage in Raw Wastewater

This SOP describes the procedure for processing and enumerating native male-specific coliphage in raw wastewater. This SOP was adapted from sections of EPA Method 1602 and EPA Method 1615. Refer to EPA 1602 for the list of the required equipment, supplies, and reagents.

1. Scope and Application

1.1. This method is designed for measuring male-specific coliphage in raw wastewater samples by a culture numeration method.

2. Summary of Method

2.1. Raw wastewater is prepared for enumeration of phage by filtration with a 0.2 μm filter. Then, a double agar layer (DAL) procedure is used to enumerate male-specific coliphage. In the DAL procedure, a tube of molten 0.7% TSA "top agar" with added host bacteria is inoculated with dilutions of the filtered raw wastewater and is poured into a 1.5% TSA "bottom agar" plate. Four dilutions of coliphage stock or sewage filtrate will be analyzed in duplicate. As a result, at least 10 double-agar layer plates will be required: two plates per dilution (undiluted, 0.1, 0.01, 0.001), one method blank (negative control) plate and at least one positive control (additional dilutions may be required to observe plaques). The phage are enumerated by counting the plaque forming units (PFU).

3. Definitions

3.1. Coliphages are viruses (bacteriophages) that infect *E. coli* and are indicators of fecal contamination.

3.2. F-factor is the fertility factor in certain strains of *E. coli*. It is a plasmid that, when present, codes for pilus formation. The pilus allows for transfer of nucleic acid from one bacterium to another.

3.3. Male-specific coliphages (F^+) are RNA or DNA viruses that infect via the F-pilus of male strains of *E. coli*.

4. Interferences

4.1. Background bacteria and inhibitory compounds can interfere with the host of target bacteriophage.

4.2. High turbidity may require the use of multiple filters.

5. Safety

5.1. The analyst/technician must know and observe the normal safety procedures required in a microbiology laboratory while preparing, using, and disposing of cultures, reagents and materials, and while operating sterilizing equipment.

5.2. Mouth pipetting is prohibited.

5.3. Wear personal protective equipment: gloves, laboratory coats, and eye protection.

6. Equipment and Supplies

6.1. Section 6 of EPA Method 1602 lists the equipment and supplies needed for this method. In addition to these supplies, the following supplies are required:

1. Luer-Loc syringe (BD 309604, or equivalent)

2. Sterile Luer-Loc 0.2 μm filter (Cole-Parmer, 02915-40 or equivalent)
3. Centrifuge Tubes, 15, and 50 mL

7. Reagents and Standards

7.1. Section 7 of EPA Method 1602 lists the reagents and standards needed for this method. This method will not be used to enumerate somatic phage in the raw wastewater. Therefore, reagents specifically related to *E. coli* CN-13 (somatic coliphage host) and nalidixic acid are not needed for this method.

7.2. In addition, the laboratory will need a stock solution of MS2 with a concentration of approximately 10^8 PFU/mL for the positive control.

8. Sample Collection, Preservation, and Storage

8.1. Samples will be collected by the wastewater treatment facility staff and delivered to the lab. Samples should be delivered cold (target 4 °C, acceptable range of 1 – 10 °C)

8.2. The sample shall be analyzed within 24 hr of collection.

9. Quality Control

9.1. The laboratory shall analyze one method blank (negative control) with each sample.

9.2. The laboratory shall analyze one positive control with each sample.

9.3. The laboratory shall perform additional dilutions if the results from the required dilutions are inconclusive (to distinguish between an overloaded plate and a plate with inhibition).

9.4. The QA/QC requirements for this project are summarized in the following table:

QC Sample	Description	Method	Frequency/Timing	Purpose	Acceptance Criteria
Negative Control (method blank)	1 culture test vessel with buffer	PFU culture assay (with *E. coli* F_{amp})	Every *E. coli* F_{amp} culture assay	To verify that external factors aren't preventing the growth of *E. coli*	If *E. coli* fails to grow, troubleshoot and repeat assay
Positive Control	At least one culture test with MS2 in buffer (appropriate number of dilutions to observe plaques)	PFU culture assay (with *E. coli* F_{amp})	Every *E. coli* F_{amp} culture assay	To verify the continued sensitivity of the *E. coli* to phage infection	If no plaques are observed, troubleshoot and repeat assay

There is no demonstration of capability or ongoing performance and recovery testing for this method.

10. Calibration and Standardization

10.1. Check thermometers at least annually against a NIST certified thermometer or one that meets the requirements of NIST Monograph SP 250-33. Check mercury columns for breaks.

10.2. Micro-pipettors are calibrated semi-annually.

10.3. Balances and scales are checked before use using Class S weights.

10.4. Biological safety cabinets and chemical hood are certified annually.

11. Procedure

11.1. Filter raw wastewater

11.1.1. Remove a 50 mL portion of the raw wastewater from the total sample delivered. Vortex.

11.1.2. Remove the plunger from a 10 mL luer-loc syringe (BD 309604, or equivalent) and aseptically fit a sterile luer-loc 0.2 μm filter (Cole-Parmer, 02915-40 or equivalent) on the end.

11.1.3. Add about 2 mL sterile Trypticase Soy Broth (TSB) (or other sterile organic solution) to the syringe and process through the filter.

11.1.4. Aseptically remove filter from end of syringe, set aside. Remove the plunger from syringe and re-attach the filter.

11.1.5. Add 10 mL of sample to the filter. Re-insert the plunger and process through the filter into a sterile 15 mL conical tube (or equivalent).

11.2. Dilutions:

11.2.1. Four dilutions will be needed for the culture method: undiluted, 0.1, 0.01, 0.001. Additional dilutions may be necessary. TSB without antibiotics is used as the diluent and is used to prepare the negative control.

11.2.2. To prepare the dilutions, aseptically add 9 mL of TSB without antibiotics into each of four (or more) sterile dilution tubes. Label them as “0.1,” “0.01,” “0.001,” “method blank,” etc.

11.2.3. Add 1 mL of the filtered raw wastewater to the tube of TSB labeled “0.1.” Cap the tube and vortex for 5 seconds on a medium-high setting (if available) or until well-mixed.

11.2.4. Add 1 mL of the well-mixed 0.1 dilution to a tube with 9 mL of TSB labeled “0.01". Cap the tube and vortex for 5 seconds on a medium-high setting (if available) or until well-mixed.

11.2.5. Add 1 mL of the well-mixed 0.01 dilution to a tube with 9 mL of TSB labeled “0.001.” Cap the tube and vortex for 5 seconds on a medium-high setting (if available) or until well-mixed.

11.2.6. Add 1 mL of TSB without antibiotics to the tube labeled “method blank.” Cap the tube and vortex for 5 seconds on a medium-high setting (if available) or until well-mixed.

11.3. Agar preparation:

11.3.1. Place 0.7% TSA top agar tubes with antibiotics in a 45°C to 48°C water bath. The top agar should remain molten in the water bath until ready for use. At least 10 tubes are necessary to enumerate four dilution volumes in duplicate with a method blank and at least one positive control. The 10 top agar tubes should contain ampicillin/streptomycin for growth of *E. coli* F_{amp}.

11.3.2. As a precaution against contamination, disinfect a workspace near the water bath with a 1 : 100 dilution of household bleach and allow to dry. If workspace can be corroded by bleach use an ethanol solution of 70% or greater.

11.3.3. Assemble 1.5% TSA bottom agar plates and label so that the following information is identifiable:

11.3.3.1. Dilution of stock filtrate, method blank, or positive control

11.3.3.2. Sample Number

11.4. Preparation of plates for enumeration:

11.4.1. Please note: The following steps are critical. To ensure viability of bacterial host and coliphage, do not add bacterial host and sample until ready to plate.

11.4.2. With the top agar tube still in the water bath, aseptically inoculate a top agar tube containing ampicillin/streptomycin with 100 μL of log-phase *E. coli* F_{amp}.

11.4.3. Immediately add 1 mL of the undiluted filtered raw wastewater sample.

11.4.4. Mix the inoculum by rolling the tube briefly in palm of hand.

11.4.5. Pour contents into one of the two bottom agar plates marked “undiluted."

11.4.6. Duplicate analysis—Repeat Sections 11.4.2 through 11.4.5 for the duplicate.

11.4.7. Repeat Sections 11.4.2 through 11.4.6 for each dilution volume.

11.5. Preparation of method blank:

11.5.1. With the top agar tube still in the water bath, aseptically inoculate a top agar tube containing ampicillin/streptomycin with 100 μL log-phase *E. coli* F_{amp}.

11.5.2. Immediately add 1 mL of TSB from the “method blank” dilution tube. Mix the inoculum by rolling the tube briefly in palm of hand.

11.5.3. Pour contents into a bottom agar plate marked “method blank.”

11.6. Preparation of the positive control(s):

11.6.1. Dilute the MS2 stock to the appropriate concentration to observe plaques. Multiple dilutions may be required.

11.6.2. With the top agar tube still in the water bath, aseptically inoculate a top agar tube containing ampicillin/streptomycin with 100 μL log-phase *E. coli* F_{amp}.

11.6.3. Immediately add 1 mL of the diluted MS2 stock. Mix the inoculum by rolling the tube briefly in palm of hand.

11.6.4. Pour contents into a bottom agar plate marked “positive”.

11.6.5. To run multiple dilutions of the positive control, repeat steps 11.6.2 – 11.6.4 with each dilution.

11.7. Incubate:

11.7.1. After the top agar hardens, invert the plates and incubate for 16 to 24 hours at 36°C ± 1.0°C.

11.8. Count and Record:

11.8.1. Circular zones of clearing (typically 1 to 10 mm in diameter) in lawn of host bacteria after 16 to 24 hours of incubation are plaques. Count the number of plaques on each plate. Please note: The use of a light box to evaluate results is recommended. Record the results in native phage sheet.

Standard Operating Procedure for Processing and Enumerating *Giardia*, *Cryptosporidium*, and the Corresponding Matrix Spikes in Raw Wastewater

EPA Method 1693 along with the following addendum shall be used to process and enumerate *Giardia*, *Cryptosporidium*, and the corresponding matrix spikes in raw wastewater. The addendum lists the method modifications and clarifies certain steps in EPA Method 1693. Follow all of the steps in EPA Method 1693 unless otherwise specified by the addendum.

Addendum to EPA Method 1693:

1.1	Method has been modified for raw wastewater
2.1.1	Not applicable (lab will not use filtration option)
6.1.7	A ~10 L sample will be sent to the lab
6.1.8	Not applicable (lab will not use filtration option)
6.1.11	Not applicable (lab will not use filtration option)
6.1.12	Hemacytometer not required because pre-enumerated spiking suspensions (ColorSeed) will be used for IPR and OPR analyses. BTF (CG-3WS-X25) and Waterborne (S100-3) well slides may also be used.
6.1.17-6.1.21	Spiking enumeration equipment not applicable because pre-enumerated spiking suspensions (ColorSeed) shall be used for OPR analyses
6.2.2	Not applicable (lab will not use filtration option)
6.2.5-6.2.7	Not applicable (lab will not use filtration option)
6.3.5	Low adhesion microcentrifuge tubes are required per step 16.2 (Fisher 02-681-320 or equivalent)
7.4	Not applicable (lab will not use filtration option)
7.5	Not applicable for filter elution (lab will not use filtration option), however, a small volume (~100 mL) of elution solution is required for pre-rinsing pipettes during the IMS procedure
7.8	The TWG will specify the brand of stains to use prior to the pre-campaign testing.
7.9.4-7.9.6	**Lab must use ColorSeed for spiking. No exceptions taken. The TWG may specify the manufacturer and batch number for interlaboratory consistency.**
8.2	Lab will not be filtering/eluting sample. Must use direct centrifugation. Centrifugation, IMS purification, and application of purified sample to the slide must be completed in one working day. Application of the purified sample to the slide should be completed immediately after IMS purification.
Table 1	Lab will not be filtering/eluting sample. Must use direct centrifugation. Centrifugation, IMS purification, and application of purified sample to the slide must be completed in one working day.
8.2.2	Lab will not be filtering/eluting sample. Must use direct centrifugation.
9.2	To be selected for this monitoring campaign, the labs are expected to have already completed the IDC and IPR for 1693 or 1623. Once contracted, the labs will perform the pre-campaign testing that will include some additional demonstration of capability testing. The scope of this testing is described in RFQ 4952.

Table 2 Replace with the following table:

Modified Table 2: Summary of Required Quality Control

QC Sample	Description	Method	Frequency/Timing	Purpose	Acceptance Criteria
Initial demonstration of capability (IDC) for 1623/1693	Initial precision and recovery (IPR) set: one sample with ColorSeed (or equivalent) spiked to reagent water, one method blank (reagent water), and a MS/MSD in field sample	Either 1623 or 1693	Once (sometime before pre-campaign testing)	To establish initial control over the analytical system and demonstrate acceptable method performance	Table 3, 4, and 5 of 1693
Demonstration of capability for this study	Three field sub-samples with blind matrix spikes (ColorSeed[1])	Modified 1693 with direct centrifugation	Once (during pre-campaign testing)	To demonstrate acceptable method performance using the modified method	Appendix D of RFQ 4952
Ongoing precision and recovery (OPR)	One positive control (ColorSeed[1] in reagent water) and one method blank (reagent water)	Modified 1693 with direct centrifugation	Once initially and then once per month for the duration of the study	To demonstrate ongoing control of the analytical system and verify continuing method performance	Appendix D of RFQ 4952
Matrix spike (MS)	ColorSeed[1] in field sample	Modified 1693 with direct centrifugation	Every sample (during full monitoring campaign)	To determine the effect of the matrix on (oo)cyst recoveries	Appendix D of RFQ 4952
Positive staining control	Positive antigen that comes with EasyStain ™ stain kit[1]	Prepare according to the manufacturer recommendations	Process each time samples are stained; examine each microscope session	To demonstrate ongoing control of the staining process and performance of reagents and microscope	Control should display fluorescence for IFA and DAPI stain
Negative staining control	PBS	Prepare according to the manufacturer recommendations	Process each time samples are stained; examine each microscope session	To demonstrate the absence of contamination through staining process	Control should not contain any fluorescent (oo)cysts

1. ColorSeed ™ and EasyStain™ from BioPoint Pty, Ltd (formerly BFT, Ltd)

9.2.1 Laboratory performance for the MS samples conducted for this study will be compared to the **acceptance criteria in Appendix D of RFQ 4952**.

9.3 IPR using this modified method is not required for this study (it is sufficient for the lab to have completed IPR using either method 1623 or 1693 sometime in the past). The lab will complete the pre-campaign testing described in RFQ 4952 (using this modified method) as an initial demonstration of capability for this study.

9.4	The lab should complete the **OPR analysis (positive control and blank) once per month** (and once initially) rather than the frequency specified in EPA 1693.
9.4.1	The lab should use ColorSeed as the spiking suspension. The sample volume should be 1 L. The sample should be processed using the direct centrifugation method.
9.4.4	Laboratory performance for the OPR samples conducted for this study will be compared to the **acceptable criteria in Appendix D of RFQ 4952**.
9.5	Labs must use ColorSeed as the spiking suspension for MS.
9.5.1	Lab must use the direct centrifugation concentration option rather than filtration
9.5.2	There will be no unspiked field samples because ColorSeed will be added to all field samples.
9.5.3	Use equation for ColorSeed not non pre-stained organisms
9.4.5	Note numbering error in method text (should be 9.5.4 not 9.4.5). Laboratory performance for the MS samples conducted for this study will be compared to the acceptance criteria in Appendix D of RFQ 4952.
Table 5	The acceptance criteria in Appendix D of RFQ 4952 will be used in place of criteria in this table for the purposes of this study.
9.6.1	Lab must us the direct centrifugation concentration option rather than filtration to process and analyze the method blank (1 L sample).
9.9	Field replicates and duplicate spiked samples are not required for the full monitoring campaign.
9.13.2	ColorSeed shall be used.
11.1	Labs must use ColorSeed as the spiking suspension for all QC samples.
11.2	This section is not applicable (lab shall not use filtration option). Proceed to 11.3.
11.3.1	**Volume should be 1 L.**
11.3.3	Labs must use ColorSeed as the spiking suspension. 100 ColorSeed *Giardia* cysts and 100 ColorSeed *Cryptosporidium* oocysts should be spiked to each 1 L sample, following the BTF instructions for the spiking procedure.
12.1, 12.2	**Skip these steps. Proceed to 12.3.**
12.3	This direct centrifugation option will be used for all samples. The sample volume must be 1 L.
12.3.1, 12.3.2	These are the same steps as 11.3.1 and 11.3.2. The sample volume should be 1 L. After completing steps 11.3.2/12.3.2, 100 ColorSeed *Giardia* cysts and 100 ColorSeed *Cryptosporidium* oocysts will be spiked to every 1 L sample. Then proceed to step 12.3.3.
12.3.6	Utilize the 1500 x g centrifuge force per 13.3.4. Do not use the higher centrifuge force (2000 x g). Ensure that the pellet is not resuspended after centrifugation.
13.2	Target sample volume is 1 L. Each subsample will be 0.5 mL of packed pellet material.
13.2.2, 13.2.3	Replace with the following: **If total packed pellet volume is > 0.5 but ≤ 4mL:** The entire pellet is analyzed (corresponding to up to 8 subsamples). Resuspend every 0.5 mL of pellet in 5 mL of fluid remaining after aspiration. For example, aspirate to 30 mL for a pellet size of 2.6 mL - 3.0 mL. Vortex the 250-mL centrifuge tube gently, stir the pellet

with a PBST-rinsed pipette, and/or swirl to resuspend. Each 5 mL subsample of resuspended pellet is transferred to individual IMS sample tubes.
If total packed pellet volume is > 4 mL: Process 4 mL of the pellet (corresponding to 8 subsamples). Resuspend 4 mL of pellet in 40 mL of fluid remaining after aspiration. Vortex the 250-mL centrifuge tube gently, stir the pellet with a PBST-rinsed pipette, and/or swirl to resuspend. Each 5 mL subsample of resuspended pellet is transferred to 8 individual IMS sample tubes.

13.3.1.2 Perform the kaolin addition step (13.3.2.6) after 13.3.1.1. In short, the 0.25 g kaolin is added prior to adding the Dynabeads and the SL buffers. Mix kaolin with sample by inversion.

13.3.2.10 and 1.3.2.11 Skip these steps. Proceed to step 13.3.2.12.

13.3.2.14 When rocking the flat-sided tubes in the MPC after the PBS rinse, steps 13.3.2.10 and 13.3.2.11 can be used instead of 13.3.2.12.

13.3.2.15 and 13.3.2.16 When analyzing multiple samples, the analyst may choose to pause at the completion of 13.3.2.15 or 13.3.2.16 before going back to rock other flat-sided tubes in the MPC. This ensures that the sample is sitting in buffer and does not dry out.

13.3.2.19 After the SL-buffer-A has been aspirated from the microcentrifuge tube, add 1 mL of PBS to each tube, resuspend, and repeat steps 13.3.2.17 to 13.3.2.19 (this additional PBS rinse is found under the fourth bullet of 13.3.4).

13.3.3.8 **Add both dissociations to a single well slide.**

13.3.3.9 The pipette used to transfer the acid dissociation sample from the microcentrifuge tube to the slide well should be pre-rinsed with PBST to minimize sample retention in the pipette.

13.3.3.10 Add both dissociations to a single well slide.

13.3.3.12 If stain manufacturer has specific slide drying instructions, follow these provided instructions.

13.3.4 The well diameter should be at least 12 mm to avoid over-crowding.

14.1.1 **Use the positive control that comes with the stain kit and perform the IFA and DAPI examination.**

14.2.1 and 14.2.2 Per BTF EasyStain instructions, the methanol step can be included prior to DAPI staining. The TWG will specify the brand of stains to use prior to the pre-campaign testing.

15.2 ColorSeed will be used so also examine each IFA-positive organism using the Texas Red filter and record the presence/absence of red fluorescence on the examination form.

17.0 The acceptance criteria in Appendix D of RFQ 4952 will be used in place of the MS criteria in EPA 1693 for the purposes of this study.

Appendix B This appendix is not applicable because the lab must use ColorSeed as the spiking suspension (ColorSeed has already been enumerated by the manufacturer).

Standard Operating Procedure for Processing and Enumerating Enterovirus, Adenovirus, Norovirus, SARS-CoV-2, and the Corresponding Matrix Spikes in Raw Wastewater

EPA Method 1615 (attached) along with the following addendum shall be used to process and analyze the virus sample by both culture and molecular methods. Many modifications have been made to EPA Method 1615, including revisions to the concentration/purification steps, addition of a culture and molecular procedure for adenovirus; a molecular procedure for SARS-CoV-2 and betacoronavirus OC43 (enveloped viruses); and revisions to the matrix spike organism, method, and frequency. The addendum lists the method modifications and clarifies certain steps in EPA Method 1615. Follow all of the steps in EPA Method 1615 unless otherwise specified by the addendum.

Refer to EPA Method 1602 (attached) for the list of required equipment, supplies, and reagents for the matrix spike assays.

In this addendum, dashed lines (------) are used to highlight sections with large additions and/or sections that are highly modified.

Addendum to EPA Method 1615:

1.1	Method has been modified to also include culture and/or molecular procedures for adenovirus, SARS-CoV-2, betacoronavirus OC43 matrix spike), MS2 (matrix spike); and, PhiX174 (matrix spike). The method has been modified specifically for use with raw wastewater.
2.0	Significant modifications have been made.
6.1	Not applicable. The sample will not be filtered.
6.2	Not applicable - WWTP will do the sample collection.
6.3.1	Not applicable. Matrix spike sample is same as field sample.
6.3.6	Not applicable. The sample will not be filtered.
6.3.7	Not applicable. Matrix spike sample is same as field sample.
6.3.8	Not applicable. The sample will not be filtered.
6.4	Not applicable. The sample will not be processed by the filtration/elution/organic flocculation method in 1615.
10.0 & 11.0	Concentration and Purification has been modified for analysis of raw wastewater.
12.0	Addition of Adenovirus and SARS-CoV-2/betacoronavirus OC43 analysis
13.0	Modification from two-step RT-qPCR procedure to a one-step RT-qPCR procedure. Some primer sets have been modified or added.

ADDITION TO SECTION 6: In addition to the equipment/supplies specified in 1615, the following equipment/supplies will be needed:

Equipment and Supplies for Concentration/Purification and Nucleic Acid Extraction:

1. Aerosol/filter barrier micropipette tips, 20, 200, 1000 uL (E&K Cat # EK 3016 and EK 3025, or equivalent)

2. Micropipettes
3. Vortex Mixer
4. Waterbath at 56 °C
5. Refrigerated large capacity swing bucket centrifuge (Beckman X-14R or equivalent) for 250 or larger conical bottom tubes.
6. High speed refrigerated centrifuge (Beckman JE or equivalent) capable of running 13,500xg using 50 or 15 mL tubes
7. pH Meter
8. Balance
9. Transfer Pipets, disposable (Fisher brand 13-711-9BM, or equivalent)
10. Centrifuge Tubes, 15, 50, 250, 500 mL including 15 mL tubes rated for high speed centrifugation (13500xg) (VWR 21008- 242, or equivalent)
11. 1, 2, 3 and/or 4-L Sterile beakers with sterile stir magnetic bars
12. "miniMag" or Magnetic Particle Concentrator (MPC)
13. Orbital Shaker/Mixer
14. Microcentrifuge Tubes, 1.5 mL
15. Micro Tubes, screw cap, 1.5 mL (VWR 89004-294, or equivalent)
16. Molecular Grade Water (MGW, VWR 82021-434 or equivalent)
17. Sodium Chloride (BDH9286-2.5KG)
18. Polyethylene Glycol MW 8000 (VWR 97061-098, or equivalent)
19. Chloroform (Sigma 496189-1L, or equivalent)
20. Butanol (Sigma B7906-500 mL, or equivalent)

21. Sodium phosphate dibasic (Na2HPO4.7H2O) (BDH9296-500G)
22. Trisodium citrate dihydrate (BDH9288-500G)
23. BBL Beef Extract Desiccated Powder (BD 211520)
24. Stir plate
25. 5% BSA (5g of albumin/bovine crystalline (United States Biochemical cat# 10856 or equivalent)
26. 0.2% BSA in PBS (Dulbecco's Phosphate buffer saline, without CaCl2 and MgCl2)
27. 0.2 uM sterilizing filters (Sigma-Millipore Cat# F-9768 for filtering up to 250 ml)
28. Amicon concentrator: Vivaspin 20 (30,000 MWCO PES) Sartorius Cat# VS022 or equivalent

Equipment and Supplies for MS2 and PhiX174 Culture

Refer to Section 6 of EPA Method 1602 (attached) for the equipment and supplies needed for the MS2 and PhiX174 culture assays. Use 1X TSA for plaque assay

Equipment and Supplies for MS2 and PhiX174 Molecular

No additional equipment or supplies are required for the MS2 and PhiX174 molecular assays

Equipment and Supplies for Adenovirus Culture

No additional equipment or supplies are required for the adenovirus culture assays

Equipment and Supplies for Adenovirus Molecular

No additional equipment or supplies are required for the adenovirus molecular assays

Equipment and Supplies for SARS-CoV-2 Molecular

No additional equipment or supplies are required for the SARS-CoV-2 molecular assays

Equipment and Supplies for OC43 Culture

Equipment and Supplies for OC43 Molecular

No additional equipment or supplies are required for the OC43 molecular assays

CONTINUE TO SECTION 7.0 AFTER COMPLETING SECTION 6 (W/ ADDITIONS)

7.2.4 Not applicable. Matrix spike will be MS2 and PhiX174 for the enteric viruses ,rather than Sabin Poliovirus 3, and OC43 for enveloped viruses,.

7.3 Not applicable. The sample will not be processed by the filtration/elution/organic flocculation method in 1615

ADDITION TO SECTION 7: In addition to the reagents, media, and standards specified in 1615, the following reagents and standards will be needed:

Reagents and Standards for Concentration/Purification and Nucleic Acid Extraction:

1. Purity of Reagents: ACS reagent grade chemicals shall be used in all tests. Unless otherwise indicated, reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society. The microbiological growth media must be of microbiological grade.
2. Chloroform:Butanol: Add equal parts chloroform and butanol together. Shake to mix. Store for three months refrigerated. **Note: do not use chloroform:butanol reagent with the enveloped virus procedure.**
3. Phosphate Buffered Saline (PBS): Add 8g NaCl, 0.2g KCl, 1.15g Na_2HPO_4 to 1000mL of molecular grade water. Mix until dissolved. Adjust the pH to 7.3. Sterilize by autoclaving. Store refrigerated for 3 months.
4. DNA/RNA purification kit (Zymo #D7020 or equivalent).

Reagents and Standards for Matrix Spike (MS2 and PhiX174)

1. Refer to Section 7 of EPA Method 1602 (attached) for the reagents and standards needed for enumeration of the matrix spikes MS2 and PhiX174.
2. Prepare MS2 and PhiX174 spiking suspensions
    a. MS2 spiking suspension: **Prepare a 10^9 PFU/mL stock virus suspension**. Measure and record the exact concentration. Repeat preparation of this

solution if is less concentrated than 10^8PFU/mL. **Verify the concentration of the stock every time a matrix spike is performed.**

b. PhiX174 spiking suspension: **Prepare a 10^9 PFU/mL stock virus suspension**. Measure and record the exact concentration. Repeat preparation of this solution if is less concentrated than 10^8 PFU/mL. **Verify the concentration of the stock every time a matrix spike is performed.**

c. The laboratory must perform a molecular assay of the MS2 and PhiX174 spiking suspensions every time a sample is spiked with these matrix spikes to determine the concentration (as genome copies per liter) of the stock.

3. Primers, probes, and standards for molecular assay:
   PhiX174 Primer and Probes (Turgeon, N. et al. 2014; Appl Environ Microbiol 80(14)**: 4242-4250**
   Forward: 5'-ACAAAGTTTGGATTGCTACTGACC-3'
   Reverse: 5'-CGGCAGCAATAAACTCAACAGG-3'
   Probe: 5'- FAM/CTCTCGTGCTCGTCGCTGCGTTGA/BHQ-3'
   MS2 Primer and Probes (Turgeon, N. et al. 2014; Appl Environ Microbiol 80(14)**:4242-4250)**
   Forward: 5'-GTCCATACCTTAGATGCGTTAGC-3'
   Reverse: 5'-CCGTTAGCGAAGTTGCTTGG-3'
   Probe: 5'-FAM/ACGTCGCCAGTTCCGCCATTGTCG/BHQ-3'

Reagents and Standards for Adenovirus Culture Assay

Adenovirus stock.

1. Adenovirus stock; Adenovirus serotype 10 (AdV10; ATCC 1504) stored at -70°C. for use as a positive control in the culture assay

Cell culture media.

1. DMEM (Corning cat. no. 10-013-CV or equivalent)
2. Fetal Bovine Serum (FBS) (Corning cat. no. 35-010-CV or equivalent)
3. Antibiotic; Penicillin (10,000 IU) and Streptomycin (10,000 µg/ml); 100X (Corning cat. no. 30-002-CI or equivalent)
4. Antimycotic Amphotericin B (25 µg/mL Amphotericin) 100X (Corning cat. No. 30-003-CF); Antimycotic can be part of the 100X Antibiotic Solution above.
5. Kanamycin (Invitrogen cat. no. 759788 or equivalent) or Gentamycin Sulfate (Corning 30-005-CR or equivalent)
6. Dulbecco's phosphate buffered saline (PBSA) (Sigma cat. no. D8537 or equivalent)
7. Trypsin-EDTA solution (Sigma cat. no. T4049 or equivalent). For long term storage, thaw upon receipt in the laboratory and dispense 5 mL aliquots to sterile conical tubes and store at –20°C until expiration date.
8. Trypan Blue, 0.4% (Invitrogen cat. no. 15250-061).
9. Growth medium preparation in Biological Safety Cabinet:
   a. Growth Medium (10% FBS in DMEM): To each 1L sterile bottle of DMEM add 115 mL FBS, 11.52 ml Antibiotic, 11.5mL Antimycotic, and 11.5 mL Kanamycin or Gentamycin. For checking of sterility, aseptically transfer 5 mL of the growth medium to a sterile tube and incubate in the CO_2 incubator at

37 ± 1.0°C for a minimum of 2-3 days. Look for contamination (turbidity) and discard if contamination is evident. Store media at 4°C for up to 6 months or earliest expiration date of reagents.

10. Maintenance medium preparation in Biological Safety Cabinet:
    a. Maintenance Medium (2% FBS in DMEM): To each 1L sterile bottle of DMEM add 20 mL FBS, 10.2 ml Antibiotic, 10.2 mL Antimycotic, and 10.2 Kanamycin or Gentamycin. For checking of sterility, aseptically transfer 5 mL of the growth medium to a sterile tube and incubate in the CO_2 incubator at 37 ± 1.0°C for a minimum of 2-3 days. Look for contamination (turbidity) and discard if contamination is evident. Store media at 4°C for up to 6 months or earliest expiration date of reagents.
    b. Optionally, maintenance medium consists of a 50/50 mixture of DMEM and Leibovitz L-15 medium with L-glutamine (Sigma-Aldrich, Cat. No. L4386), 1 mL/L of 7.5% sodium bicarbonate, 10 mL/L of antibiotic-antimycotic solution, and 20 mL/L of fetal bovine serum and may be used for incubation of inoculated flasks at 37 ± 1.0°C without CO2.

A549 cell culture.

1. Warm all reagents to 37°C prior to use in this procedure.
2. Growth media and maintenance media are as described for BGM cells, briefly:
    a. Growth Medium (10% FBS in DMEM): To each 1L sterile bottle of DMEM add 115 mL FBS, 11.52 ml Antibiotic, 11.5mL Antimycotic, and 11.5 mL Kanamycin or Gentamycin.
    b. Maintenance Medium consists of 2% FBS in DMEM or a 50/50 mixture of DMEM and Leibovitz L-15 medium with L-glutamine (Sigma-Aldrich, Cat. No. L4386), 1 mL/L of 7.5% sodium bicarbonate, 10 mL/L of antibiotic-antimycotic solution, and 20 mL/L of fetal bovine serum and may be used for incubation of inoculated flasks at 37 ± 1.0°C without CO2.
3. Turn on UV light and blower in biosafety cabinet (BSC) for a minimum of ½ hr before working in hood. Be sure that tube racks that will be used are placed in the BSC while the UV light is on. Immediately prior to working in hood switch the UV light off. If the UV light is left on, exposed skin will burn. NOTE: BEFORE PLACING REAGENTS IN BIOSAFETY CABINET, SPRAY ALL CONTAINERS AROUND THE CAP WITH 70% ETHANOL SOLUTION PRIOR TO OPENING THROUGHOUT THE ENTIRE PROCEDURE. USE GLOVES THAT HAVE BEEN SPRAYED WITH 70% ETHANOL SOLUTION THROUGHOUT PROCEDURE.
4. Remove the cells to be split from the incubator and spray the cap and neck of the flask with 70% ethanol before placing the flask in the BSC. Place pre-wrapped sterile pipettes in the BSC. Remove the PBSA and the trypsin-EDTA solution from 37°C and spray the outside and cap of each vial with 70% ethanol. Place the tubes in a rack in the BSC and loosen caps.
5. Remove the spent growth medium from the culture flask. Tilt the flask away from the cell monolayer to avoid disturbing it. Place spent media in waste beaker located in the BSC.

6. Aseptically add pre-warmed PBSA to flask. Do not put the pipet down or touch the tip to anything. Gently tilt flask to rinse residual growth medium from cells. Using the same pipette or a new pipette, remove the PBSA from flask without disturbing cells and discard in waste beaker. Repeat once more. This removes any media that contains serum, a trypsin inhibitor. If the pipette is put down or the tip touches any object use a new sterile pipette to remove PBS rinse from culture flask.

Flask Size	Amount of PBS to Add (mL)	Amount of Growth Medium (mL)
25	5-10	10-15
75	10-15	20-30
225	15-25	60-100
6-well plate	1 mL per well	3-5

7. Immediately add enough pre-warmed trypsin-EDTA solution to cover the bottom of the flask (1- 12 mL) cap and place in incubator for 1-5 min. Check flask to see if the cells have lifted from the flask surface after 1 min. Rock the flask to dislodge the cells from the flask surface. If there are still cells attached to the flask put back in incubator for an additional 1 minute and recheck. Continue checking frequently until all cells have lifted from the flask surface. Place 25 mL and 10 mL sterile pipettes in the BSC. In addition, place a sterile 15 mL conical tube and if needed a new cell culture flask into the BSC and loosen the caps. NOTE: It is important to minimize the time the cells are exposed to the trypsin-EDTA solution.
8. Remove growth media from 37°C and spray with 70% ethanol solution and place in BSC. Be sure to loosen the cap.
9. When all cells have been lifted from the flask surface, spray around the cap of the flask with 70% ethanol solution and place in the BSC. Using an appropriate size pipette, aseptically add pre-warmed growth media (equivalent to the amount of trypsin in the flask) and rinse bottom of flask with cell suspension several times.
10. If enumeration is necessary, transfer the entire content to a sterile tube and tightly cap the tube. Concentrate the cells by centrifugation (400 x *g*, 2 min or 175 x *g*, 6 min) and discard supernatant. Re-suspend cells in 10 mL of pre-warmed growth media. Be sure to break up clumps by vigorously pipetting suspension up and down in tube.
11. Allow the suspension to settle for 1 min and remove a 50 µL aliquot with a sterile pipette tip and place in 1.5 mL Eppendorf tube containing 50 µL Trypan Blue. Flick tube to mix and perform a hemocytometer count to determine the concentration of viable cells.
12. Transfer an appropriate amount of cells to the culture flask containing pre-warmed growth media

Flask Size	Seeding Density	Days until Confluent
75	0.75×10^6	3-4
225	1×10^6	4-7
6-well plate	1×10^6 ($\sim 3 \times 10^5$ per well)	1-2

13. Place flask in incubator at 37.0 ± 1°C under 5% CO_2. Place well plates into a Ziploc™ bag before placing into incubator.
14. If cells are not confluent within the expected time frame, replace media. Aseptically remove old media using a sterile pipette. Replace with pre-warmed maintenance media. Place flask back in incubator until cells are confluent.

15. If cells used for bioassay are not confluent within 1-4 days the flasks should be discarded. An older monolayer will not optimally support an infection.
16. Autoclave media waste and any media that have had contact with cells or serum before discarding.
17. Discard cells after they have been passaged a total of 200 times.

Reagents and Standards for Adenovirus Molecular Assay

1. Human Adenovirus Primers/Probe (Integrated DNA Technologies or equivalent) (Ko *et al*., J. Virol Methods, 2005; 127: 148-153)

Adenovirus
Forward Primer: 5'-AACTTTCTCTCTTAATAGACGCC-3'
Reverse Primer: 5'-AGGGGGCTAGAAAACAAAA-3'
Probe: 5'-**CTGACACGGGCACTCTTCGC**-3'

2. Zymo Quick-DNA/RNA Viral Kit # D7020 or equivalent
3. QuantaBio qScript™ XLT One-Step RT-qPCR ToughMix® or equivalent
4. ABI real-time PCR Machine (or equivalent)
5. strip tubes and caps
6. Standards for preparing the standard curve.

Reagents and Standards for OC43 Matrix Spike

OC43 stock.

1. OC43 (Betacoronavirus 1 (ATCC® VR-1558™) stored at -70°C for use as a positive control in the culture assay

Cell culture media.

1. DMEM Media **(**Corning cat. no. 10-013-CV or equivalent)
2. Fetal Bovine Serum (FBS) (Corning cat. no. 35-010-CV or equivalent)
3. Antibiotic; Penicillin (10,000 IU) and Streptomycin (10,000 µg/ml); 100X (Corning cat. no. 30-002-CI or equivalent)
4. Antimycotic Amphotericin B (25 µg/mL Amphotericin) 100X (Corning cat. No. 30-003-CF); Antimycotic can be part of the 100X Antibiotic Solution above.
5. Kanamycin (Invitrogen cat. no. 759788 or equivalent) or Gentamycin Sulfate (Corning 30-005-CR or equivalent)
6. Dulbecco's phosphate buffered saline (PBSA) (Sigma cat. no. D8537 or equivalent)
7. Trypsin-EDTA solution (Sigma cat. no. T4049 or equivalent). For long term storage, thaw upon receipt in the laboratory and dispense 5 mL aliquots to sterile conical tubes and store at –20°C until expiration date.
8. Trypan Blue, 0.4% (Invitrogen cat. no. 15250-061).
9. Growth medium preparation in Biological Safety Cabinet:
    i. Growth Medium (10% FBS in DMEM): To each 1L sterile bottle of DMEM add 115 mL FBS, 11.52 ml Antibiotic, 11.5mL Antimycotic, and 11.5 mL Kanamycin or Gentamycin. For checking of sterility, aseptically transfer 5 mL of the growth medium to a sterile tube and incubate in the CO_2 incubator at 37 ± 1.0°C for a minimum of 2-3 days. Look for contamination (turbidity) and

discard if contamination is evident. Store media at 4°C for up to 6 months or earliest expiration date of reagents.

10. Maintenance medium preparation in Biological Safety Cabinet:
    i. Maintenance Medium (2% FBS in DMEM): To each 1L sterile bottle of DMEM add 20 mL FBS, 10.2 ml Antibiotic, 10.2 mL Antimycotic, and 10.2 Kanamycin or Gentamycin. For checking of sterility, aseptically transfer 5 mL of the growth medium to a sterile tube and incubate in the CO_2 incubator at 37 ± 1.0°C for a minimum of 2-3 days. Look for contamination (turbidity) and discard if contamination is evident. Store media at 4°C for up to 6 months or earliest expiration date of reagents.
    ii. Optionally, maintenance medium consists of a 50/50 mixture of DMEM and Leibovitz L-15 medium with L-glutamine (Sigma-Aldrich, Cat. No. L4386), 1 mL/L of 7.5% sodium bicarbonate, 10 mL/L of antibiotic-antimycotic solution, and 20 mL/L of fetal bovine serum and may be used for incubation of inoculated flasks at 37 ± 1.0°C without CO2.

HRT cell culture (HRT-18G (ATCC CRL-11663)).

1. Warm all reagents to 37°C prior to use in this procedure.
2. Growth media and maintenance media are as described for BGM cells, briefly:
    i. Growth Medium (10% FBS in DMEM): To each 1L sterile bottle of DMEM add 115 mL FBS, 11.52 ml Antibiotic, 11.5mL Antimycotic, and 11.5 mL Kanamycin or Gentamycin.
    ii. Maintenance Medium consists of 2% FBS in DMEM or a 50/50 mixture of DMEM and Leibovitz L-15 medium with L-glutamine (Sigma-Aldrich, Cat. No. L4386), 1 mL/L of 7.5% sodium bicarbonate, 10 mL/L of antibiotic-antimycotic solution, and 20 mL/L of fetal bovine serum and may be used for incubation of inoculated flasks at 37 ± 1.0°C without CO2.
3. Turn on UV light and blower in biosafety cabinet (BSC) for a minimum of ½ hr before working in hood. Be sure that tube racks that will be used are placed in the BSC while the UV light is on. Immediately prior to working in hood switch the UV light off. If the UV light is left on, exposed skin will burn. NOTE: BEFORE PLACING REAGENTS IN BIOSAFETY CABINET, SPRAY ALL CONTAINERS AROUND THE CAP WITH 70% ETHANOL SOLUTION PRIOR TO OPENING THROUGHOUT THE ENTIRE PROCEDURE. USE GLOVES THAT HAVE BEEN SPRAYED WITH 70% ETHANOL SOLUTION THROUGHOUT PROCEDURE.
4. Remove the cells to be split from the incubator and spray the cap and neck of the flask with 70% ethanol before placing the flask in the BSC. Place pre-wrapped sterile pipettes in the BSC. Remove the PBSA and the trypsin-EDTA solution from 37°C and spray the outside and cap of each vial with 70% ethanol. Place the tubes in a rack in the BSC and loosen caps.
5. Remove the spent growth medium from the culture flask. Tilt the flask away from the cell monolayer to avoid disturbing it. Place spent media in waste beaker located in the BSC.

6. Aseptically add pre-warmed PBSA to flask. Do not put the pipet down or touch the tip to anything. Gently tilt flask to rinse residual growth medium from cells. Using the same pipette or a new pipette, remove the PBSA from flask without disturbing cells and discard in waste beaker. Repeat once more. This removes any media that contains serum, a trypsin inhibitor. If the pipette is put down or the tip touches any object use a new sterile pipette to remove PBS rinse from culture flask.

Flask Size	Amount of PBS to Add (mL)	Amount of Growth Medium (mL)
25	5-10	10-15
75	10-15	20-30
225	15-25	60-100
6-well plate	1 mL per well	3-5

7. Immediately add enough pre-warmed trypsin-EDTA solution to cover the bottom of the flask (1- 12 mL) cap and place in incubator for 1-5 min. Check flask to see if the cells have lifted from the flask surface after 1 min. Rock the flask to dislodge the cells from the flask surface. If there are still cells attached to the flask put back in incubator for an additional 1 minute and recheck. Continue checking frequently until all cells have lifted from the flask surface. Place 25 mL and 10 mL sterile pipettes in the BSC. In addition, place a sterile 15 mL conical tube and if needed a new cell culture flask into the BSC and loosen the caps. NOTE: It is important to minimize the time the cells are exposed to the trypsin-EDTA solution.
8. Remove growth media from 37°C and spray with 70% ethanol solution and place in BSC. Be sure to loosen the cap.
9. When all cells have been lifted from the flask surface, spray around the cap of the flask with 70% ethanol solution and place in the BSC. Using an appropriate size pipette, aseptically add pre-warmed growth media (equivalent to the amount of trypsin in the flask) and rinse bottom of flask with cell suspension several times.
10. If enumeration is necessary, transfer the entire content to a sterile tube and tightly cap the tube. Concentrate the cells by centrifugation (400 x *g*, 2 min or 175 x *g*, 6 min) and discard supernatant. Re-suspend cells in 10 mL of pre-warmed growth media. Be sure to break up clumps by vigorously pipetting suspension up and down in tube.
11. Allow the suspension to settle for 1 min and remove a 50 µL aliquot with a sterile pipette tip and place in 1.5 mL Eppendorf tube containing 50 µL Trypan Blue. Flick tube to mix and perform a hemocytometer count to determine the concentration of viable cells.
12. Transfer an appropriate amount of cells to the culture flask containing pre-warmed growth media

Flask Size	Seeding Density	Days until Confluent
75	0.75×10^6	3-4
225	1×10^6	4-7
6-well plate	1×10^6 ($\sim 3 \times 10^5$ per well)	1-2

13. Place flask in incubator at 37.0 ± 1°C under 5% CO_2. Place well plates into a Ziploc™ bag before placing into incubator.
14. If cells are not confluent within the expected time frame, replace media. Aseptically remove old media using a sterile pipette. Replace with pre-warmed maintenance media. Place flask back in incubator until cells are confluent.
15. If cells used for bioassay are not 80-90% confluent within 1-3 days the flasks should be discarded. An older monolayer will not optimally support an infection.
16. Autoclave media waste and any media that have had contact with cells or serum before discarding.
17. Discard cells after they have been passaged a total of 200 times.

OC43 Primers/Probe for Molecular Assay.

1. OC43 (Integrated DNA Technologies or equivalent):
   Forward Primer: 5'- CGATGAGGCTATTCCGACTAGGT -3'
   Reverse Primer: 5'- CCTTCCTGAGCCTTCAATATAGTAACC -3'
   Probe: 5'- TCCGCCTGGCACGGTACTCCCT -3'

Reagents and Standards for Enveloped Virus Molecular Assay:

1. Coronavirus SARS-CoV - Primers/Probe (2019-nCoV CDC EUA Kit, 500 rxn Cat# 1000660 from Integrated DNA Technologies or equivalent)
2. Zymo Quick- RNA Viral Kit # R2040 or equivalent
3. QuantaBio qScript™ XLT One-Step RT-qPCR ToughMix® or equivalent
4. ABI real-time PCR Machine (or equivalent)
5. strip tubes and caps
6. Standards for preparing the standard curve: Positive Control provided by IDT Kit is 2E+05 GC/ul or 1E+06/5ul is serially diluted to 1E+01 and used as standard curve
7. TaqMan™ Exogenous Internal Positive Control Reagents- VIC probe (Thermofisher Cat # 4308321 or equivalent)

CONTINUE TO SECTION 8.0 AFTER COMPLETING SECTION 7 (W/ ADDITIONS)

8.0 The QA/QC requirements for this project are summarized in the following table:

QC Sample	Description	Method	Frequency/Timing	Purpose	Acceptance Criteria
Demonstration of capability for this study	Three field sub-samples with blind spikes (poliovirus) and three field sub-samples without blind spikes; both shall include matrix spikes (MS2 and PhiX174)	Modified 1615 (PEG/chloroform extraction, culture, and molecular) (Do not need to run adenovirus or norovirus assays)	Once (during pre-campaign testing)	To demonstrate acceptable method performance using the modified method	Appendix D of RFQ 4952

QC Sample	Description	Method	Frequency/Timing	Purpose	Acceptance Criteria
QC sample set-Enteric viruses	One positive control (poliovirus in reagent water) and one negative control	Modified 1615 (PEG/chloroform extraction, culture, and molecular) (For positive control, do not need to run adenovirus or norovirus assays)	Once initially and then once per month for the duration of the study	To demonstrate ongoing control of the analytical system and verify continuing method performance	Appendix D of RFQ 4952
Matrix spike Enteric viruses	MS2 and PhiX174 in field sample	Modified 1615 (PEG/chloroform extraction, culture, and molecular) Verify the concentration of the spiking suspension in terms of both PFU/L and GC/L every time a matrix spike is performed	Every other field sample from each WWTP during full monitoring campaign; every field sample during pre-campaign testing	To determine the effect of the matrix on virus recoveries	Appendix D of RFQ 4952
QC sample set – Enveloped Viruses	One positive control (OC43 in reagent water) and one negative control	Direct incubation in beef extract Amicon filter Concentration, RNA extraction, molecular assay	Once initially and then once per month for the duration of the study	To demonstrate ongoing control of the analytical system and verify continuing method performance	To Be Determined
Matrix Spike Enveloped Viruses	OC43 in field sample	Direct incubation in beef extract Amicon filter Concentration, RNA extraction, molecular assay Verify the concentration of the spiking suspension (GC/L) every time a matrix spike is performed	Every field sample from each WWTP during campaign testing	To determine the effect of the matrix on enveloped virus recovery	To Be Determined
Positive Control: Enterovirus Culture Assay	3 culture test vessels with poliovirus in buffer	MPN culture assay with BGMK cells	Every BGMK culture assay	To verify the continued sensitivity of the cell cultures to virus infection	EPA 1615 Section 12.1.2.5.2
Positive Control: Adenovirus Culture Assay	3 culture test vessels with adenovirus in buffer	MPN culture assay with A549 cells	Every A549 culture assay	To verify the continued sensitivity of the cell cultures to virus infection	EPA 1615 Section 12.1.2.5.2

QC Sample	Description	Method	Frequency/Timing	Purpose	Acceptance Criteria
Negative Control: Enterovirus Culture Assay	3 culture test vessels with buffer	MPN culture assay with BGMK cells	Every BGMK culture assay	To verify that external factors aren't harming the cells	EPA 1615 Section 12.1.2.4.2
Negative Control: Adenovirus Culture Assay	3 culture test vessels with buffer	MPN culture assay with A549 cells	Every A549 culture assay	To verify that external factors aren't harming the cells	EPA 1615 Section 12.1.2.4.2
Negative Control: MS2 Culture Assay	1 culture test vessel with buffer	PFU culture assay (with *E. coli* F_{amp})	Every *E. coli* F_{amp} culture assay	To verify that external factors aren't preventing the growth of *E. coli*	If *E. coli* fails to grow, troubleshoot and repeat assay
Negative Control: PhiX174 Culture Assay	1 culture test vessel with buffer	PFU culture assay (with *E. coli* CN-13)	Every *E. coli* CN-13 culture assay	To verify that external factors aren't preventing the growth of *E. coli*	If *E. coli* fails to grow, troubleshoot and repeat assay
Negative Control: Molecular Assay	1 sample with PCR grade water	(rt)-qPCR	One per every fourth sample on a plate	To verify the lack of contamination	EPA 1615 Section 13.4.4.2
Inhibition Control (Positive Control): Molecular Assay – Enteric Viruses	1 test sample with hepatitis G armored RNA (compare to hepatitis G in reagent water)	rt-qPCR	Every molecular assay (one per field sample)	To identify any matrix inhibition	EPA 1615 Section 13.6.2
Inhibition Control (Positive Control): Molecular Assay, Enveloped Viruses	TaqMan™ Internal Controls run with OC43 assay	rt-qPCR	Every molecular assay (one per field sample)	To identify any matrix inhibition	To Be Determined

8.3.1 **PT and PE samples not required. Once contracted, the labs will perform the pre-campaign testing that will include some demonstration of capability testing. The scope of this testing is described in RFQ 4952. One QC sample set will be processed initially and then one QC sample set per month thereafter.**

8.4 One QC sample set (poliovirus and OC43 betacoronavirus in reagent water and a negative control) should be conducted per month during the duration of this monitoring campaign (and one set initially). This QC sample set should be processed and analyzed using the modified 1615 method for enteric viruses and the enveloped virus method for coronaviruses.

8.4.1.1 Sample volume is 1100 mL

8.4.1.2-8.4.1.4 Skip these steps (filtration has been eliminated from the sample prep)

8.4.1.5 Process and analyze the negative QC sample using the PEG flocculation/chloroform extraction procedure (see the modified concentration/purification procedure, Sections 10 and 11, below), enterovirus culture assay, adenovirus culture assay, MS2 culture assay, PhiX174 culture, and molecular assay procedures (enterovirus, adenovirus, norovirus, MS2, and PhiX174).

8.4.2 Sample volume is 1100 mL

8.4.2.2 Skip this step (filtration has been eliminated from the sample prep)

8.4.2.5 Skip this step (filtration has been eliminated from the sample prep)

8.4.2.6 Enteric Viruses: Process and analyze the positive QC sample using the PEG flocculation/chloroform extraction procedure (see the modified concentration/purification procedure; Sections 10 and 11, below), enterovirus culture assay, and enterovirus molecular assay.

8.4.2.7 Enveloped Viruses: Process and analyze the positive QC sample by adding 1ml of 10^6 GCMPN/ml OC43 virus to 1100 ml of laboratory deionized water and process (see the modified concentration/purification procedure; Sections 10 and 11, below), OC43 molecular assay.

8.4.3 **Laboratory performance for the positive QC samples conducted for this study will be compared to the acceptance criteria in Appendix D of RFQ 4952**

8.5 PT and PE samples not required. Once contracted, the labs will perform the pre-campaign testing that will include some demonstration of capability testing. The scope of this testing is described in RFQ 4952.

8.6.1 Enteric viruses: Run a matrix spike for <u>every other</u> field sample from <u>each site</u> (during the pre-campaign testing, a matrix spike will be run with every field sample).

8.6.1.1 Enveloped viruses: Run a matrix spike for <u>every</u> field sample from <u>each site.</u>

8.6.2 **Replace with the following steps:**

8.6.2 REPLACEMENT: To the samples that will include a matrix spike, complete the following steps:

1. **Spike 10^8 PFU of MS2 to 1100 mL of the field sample** (the same sample that is used for measuring the native virus). This number can be achieved by adding approximately 0.1 mL of a MS2 spiking suspension with a concentration of approximately 10^9 PFU/mL (may adjust the volume of the MS2 spiking suspension added based on the actual concentration of the spiking suspension to achieve a minimum concentration of MS2 in the field sample of 10^8 PFU/L).
2. To this same sample, **spike 10^8 PFU of PhiX174**. This number can be achieved by adding approximately 0.1 mL of a PhiX174 spiking suspension with a concentration of approximately 10^9 PFU/mL (may adjust the volume of the PhiX174 spiking suspension added based on the actual concentration of the spiking suspension to achieve a minimum concentration of PhiX174 in the field sample of 10^8 PFU/L).
3. Spike 10^6 GCMPN/ml of OC43 to 1100 ml of the field sample (the same sample that has been spiked with MS2 and PhiX174 and is used for measuring the native virus). This number can be achieved by adding approximately 1ml of an OC43 spiking suspension with a concentration of approximately 10^6 GCMPN/ml (may adjust the volume of the OC43 spiking suspension added based on the actual concentration of the spiking suspension to achieve a minimum concentration of OC43 in the field sample of 10^6 GCMPN/L).

4. Process and analyze the sample using the sample procedures that are used for the samples without the matrix spike with the exception that this sample will also be analyzed for MS2 and Phix174 using both culture and molecular procedures; and, for OC43 use molecular procedures only.
5. Laboratories are to report PFU in all dilutions where there are countable plaques (zero to 300 PFU per plate for male-specific (F^+) coliphage and zero to 100 PFU per plate for somatic coliphage. For too numerous to count plates, please enter TNTC in the comment section of the report associated with that dilution. Make the final calculation based on all dilutions with countable plaques (refer to equation in section 13.1.5 from EPA 1602).
6. For enveloped viruses, report the concentration as determined from rt-qPCR standard curve.

CONTINUE TO SECTION 8.6.3

8.6.3 **Laboratory performance for the MS samples conducted for this study will be compared to the acceptance criteria in Appendix D of RFQ 4952.**

9.1 Replace this step with the following:
A 4 L raw wastewater sample will be collected by the wastewater treatment facility staff and will be delivered to the lab. The sample should be labeled with a unique sample number. The facility staff should record the unique sample number, facility name, sampler's name, location at sampling site, date, and time on a sample data sheet

9.2. Procedure is same only the 4 L sample will not be filtered so there is no cartridge housing or intake/discharge module. The sample should be shipped cold (1-10 °C) not frozen.

9.3.2 Labs will receive a 4 L sample (using a portion for measuring virus, a portion for protozoa, and a portion for phage). The samples will not be filtered, so the lab will not receive a cartridge filter.

10 and 11 **Skip these steps. Instead complete the following modified concentration/purification procedure:**

MODIFIED CONCENTRATION/PURIFICATION PROCEDURE (to replace Section 10 and 11 of EPA 1615)

1. Transfer 1100 ml of raw sewage into sterile beaker. Record this volume (designated Total Sample Volume, TSV) on the virus data sheet.
2. After spiking the virus matrix spikes, incubate for 30 min at Room temperature.
3. Add 1.43 g Sodium phosphate dibasic, 1.32 g Trisodium citrate dihydrate and 11 g beef extract to the sample and stir to dissolve.
4. Stir for 30 minutes at room temperature. Record pH (should be ambient 7.2-7.5). Remove 100 ml aliquot and process the aliquot according to the following steps:

a. Load 15 mL of the aliquot into Amicon ultrafiltration-Vivaspin 20 (pre-soaked in PBS plus 0.2% BSA 2hr to 24hr). Freeze the remaining 85 mL of the aliquot at -70°C for backup and archival purposes.
b. Centrifuge the Amicon filter at 4500-5000 x g for 30 minutes or until the concentrate is reduced to less than 1 mL. Reload the column as many times as possible until filter begins to show signs of clogging. Typically, 45-60 mL can be concentrated.
c. Record initial and final volume. Add 1 mL of RNA lysis buffer directly to the sample in the Amicon and resuspend. Store at 4 +/- 3° C until extraction (up to 24 hours). Long term storage at -80°C has not been evaluated. Proceed to SECTION 13 (Molecular Analysis).

5. Slowly adjust pH of the remaining sample (approximately 1 L) for enteric virus analysis to 9.5 +/- 0.1 using 1-3 N NaOH. Stir at room temperature for an additional 60 minutes, confirm pH is still in range after 30 minutes.
6. Transfer into 250- or 500-mL centrifuge tubes. Centrifuge at 3,000 x g for 20 minutes (brake no more than 20%).
7. Decant or pipet supernatant into 1 L container and record the volume. Discard pellets.
8. Check pH of supernatant using pH meter and adjust to 7.2+/- 0.2 using 1-3 N HCL.
9. Add 52 g of Sodium chloride (NaCl) and mix/stir to dissolve then add 120 g PEG 8000and Mix by inversion or stirring for minimum 2 hours/ or up to 20 hours at 4 +/- 3° C
10. If necessary, transfer to appropriate centrifuge tube. Centrifuge at a minimum of 4,000 x g for 45 mins at 4°C (Brake at no more than 20%).
11. Carefully decant and discard the supernatant. Invert tube over a paper towel for up to 30 seconds to ensure liquid removal from tube.
12. Resuspend the pellet in 1-2 mL of cold PBS. Mix with pipet tip. Avoid making bubbles. Do not vortex.
13. <u>Quantitatively</u> Transfer (using additional 0.5 mL cold PBS) the re-dissolved pellet to a 15 mL chemical resistant centrifuge tube
14. <u>Record the re-dissolved pellet final volume resulting from the previous step (designated Final Concentrated Sample Volume, FCSV) on the virus data sheet.</u>
15. Add 4 mL (or twice the volume of the transferred volume if it is greater than 2.5 mL) of chloroform/butanol solution. Vortex and let stand for 5 minutes.
16. Centrifuge at minimum of 13500 x g for 15 minutes at 4°C
17. Using a micropipette, transfer the top layer (the aqueous phase) to a sterile 15mL centrifuge tube (approximately 2 mL). Chloroform will be on the bottom. Avoid transferring chloroform, go slowly and do not slant tube while transferring.
18. Record the weight of the 15 mL centrifuge tube before and after transfer. Record both and calculate saved volume. Record this volume (designated FCSV recovered after extraction) on the virus data sheet.
19. The pellet/concentrate can be held for up to 18 hours in refrigerator or frozen at -70°C for longer storage. Freezing and thawing can lead to virus losses so avoid multiple freeze/thaw cycles.

20. A portion of the pellet/concentrate will proceed to nucleic acid extraction, a portion of the pellet/concentrate will be diluted and then proceed to the culture assays, and the remaining portion of the pellet/concentrate will be frozen for backup/archival purposes.
    a. 0.4 mL of the pellet shall be diluted to a total volume of 4 mL using cold PBS.
        i. 1.35 mL of the 4 mL re-dissolved pellet/concentrate will be used for the enterovirus culture assay (called subsample 1)
        ii. 1.35 mL of the 4 mL re-dissolved pellet/concentrate will be used for the adenovirus culture assay (called subsample 1B)
        iii. 0.3 mL of the 4 mL re-dissolved pellet/concentrate will be used for the MS2 and PhiX174 culture assay (called subsample 1C)
        iv. The rest of the 4 mL re-dissolved pellet/concentrate will be frozen at -70°C for backup and archival purposes
    b. 1 mL of the pellet will be used for nucleic acid extraction (called subsample 2)
    c. The remaining pellet will use frozen at -70°C for backup and archival purposes (called subsample 3)

Proceed to Section 12 (with the addition of adenovirus, MS2, and PhiX174 culture) and Section 13 (with the addition of adenovirus, SARS-CoV-2, OC43, MS2, PhiX174 molecular). Section 12 and Section 13 may be completed in parallel.

SUBSAMPLE 1 SHALL PROCEED TO SECTION 12.0 (culture analysis of enterovirus)

12.1.2.2 **The inoculum volume is 0.1 mL**. Record the Inoculum Volume on the virus data sheet. The number of cell culture **replicates should be 10**.

12.1.2.3 The laboratories should prepare **10- and 100-fold dilutions for every analysis** of the raw wastewater samples.

12.1.2.3.1 The 10-fold dilution is prepared by diluting 0.3 mL of subsample 1 (from step 17.a.i; Section 10 and 11, above) to a volume of 3.0 mL with cold PBS.

12.1.2.3.2 The 100-fold dilution is prepared by diluting 0.3 mL of the 10-fold dilution to a volume of 3.0 mL with cold PBS

12.1.2.4 **The lab must perform these negative controls**

12.1.2.5 **The lab must perform these positive controls**

12.1.4 **Lab must perform a second passage for all negative flasks; not positives. However, if positive flasks are suspected to be due to contamination or cell toxicity, conduct a second passage.**

Note: Freeze all negative flasks and suspect positives prior to performing the second passage

12.1.4.3 Skip this step

12.2.5 The Volume of Original Water Sample Assayed (D) is calculated by the following equation:

$$D = S \times \frac{TSV}{FCSV}$$

12.2.9 The QC samples are the negative and positive controls

ADDITION to Section 12: CULTURE ANALYSIS ADENOVIRUS (SUBSAMPLE 1B)

The adenovirus culture assay involves an MPN approach similar to what is specified in 1615 for enterovirus, with 10 replicates at each dilution and three dilutions (undiluted, 10-fold, and 100-fold). **Lab must perform a second passage for all negative flasks; not positive flasks. However, if positive flasks are suspected to be due to contamination or cell toxicity, conduct a second passage.** Run negative and positive controls (3 vessels as positive control and 3 vessels as negative controls) with each culture assay.

Note: Freeze all negative flasks and suspect positives prior to performing the second passage

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ADDITION to Section 12: CULTURE ANALYSIS MS2 and PHIX174 (SUBSAMPLE 1C)
(Adapted from EPA Method 1602, Section 12)
Only complete this step for samples that contain matrix spikes of MS2 and PhiX174 (every other virus sample from a given site should contain these matrix spikes)

1. **Four dilutions** of concentrate will be analyzed in **duplicate** for each coliphage type. As a result, nine double-agar layer plates will be required for each coliphage type: two plates per dilution (0.1, 0.01, 0.001, 0.0001) and one method blank plate.
2. Dilutions:
   a. Four dilutions will be needed: 0.1, 0.01, 0.001, 0.0001. **Note: Subsample 1C (prepared in step 17.a.iii of the modified concentration/purification procedure; section 10 and 11, above) is already 0.1 dilution.** Additional dilutions (beyond 0.0001) may be necessary. PBS (or PBW) is used as the diluent and is used to prepare the negative control.
   b. To prepare the dilutions, aseptically add 0.9 mL of PBS (or PBW) into each of four (or more) sterile dilution tubes. Label them as “0.01,” “0.001,” “0.0001,” “method blank,” etc.
   c. Add 0.1 mL of the subsample 1C (from step 17.a.iii of the modified concentration/purification procedure; section 10 and 11, above) to the tube of PBS (or PBW) labeled “0.01.”
   d. Cap the tube and vortex for 5 seconds on a medium-high setting (if available) or until well-mixed.
   e. Add 0.1 mL of the well-mixed 0.01 dilution to a tube with 0.9 mL of PBS (or PBW) labeled “0.001".
   f. Cap the tube and vortex for 5 seconds on a medium-high setting (if available) or until well-mixed.
   g. Add 0.1 mL of the well-mixed 0.001 dilution to a tube with 0.9 mL of PBS (or PBW) labeled “0.0001.” Cap the tube and vortex for 5 seconds on a medium-high setting (if available) or until well-mixed.

h. Add 0.1 mL of PBS (or PBW) the tube labeled “method blank.” Cap the tube and vortex for 5 seconds on a medium-high setting (if available) or until well-mixed.

3. Agar preparation:

a. Place TSA molten agar with respective antibiotics in a 48-53°C water bath. The agar should remain molten in the water bath until ready for use. 18 tubes are necessary to enumerate four dilution volumes in duplicate for each phage. The 18 tubes also include an additional method blank tube for each phage type. Nine of the top agar tubes should contain nalidixic acid for growth of *E. coli* CN-13; the other nine should contain ampicillin/streptomycin for growth of *E. coli* F_{amp}.

b. As a precaution against contamination, disinfect a workspace near the water bath with a 1:100 dilution of household bleach and allow to dry. If workspace can be corroded by bleach use an ethanol solution of 70% or greater.

c. Assemble plates and label so that the following information is identifiable:

i. Dilution of stock filtrate or method blank
ii. Bacterial host (*E. coli* CN-13 or *E. coli* F_{amp})
iii. Coliphage type (somatic for the *E. coli* CN-13 bacterial host or male-specific for the *E. coli* F_{amp} bacterial host)
iv. Date
v. Time

4. Please note: The following steps are critical. To ensure viability of bacterial host and coliphage, do not add bacterial host and concentrate (re-dissolved pellet) until ready to plate.

5. Preparation of plates for enumeration of somatic coliphage:

a. With the agar still in the water bath, aseptically inoculate tube containing nalidixic acid with 100 μL of log-phase *E. coli* CN-13.

b. Immediately add 100 μL (0.1 mL) of subsample 1c (from step 17.a.iii of the modified concentration/purification procedure; section 10 and 11, above).

c. Add 12-15 mL molten agar

d. Mix the inoculum by rolling the tube briefly in palm of hand.

e. Pour contents into one of the two petri plates marked “undiluted, *E. coli* CN-13, somatic."

f. Duplicate analysis—Repeat Sections 5a through 5d for the duplicate.

g. Repeat Sections 5a through 5e for each dilution volume.

6. Preparation of plates for enumeration of male-specific (F^+) coliphage—Repeat Section 5 using agar containing ampicillin/streptomycin and log-phase *E. coli* F_{amp}

7. Preparation of somatic coliphage method blank:

a. With the agar still in the water bath, aseptically inoculate a tube containing nalidixic acid with 100 μL of log-phase *E. coli* CN- 13.

b. Immediately add 100 μL (0.1 mL) of TSB from the “method blank” dilution tube. Add 12-15 mL molten agar.
Mix the inoculum by rolling the tube briefly in palm of hand.

c. Pour contents into a petri plate marked “method blank, *E. coli* CN-13, somatic."

8. Preparation of the male-specific (F^+) coliphage method blank—Repeat Section 7 using agar containing ampicillin/streptomycin and log-phase *E. coli* F_{amp}.
9. After the agar hardens, invert the plates and incubate for 16 to 24 hours at 36°C ± 1.0°C.
10. Circular zones of clearing (typically 1 to 10 mm in diameter) in lawn of host bacteria after 14 to 24 hours of incubation are plaques. Count the number of plaques on each plate. Please note: The use of a light box to evaluate results is recommended. Record the results in the virus sheet.

SUBSAMPLE 2 SHALL PROCEED TO SECTION 13 (Molecular Analysis)

13.1 – 13.3 **Replace these steps with the following modified nucleic acid extraction procedure:**

MODIFIED NUCLEIC ACID EXTRACTION PROCEDURE (replace 13.1 – 13.3) or

1. **Follow manufacturer instructions in commercial kit (Zymo Quick-DNA/RNA Viral Kit #** D7020) or equivalent.
2. Add 1 mL of 2X Viral DNA/RNA Buffer to 1 mL of sample and mix well (DNA/RNA Shield step is not necessary).
3. Transfer 700 µl of the mixture into one IIC-XL Column in a collection tube and centrifuge @ 10,000 x g for 2 minutes. Transfer the column into a new collection tube.
4. Reload the column with another 700 µl of the mixture and repeat centrifuge. Repeat until all of the mixture has been passed through the column.
5. Add 500 µl Viral Wash Buffer to the column, centrifuge for 30 seconds @ 10,000 x g and discard the flow-through. Repeat this step.
6. Add 500 µl ethanol (95-100%) to the column and centrifuge for 1 minute @ 10,000 x g to ensure complete removal of the wash buffer. Carefully, transfer the column into a 1.5 mL nuclease-free tube.
7. Add 200 µl DNase/RNase-Free Water directly to the column matrix and centrifuge for 30 seconds @ 10,000 x g.
8. The final extraction concentrate volume is 0.2 mL (enteric viruses) and 0.05 mL (coronaviruses)
9. Refrigerate at 4°C for a maximum of 24 hours or <-20°C for up to one week until PCR analysis. Indefinite storage @ -80°C.
10. Extract RNA from poliovirus, MS2, PHIX174, and OC43 stocks following the protocol above to calculate percent recoveries.

CONTINUE TO SECTION 13.4 of EPA 1615 for Molecular Enumeration of Enterovirus, Norovirus, Adenovirus, OC43, SARS-CoV-2, MS2, and PhiX174

13.4, 13.5 **These two steps may be completed as a one-step reaction (i.e., reverse transcription should be completed in the same tube as qPCR); Each assay will be completed in triplicate**. The test sample requires 30 plate wells/tubes (i.e., 3

replicates x 10 qPCR assay). The 10 assays are enterovirus, norovirus GIA, norovirus GIB, norovirus GII, hepatitis G, adenovirus, MS2, and PhiX174, SARS-Cov-2, and OC43. 5-6 μL of the concentrate from nucleic acid extraction will be used in each of plate wells/tubes. Note: the assays for MS2 and PhiX174 should only be run if the sample contains these matrix spikes (every other virus sample from each site should contain these matrix spikes)

For 1-step: set up the thermocycler program and run with a setting of 1 cycle hold at 50 °C for 10 min, 1 cycle hold at 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 sec, and 60 °C for 60 sec (capture fluorescence).

For 1-step RTqPCR HepG inhibition assay: depending on the instrument used , first equilibrate the thermocycler to 50 °C , then set up the thermocycler program and run with a setting of 1 cycle hold at 50 °C for 35-50 min, 1 cycle hold at 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 sec, and 60 °C for 60 sec (capture fluorescence).

13.5.6 Use the following equation to calculate genome copies per liter in the original sample rather than Eq. 5 in EPA 1615:

$$GC_{original\ sample\ per\ L} = \frac{GC_{assay} \times V_{nucleic\ acid\ extract} \times FCSV \times DF}{V_{assay} \times V_{pellet\ for\ molecular} \times TSV}$$

Where,

$GC_{original\ sample\ per\ L}$ = Calculate genome copies per liter in the original sample

GC_{assay} = Genome copies measured from the qPCR assay

$V_{nucleic\ acid\ extract}$ = Volume of the concentrate after nucleic acid extraction (should be 0.2 mL)

FCSV = Final concentrated sample volume (before chloroform extraction) (should be 2.0 mL)

DF = dilution factor = the reciprocal of any dilution performed to compensate for inhibition (e.g., 10 and 100 for the 1:10 and 1:100 dilutions)

V_{assay} = volume of the nucleic acid extraction used for each (rt)qPCR assay (should be 0.006 mL)

$V_{pellet\ for\ molecular}$ = volume of the re-dissolved pellet/concentration used for nucleic acid extraction (should be 1 mL)

TSV = total sample volume (should be 1 L)

13.5.7 skip

13.6 **The laboratory must use hepatitis G armored RNA as an inhibition control in all assays.** The laboratory may option 1, 2, or 3 of 1615 as long as the inhibition control is run for all assays. See section 13.7.3 below that include an exogenous internal positive control for OC43/Sars-Cov2 assays

13.7 Armored RNA containing the enterovirus, norovirus GI, and norovirus GII sequences, (Armored RNA 1615 from Asuragen) will be used for preparing the standard curves. For OC43 assay, standard Curve is prepared from OC43 virus serially

diluted from 10^6 GCMPN /5 uL to 10^1 GCMPN / 5uL; for SARS-Cov2 assay Standards are prepared from Positive Control provided by IDT Kit is 2E+05 GC/ul or 1E+06/5ul serially diluted to 1E+01 and used as standard curve

Note: No RNA extraction is necessary for the armored RNA
However, due to variability in stock Armored RNA Gene copies (up to ±20%), it is highly recommended to determine the GC-MPN/ml titer of the stock armored RNA using the following steps;

13.7.1 Prepare 10-fold serial dilution (in TSM buffer)

13.7.2 Use the highest three dilutions (10, 100,1000) and 10 replicates per dilution.

13.7.3 Perform RTqPCR (section 13.5) using primers and probe. Include TaqMan™ Exogenous Internal Positive Control Reagents in OC43 assay and SARS-CoV-2 assay.

13.7.4 Obtain GC-MPN/ml for Armored RNA using EPA most probable Number Calculator or equivalent. 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.

13.8 **For this project, a full standard curve will be required for every sample. It is not acceptable to use stored standard curves and calibrators.**

14.0 While the information in this section provides good perspective, **the acceptance criteria given in Appendix D of the RFQ will be used for this study instead of the acceptance criteria in EPA 1615 (for both matrix spikes and positive QC samples).**

16, Table 2 Not applicable

16, Revised Table 4

Table 4: Primers and TaqMan® Probes for Virus Detection by RT-qPCR

Virus Group	Primer/Probe Name/Sequence (1,2,3,4**)
Enterovirus	
	EntF: CCTCCGGCCCCTGAATG
	EntR: ACCGGATGGCCAATCCAA
	EntP: 6FAM-CGGAACCGACTACTTTGGGTGTCCGT-TAMRA
Norovirus GIA	
	NorGIAF (JJVMF): CCA TGT TCC GTT GGA TGC**
	NorGIAR: TCCTTAGACGCCATCATCAT
	NorGIAP (RING1): FAM-AGA TYG CGI TCI CCT GTC CA-BHQ **
Norovirus GIB	
	NorGIBF: CGCTGGATGCGNTTCCAT

Virus Group	Primer/Probe Name/Sequence (1,2,3,4**)
	NorGIBR: CCTTAGACGCCATCATCATTTAC
	NorGIBP: 6FAM-TGGACAGGAGAYCGCRATCT-TAMRA
Norovirus GII	
	NorGIIF: ATGTTCAGRTGGATGAGRTTCTCWGA
	NorGIIR: TCGACGCCATCTTCATTCACA
	NorGIIP: 6FAM-AGCACGTGGGAGGGCGATCG-TAMRA
Hepatitis G	
	HepF: CGGCCAAAAGGTGGTGGATG
	HepR: CGACGAGCCTGACGTCGGG
	HepP: 6FAM-AGGTCCCTCTGGCGCTTGTGGCGAG-TAMRA
OC43	OC43F: CGATGAGGCTATTCCGACTAGGT
	OC43R: CCTTCCTGAGCCTTCAATATAGTAACC
	OC43P : TCCGCCTGGCACGGTACTCCCT
SARS-CoV-2	2019-nCoV CDC EUA Kit, 500 rxn IDTCat# 10006606
	2019-nCoV_N1-F: 5'-GAC CCC AAA ATC AGC GAA AT-3'
	N1-R: 5'-TCT GGT TAC TGC CAG TTG AAT CTG-3'
	N1-P: 5'-FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1-3'
	N2-F: 5'-TTA CAA ACA TTG GCC GCA AA-3'
	N2-R: 5'-GCG CGA CAT TCC GAA GAA-3'
	N2-P: 5'-FAM-ACA ATT TGC CCC CAG CGC TTC AG-BHQ1-3'
SARS CoV-2 WHO Primers/Probes:	
	E_Sarbeco_F1: ACAGGTACGTTAATAGTTAATAGCGT
	E_Sarbeco_R2: ATATTGCAGCAGTACGCACACA
	E_Sarbeco_P1: FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ

(1) 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.
(2) All primer and probe sequences are 5' to 3'
(3) 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 equal inosine.
(4) ** NorG1A forward primer and NorGIA probe differ from EPA 1615 recommended NorG1A primer/probe. These new sequences are more efficient in one-step RT PCR. Reference: Vincent R. Hill • Bonnie Mull •Narayanan Jothikumar • Karen Ferdinand •Jan Vinje´Food Environ Virol (2010) 2:218–224: Detection of GI and GII Noroviruses in Ground Water Using Ultrafiltration and TaqMan Real-time RT-PCR:

ADDITION to Table 4: Primers and probes for Adenovirus, MS2, PhiX174, Coronavirus OC43, and SARS-CoV-2.

4.1 PhiX174 Primer and Probes

Sequence	Reference
Forward: 5'-ACAAAGTTTGGATTGCTACTGACC-3'	Turgeon, N. et al. 2014; Appl Environ Microbiol 80(14):424-4250
Reverse: 5'-CGGCAGCAATAAACTCAACAGG-3'	
Probe: 5'- FAM/CTCTCGTGCTCGTCGCTGCGTTGA/BHQ-3'	

PhiX174

Target sequence length: 123 bp

ACAAAGTTTGGATTGCTACTGACCG**CTCTCGTGCTCGTCGCTGCGTTGA**GGCTTGCGTTTATGGTACGCTGGACTTT
GTGGGATACCCTCGCTTTCCTGCTCCTGTTGAGTTTATTGCTGCCG

Total length with pUCIDT-Amp Plasmid- 2875 bp
For Standard curve: linearize with PvuI enzyme

Plasmid is linearized with PVUI High Fidelity Enzyme from NEB and PCR cleaned Using QIAGEN PCR Cleanup Kit, Linear DNA conc. is measured on nanodrop and copy number determined using the following formula:

PhiX 174 number of copies = (amount * 6.022×10^{23}) / (length * 1×10^{9} * 650)

4.2 MS2 Primer and Probes

Sequence	Reference
Forward: 5'-GTCCATACCTTAGATGCGTTAGC-3'	Turgeon, N. et al. 2014; Appl Environ Microbiol 80(14):4242 – 4250.
Reverse: 5'-CCGTTAGCGAAGTTGCTTGG-3'	
Probe: 5'-FAM/ACGTCGCCAGTTCCGCCATTGTCG/BHQ-3'	

MS2 RNA sequence for Standard curve;
-Length 3559 bp
-Concentration: 10 A260 Unit or 400ug/ul
(each 1 OD_{260} Unit = 40µg/ml ssRNA)
number of copies = (amount * 6.022×10^{23}) / (length * 1×10^{9} * 650/2)
= 800ng/ul* 6.022×10^{23}) / (length * 1×10^{9} * 650/2)
= 4.16E+11 copies

4.3 Adenovirus Primer and Probes

Sequence	Reference
Forward Primer: 5'-AACTTTCTCTCTTAATAGACGCC-3'	Ko *et al.*, J. Virol Methods, 2005, 127: 148-153
Reverse Primer: 5'-AGGGGGCTAGAAAACAAAA-3'	
Probe: 5'-6FAM- **CTGACACGGGCACTCTTCGC** -BHQ-3'	

Standard Curve Target sequence: 118 bp

AACTTTCTCTCTTAATAGACGCCCCACTTAATG**CTGACACGGGCACTCTTCGC**CTTCAAAGTGCTGCACCTCTTGGAC TAGTGGACAAAACACTAAAAGTTTTGTTTTCTAGCCCCCT

Total length with pUCIDT-Amp Plasmid - 2870 bp
For Standard curve: linearize with PvuI enzyme

Plasmid is linearized with PVUI High Fidelity Enzyme from NEB and PCR cleaned Using QIAGEN PCR Cleanup Kit, Linear DNA conc is measured on nanodrop and copy number determined using the following formula:

number of copies = (amount * 6.022x10^{23}) / (length * 1x10^{9} * 650)

4.4 Coronavirus OC43 Primers and Probe

Sequence	Reference
Forward Primer: 5'- CGATGAGGCTATTCCGACTAGGT-3 '	Dare, R.K. et al. J Infect. Diseases, 2007, 196: 1321-8
Reverse Primer: 5'-CCTTCCTGAGCCTTCAATATAGTAACC -3'	
Probe: 5'-6FAM- TCCGCCTGGCACGGTACTCCCT -BHQ-3'	

Standard Curve use OC43 virus serially diluted from 10^{6} GCMPN /5 uL to 10^{1} GCMPN / 5uL

4.5 SARS -oV-2 Primers and Probes

Sequence	Reference
2019-nCoV_N1-F: 5'-GAC CCC AAA ATC AGC GAA AT-3'	2019-nCoV CDC EUA Kit, 500 rxn IDTCat# 10006606
N1-R: 5'-TCT GGT TAC TGC CAG TTG AAT CTG-3'	
N1-P: 5'-FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1-3'	
N2-F: 5'-TTA CAA ACA TTG GCC GCA AA-3'	
N2-R: 5'-GCG CGA CAT TCC GAA GAA-3'	
N2-P: 5'-FAM-ACA ATT TGC CCC CAG CGC TTC AG-BHQ1-3'	

Standards for preparing the standard curve: Positive Control provided by IDT Kit is 2E+05 GC/ul or 1E+06/5ul is serially diluted to 1E+01 and used as standard curve.

4.6 WHO SARS-CoV-2 Primers and Probe

Sequence	Reference
E_Sarbeco_F1: ACAGGTACGTTAATAGTTAATAGCGT	WHO Protocol: Diagnostic detection of 2019-nCoV by real-time RT-PCR on Jan 17, 2020
E_Sarbeco_R2: ATATTGCAGCAGTACGCACACA	
E_Sarbeco_P1: FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ	

Standard curve Target sequence for the E gene: 114 bp

ACAGGTACGT TAATAGTTAA TAGCGTACTT CTTTTTCTTG CTTTCGTGGT ATTCTTGCTA GTTACACTAG CCATCCTTAC TGCGCTTCGA TTGTGTGCGT ACTGCTGCAA TATT

Plasmid is linearized with PVUI High Fidelity Enzyme from NEB and PCR cleaned Using QIAGEN PCR Cleanup Kit, Linear DNA conc is measured on nanodrop and copy number determined using the following formula:

number of copies = (amount * 6.022x10^{23}) / (length * 1x10^{9} * 650)

QPCR MASTER MIX TABLES
The Following Tables provide the reaction volume for each RT qPCR/qPCR reactions.

Table 5 -Enterovirus RT-qPCR reaction

Master Mix - Enterovirus	**Option 1**	**Option 2**
Reagent	**Volume per rxn (uL)**	**Volume per rxn (uL)**
Quanta Bio qScript XLT 1-**Step** RT-qPCR ToughMix	12.5	12.5
PCR grade water	3.25	4.25
10 uM EntF	0.75	0.75
10 uM EntR	2.25	2.25
10 uM EntP	0.25	0.25
Total MM	19	20
Sample Volume	6	5
Total reaction Vol	25	25

For 1-step: set up the thermocycler program and run with a setting of 1 cycle hold at 50 °C for 10 min, 1 cycle hold at 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 sec, and 60 °C for 60 sec (capture fluorescence).
Use Option 1 if standards are prepared to correspond to 6 ul per reaction- Use Option 2 if standards are prepared to correspond to 5 ul per reaction volume.

Table 6 -Norovirus GIA RTqPCR reaction

Master Mix - Norovirus GIA	**Option 1**	**Option 2**
Reagent	**Volume per rxn (uL)**	**Volume per rxn (uL)**
Quanta Bio qScript XLT 1-**Step** RT-qPCR ToughMix	12.5	12.5
PCR grade water	3.75	4.75
10 uM NorV GIA F(JJVMF)	1.25	1.25
10 uM NorV GIA R	1.25	1.25
10 uM NorV GIA P (RING1)	0.25	0.25
Total MM	19	20
Sample Volume	6	5
Total reaction Vol	25	25

For 1-step: set up the thermocycler program and run with a setting of 1 cycle hold at 50 °C for 10 min, 1 cycle hold at 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 sec, and 60 °C for 60 sec (capture fluorescence).
Use Option 1 if standards are prepared to correspond to 6 ul per reaction- Use Option 2 if standards are prepared to correspond to 5 ul per reaction volume.

Table 7 -Norovirus GIB RTqPCR reaction

Master Mix – Norovirus GIB	**Option 1**	**Option 2**
Reagent	**Volume per rxn (uL)**	**Volume per rxn (uL)**
Quanta Bio qScript XLT 1-**Step** RT-qPCR ToughMix	12.5	12.5
PCR grade water	2.375	3.375
10 uM NorV GIB F	1.25	1.25
10 uM NorV GIB R	2.25	2.25
10 uM NorV GIB P	0.625	0.625
Total MM	19	20
Sample Volume	6	5
Total reaction Vol	25	25

For 1-step: set up the thermocycler program and run with a setting of 1 cycle hold at 50 °C for 10 min, 1 cycle hold at 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 sec, and 60 °C for 60 sec (capture fluorescence).
Use Option 1 if standards are prepared to correspond to 6 ul per reaction- Use Option 2 if standards are prepared to correspond to 5 ul per reaction volume.

Table 8 -Norovirus GII RTqPCR reaction

Master Mix - Norovirus GII	**Option 1**	**Option 2**
Reagent	**Volume per rxn (uL)**	**Volume per rxn (uL)**
Quanta Bio qScript XLT 1-**Step** RT-qPCR ToughMix	12.5	12.5
PCR grade water	2.375	3.375
10 uM NorV GII F	1.25	1.25
10 uM NorV GII R	2.25	2.25
10 uM NorV GII P	0.625	0.625
Total MM	19	20
Sample Volume	6	5
Total reaction Vol	25	25

For 1-step: set up the thermocycler program and run with a setting of 1 cycle hold at 50 °C for 10 min, 1 cycle hold at 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 sec, and 60 °C for 60 sec (capture fluorescence).
Use Option 1 if standards are prepared to correspond to 6 ul per reaction- Use Option 2 if standards are prepared to correspond to 5 ul per reaction volume.

Table 9 -Hepatitis G RTqPCR Inhibition Assay reaction

Master Mix - Hepatitis G	**Option 1**	**Option 2**
Reagent	**Volume per rxn (uL)**	**Volume per rxn (uL)**
Quanta Bio qScript XLT 1-**Step** RT-qPCR ToughMix	12.5	12.5
HepG	2	2.5
PCR grade water	1.75	2.25
10 uM HepF	1.25	1.25
10 uM HepR	1.25	1.25
10 uM HepP	0.25	0.25
Total MM	19	20
Sample Volume	6	5
Total reaction Vol	25	25

For 1-step: set up the thermocycler program and run with a setting of 1 cycle hold at 50 °C for 10 min, 1 cycle hold at 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 sec, and 60 °C for 60 sec (capture fluorescence).

Notes
-depending on the instrument used, a longer cDNA extension of 35-50 minutes (instead of 15 min) may be required.

-Use Option 1 if standards are prepared to correspond to 6 ul per reaction- Use Option 2 if standards are prepared to correspond to 5 ul per reaction volume.

Table 10 - PhiX174 qPCR reaction

Master Mix - PhiX174	**Option 1**	**Option 2**
Reagent	**Volume per rxn (uL)**	**Volume per rxn (uL)**
2X qPCR MM (1)	12.5	12.5
PCR grade water	3.5	4.5
10 uM PhiX174F	1.25	1.25
10 uM PhiX174R	1.25	1.25
10 uM PhiX174P	0.5	0.5
Total MM	19	20
Sample Volume	6	5
Total reaction Vol	25	25

PhiX174 Thermal cycler qPCR program: 5 min at 94°C and then 40 amplification cycles including denaturation at 94°C for 15 s and annealing and elongation at 60°C for 60 s, followed by fluorescence measurement.
-Use Option 1 if standards are prepared to correspond to 6 ul per reaction- Use Option 2 if standards are prepared to correspond to 5 ul per reaction volume.

1. **qPCR master mixes: Roche 480 Probe Master or Quantabio PerfeCTa® qPCR ToughMix**

Table 11 - MS2 phage RTqPCR Assay reaction

Master Mix - MS2 phage	**Option 1**	**Option 2**
Reagent	**Volume per rxn (uL)**	**Volume per rxn (uL)**
Quanta Bio qScript XLT 1-**Step** RT-qPCR ToughMix	12.5	12.5
PCR grade water	3.5	4.5
10 uM MS2	1.25	1.25
10 uM MS2	1.25	1.25
10 uM MS2	0.5	0.5
Total MM	19	20
Sample Volume	6	5
Total reaction Vol	25	25

MS2 _RT-PCR (One step): 15 min at 50°C, 5 min at 95°C, and then 40 cycles including denaturation at 94°C for 15 s and annealing and elongation at 60°C for 60 s, followed by fluorescence measurement.
Use Option 1 if standards are prepared to correspond to 6 ul per reaction- Use Option 2 if standards are prepared to correspond to 5 ul per reaction volume.

Table 12 – Adenovirus qPCR reaction

Master Mix - Adenovirus	**Option 1**	**Option 2**
Reagent	**Volume per rxn (uL)**	**Volume per rxn (uL)**
2x qPCR MM [1]	12.5	12.5
PCR grade water	4	5
10 uM AdvF	1	1
10 uM AdvR	1	1
10 uM AdvP	0.5	0.5
Total MM	19	20
Sample Volume	6	5
Total reaction Vol	25	25

Adenovirus Thermal cycler qPCR:
Hot-start denaturation step at 95°C for 15 min, followed by 45 cycles with a 95°C denaturation for 10 s, 55°C annealing for 30 s, and 72°C elongation for 15 s

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-Use Option 1 if standards are prepared to correspond to 6 ul per reaction- Use Option 2 if standards are prepared to correspond to 5 ul per reaction volume.

Table 13 – Coronavirus OC43 RTqPCR Assay reaction

Master Mix – OC43	**Option 1**	**Option 2**
Reagent	**Volume per rxn (uL)**	**Volume per rxn (uL)**
Quanta Bio qScript XLT 1-**Step** RT-qPCR ToughMix	12.5	12.5
PCR grade water	0.5	1.5
10 uM OC43F	1.25	1.25
10 uM OC43R	1.25	1.25
10 uM OC43P	0.5	0.5
10X IPC Mix- VIC	2.5	2.5
50X Exo IPC DNA	0.5	0.5
Total MM	19	20
Sample Volume	6	5
Total reaction Vol	25	25

OC43 _RT-PCR (One step): 15 min at 50°C, 5 min at 95°C, and then 40 cycles including denaturation at 94°C for 15 s and annealing and elongation at 60°C for 60 s, followed by fluorescence measurement.

Table 14 – SARS-CoV-2 RTqPCR Assay reaction

Master Mix – CoV-2	**CoV-n1**	**CoV-2 n2**
Reagent	**Volume per rxn (uL)**	**Volume per rxn (uL)**
Quanta Bio qScript XLT 1-**Step** RT-qPCR ToughMix	10	10
PCR grade water	3.5	3.5
Combined Primer/Probe Mix n1	1.5	
Combined Primer/Probe Mix n2		1.5
Sample Volume	5	5
Total Reaction Vol	20	20

SARS-CoV-2 RT-PCR (One step): 15 min at 50°C, 2 min at 95°C, and then 45 cycles including denaturation at 95°C for 3 s and annealing and elongation at 55°C for 30 s, followed by fluorescence measurement.

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1. **qPCR master mixes: Roche 480 Probe Master or Quantabio PerfeCTa® qPCR ToughMix**

Appendix 2

WRF-Data Deliverable Format

Appendix 3

WWTPs-Sampling Protocol

DPR-2 Pathogen Monitoring in Raw Wastewater: Sampling Schedule and Protocol

1. Project Overview

The California State Water Board (SWB) has recommended that research be conducted to address knowledge gaps for developing criteria for direct potable reuse (DPR). Six research projects have been identified to address these gaps. The focus of the second of these research projects, DPR-2, is to assess the concentration of relevant pathogen and selected indicators in raw wastewater via a 14-month monitoring campaign. To provide the SWB with the most relevant data for potable reuse regulations, five California wastewater agencies were selected that have either demonstrated interest in a future direct potable reuse project or have already committed to an indirect potable reuse project. The five wastewater agencies that were selected to participate in this study and the corresponding wastewater treatment plant (WWTP) are as follows:

- Sanitation District of Los Angeles County (LACSD) – Joint Water Pollution Control Plant
- City of Los Angeles Sanitation (LASAN) – Hyperion Water Reclamation Plant
- City of San Diego – North City Water Reclamation Plant
- San Francisco Public Utilities Commission (SFPUC) – San Francisco Southeast Treatment
- Orange County Sanitation District (OCSD) – Plant 1

Each of the five WWTPs will collect and ship 24 raw wastewater grab samples during the full-scale campaign (beginning in December 2019 and ending January 2021), at a frequency of approximately one sample every two weeks. Three labs have been selected to analyze the samples:

- Cel Analytical
- Biological Consulting Services (BCS)
- Scientific Methods

The lab to which the WWTP sends a sample will rotate with each sampling event.

2. Project Organization

DPR-2 is a collaborative effort among various groups and will include numerous checks for quality assurance and quality control (QA/QC):

- A **Technical Work Group (TWG)** is developing the project deliverables and providing oversight of the monitoring campaign.
- **Five wastewater treatment agencies** will provide raw wastewater samples during the duration of the study.
- Three labs will analyze the raw wastewater samples. One of these labs, Cel Analytical, will be the **lead lab** and will coordinate with the other labs and the QA/QC officer. The **two sub-labs** will report to the lead lab.

- An independent **QA/QC officer** will review all of the results and check if the results meet the QA/QC acceptance criteria established by the TWG.

The WWTPs will collect a sample according to the schedule and protocol specified in this document and will ship the sample to the specified lab. The labs will provide the WWTPs with the necessary sampling and shipping materials (including a pre-paid shipping label); the labs should ensure that the WWTPs receive these materials **at least one week prior** to the date of the scheduled sample collection.

There may be occasions where the WWTP will be asked to send an additional sample to a lab. In this case, Brian Pecson or Emily Darby of the DPR-2 TWG will coordinate with the WWTP and provide details about the sample day, time of day, and shipping destination.

3. Schedule for Full-Scale Campaign

The sampling schedule was developed with the intent to collect samples on **different days of the week** and at **different times of day** to capture as close to the full range of pathogen concentrations as possible. The labs can receive samples Monday through Thursday. Therefore, the WWTPs will collect samples Sunday through Wednesday. Samples will be collected between the hours of 8:00 (8 am) and 15:00 (3 pm) to ensure that the samples can be collected and shipped the same day (except Sunday samples). Samples collected on Sunday will be kept refrigerated and will be shipped on Monday morning.

The WWTP sampling schedules, with the dates, time of day, and lab to ship to are attached:

Figure 1: LACSD Sampling Schedule Overview
Table 2: LACSD Sampling Schedule Details
Figure 2: LASAN Sampling Schedule Overview
Table 3: LASAN Sampling Schedule Details
Figure 3: City of San Diego Sampling Schedule Overview
Table 4: City of San Diego Sampling Schedule Details
Figure 4: SFPUC Sampling Schedule Overview
Table 5: SFPUC Sampling Schedule Details
Figure 5: OCSD Sampling Schedule Overview
Table 6: OCSD Sampling Schedule Details

The corresponding lab schedules, with the dates, time of day, and WWTP to ship supplies to and receive a sample from are also attached:

Table 7: Cel Analytical Schedule
Table 8: BCS Lab Schedule
Table 9: Scientific Methods Schedule

4. Sampling and Shipping Protocol

Raw wastewater samples should be collected as grab samples on the scheduled sampling date and shipped overnight to the laboratory. The sampling location should be upstream of primary treatment. The Laboratory will send the supplies (cooler, 4-L sample bottle, ice packs, chain of custody documents, pre-paid shipping label) to the WWTP at least one week in advance of the scheduled sampling date. The WWTP staff will collect, pack, and ship the sample according to the following procedure:

1. At least one night before: Freeze the ice packs.
2. Collect approximately 4-L of raw wastewater into a 4-L sample bottle. Place label on the sample container including facility name, sample number, date, and time. It is not necessary to measure the pH and temperature.
3. Place sample in two large trash bags. Knot the two large plastic trash bags, and seal with tape.
4. Sign and date sample collection on the chain of custody. Place the chain of custody in another Ziploc bag to avoid getting wet during shipping. The Chain of custody may be taped securely to the inside lid of the cooler.
5. Pack the cooler with the frozen ice packs.
6. Complete air bill and attach to cooler; retain the shipper's copy.
7. Ship the cooler to the laboratory via overnight delivery with the pre-paid shipping label.

NOTE on transportation precautions: Chill all samples to reduce biological activity and preserve the state of wastewater samples between collection and analysis. Sample should arrive at the laboratory COLD (temperature between 1°C and 10°C)

If samples are collected early in the day, chill samples by storing in a refrigerator between 1°C and 10°C. Sunday samples should be kept in a refrigerator between 1°C and 10°C and shipped on Monday morning using protocol above.

5. Contact Information

Brian Pecson and Emily Darby (Trussell Technologies)—representing the DPR-2 TWG—will be the primary points of contact for the agencies.

Brian Pecson
brianp@trusselltech.com
Work: 510-457-2201
Cell: 510-502-0448

Emily Darby
emilyd@trusselltech.com
Work: 510-457-2211

Cel Analytical is the lead laboratory for the testing and will also help to coordinate the collection of samples to and from the WWTPs. Laboratory-specific questions may also be

addressed to the contact person at the individual laboratories. Contact information for the laboratories is provided below.

Cel Analytical
Yeggie Dearborn
Yeggie@celanalytical.com
Work: 415-882-1690
Cell: 415-244-3170

Steven Tan
steven@celanalytical .com
Cell: 415-810-2177

BCS Laboratories
Bonnie Mull
bmull@microbioservices.com
Work: 352-377-9272

Scientific Methods
Anne Petersen
anne@scientificmethods.com
Work: 574-277-4078

The contact information and shipping address for the five WWTPs is provided in Table 1 below. The labs will ship all sample collection and shipping materials to the addresses provided.

Table 1: Wastewater Agency Contact Information and Shipping Address

Agency	Contact Information	Shipping Address
Sanitation District of Los Angeles County (LACSD)	**Eric Krikorian** erickrikorian@lacsd.org 562-908-4288 Cell: 847-454-5167	Joint Water Pollution Control Plant 24501 South Figueroa Street Carson, CA 90745 Attention: Wastewater Research / Eric Krikorian
City of Los Angeles Sanitation (LASAN)	**Nasir Emami** 310-648-5646 nasir.emami@lacity.org **Hamed Haddad Zadegan** 310-648-5223 cell: 323-696-5292 hamed.haddad-zadegan@lacity.org	Hyperion Water Reclamation Plant 12000 Vista del Mar Los Angeles, CA 90293 Attention: Nasir Emami
City of San Diego	**Mitch Bartolo** 858-458-1030 mitchb@trusselltech.com **Aviv Kolakovsky** (858) 314-4390 Avivk@trusselltech.com **Lauren Breitner** (858) 314-4391 laurenb@trusselltech.com	North City Water Reclamation Plant 4949 Eastgate Mall San Diego, CA 92121 Attn: Trussell (Aviv, Mitch, Lauren)
San Francisco Public Utilities Commission (SFPUC)	**John O'Connell** Cell: 628-249-8939 Office: 415-920-4880 **Andrew Clark** 415-920-4944 Cell: 415-750-9324 AClark@sfwater.org	San Francisco Southeast Treatment 750 Phelps St San Francisco, CA 94124 Attention: John O'Connell
Orange County Sanitation District (OCSD)[1] and Orange County Water District (OCWD)	**Samuel Choi** 714-593-7497 SChoi@OCSD.COM **Margil Jimenez** mjjimenez@OCSD.COM **Julio Polanco** 714-378-3313 jpolanco@ocwd.com	Orange County Sanitation District 10844 Ellis Ave Fountain Valley, CA 92708 Attention: Margil Jimenez (Division 630) Orange County Water District 18700 Ward St Fountain Valley, CA 92708 Attention: Julio Polanco

[1]The sampling effort at the OCSD Plant 1 will be divided between the OCSD staff and the OCWD staff. The schedule indicates where the sampling materials should be shipped (OCWD or OCSD) for each sample.

Figure 1: LACSD Sampling Schedule Overview

Table 2: LACSD Sampling Schedule Details

Sample #	Date	Time of Day	Shipping Destination (lab)
1	Sunday, December 1, 2019	8	Cel Analytical
2	Monday, December 30, 2019	13	BCS Labs
3	Tuesday, January 21, 2020	12	Scientific Methods
4	Wednesday, January 29, 2020	9	BCS Labs
5	Sunday, February 9, 2020	14	Cel Analytical
6	Monday, March 2, 2020	11	Scientific Methods
7	Tuesday, March 17, 2020	8	Cel Analytical
8	Wednesday, April 8, 2020	14	BCS Labs
9	Sunday, April 26, 2020	12	Cel Analytical
10	Monday, May 11, 2020	8	Cel Analytical
11	Tuesday, May 26, 2020	12	BCS Labs
12	Wednesday, June 10, 2020	9	Cel Analytical
13	Sunday, June 28, 2020	14	Cel Analytical
14	Monday, July 20, 2020	13	BCS Labs
15	Tuesday, August 11, 2020	10	BCS Labs
16	Wednesday, September 2, 2020	15	Cel Analytical
17	Sunday, September 13, 2020	10	BCS Labs
18	Monday, October 5, 2020	8	BCS Labs
19	Tuesday, October 27, 2020	14	Cel Analytical
20	Wednesday, November 11, 2020	10	BCS Labs
21	Sunday, November 29, 2020	14	BCS Labs
22	Monday, December 14, 2020	11	Cel Analytical
23	Tuesday, January 5, 2021	15	BCS Labs
24	Wednesday, January 20, 2021	11	BCS Labs

Figure 2: LASAN Sampling Schedule Overview

Table 3: LASAN Sampling Schedule Details

Sample #	Date	Time of Day	Shipping Destination (lab)
1	Monday, December 9, 2019	9	BCS Labs
2	Tuesday, January 7, 2020	9	Scientific Methods
3	Wednesday, January 22, 2020	14	Cel Analytical
4	Sunday, February 2, 2020	11	BCS Labs
5	Monday, February 17, 2020	8	Scientific Methods
6	Wednesday, March 4, 2020	12	Cel Analytical
7	Wednesday, March 18, 2020	9	BCS Labs
8	Sunday, March 29, 2020	12	BCS Labs
9	Monday, April 20, 2020	10	Cel Analytical
10	Tuesday, May 5, 2020	14	BCS Labs
11	Wednesday, June 3, 2020	13	BCS Labs
12	Sunday, June 14, 2020	10	Cel Analytical
13	Monday, June 29, 2020	15	BCS Labs
14	Tuesday, July 14, 2020	10	Cel Analytical
15	Wednesday, August 5, 2020	8	Cel Analytical
16	Sunday, August 23, 2020	13	BCS Labs
17	Monday, September 14, 2020	11	BCS Labs
18	Tuesday, September 29, 2020	14	Cel Analytical
19	Wednesday, October 21, 2020	12	BCS Labs
20	Sunday, November 1, 2020	8	Cel Analytical
21	Monday, November 30, 2020	15	Cel Analytical
22	Tuesday, December 15, 2020	12	BCS Labs
23	Wednesday, January 6, 2021	8	Cel Analytical
24	Sunday, January 24, 2021	14	Cel Analytical

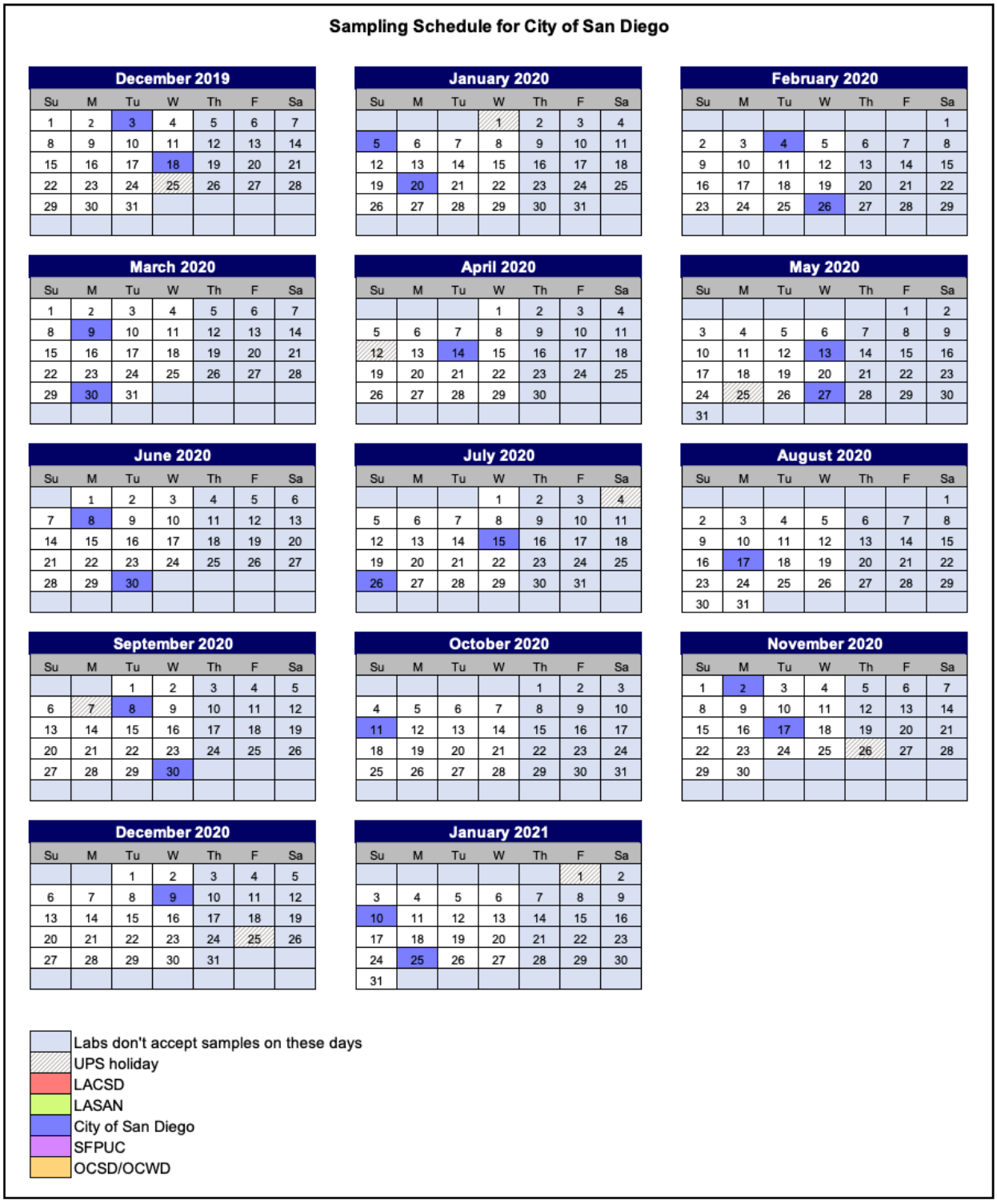

Figure 3: City of San Diego Sampling Schedule Overview

Table 4: City of San Diego Sampling Schedule Details

Sample #	Date	Time of Day	Shipping Destination (lab)
1	Tuesday, December 3, 2019	10	Scientific Methods
2	Wednesday, December 18, 2019	14	Cel Analytical
3	Sunday, January 5, 2020	10	BCS Labs
4	Monday, January 20, 2020	15	Scientific Methods
5	Tuesday, February 4, 2020	12	Cel Analytical
6	Wednesday, February 26, 2020	10	BCS Labs
7	Monday, March 9, 2020	14	Cel Analytical
8	Monday, March 30, 2020	13	Cel Analytical
9	Tuesday, April 14, 2020	8	BCS Labs
10	Wednesday, May 13, 2020	9	BCS Labs
11	Wednesday, May 27, 2020	14	Cel Analytical
12	Monday, June 8, 2020	11	BCS Labs
13	Tuesday, June 30, 2020	8	BCS Labs
14	Wednesday, July 15, 2020	11	Cel Analytical
15	Sunday, July 26, 2020	14	BCS Labs
16	Monday, August 17, 2020	11	Cel Analytical
17	Tuesday, September 8, 2020	9	Cel Analytical
18	Wednesday, September 30, 2020	15	BCS Labs
19	Sunday, October 11, 2020	10	Cel Analytical
20	Monday, November 2, 2020	9	Cel Analytical
21	Tuesday, November 17, 2020	12	BCS Labs
22	Wednesday, December 9, 2020	9	Cel Analytical
23	Sunday, January 10, 2021	10	Cel Analytical
24	Monday, January 25, 2021	15	BCS Labs

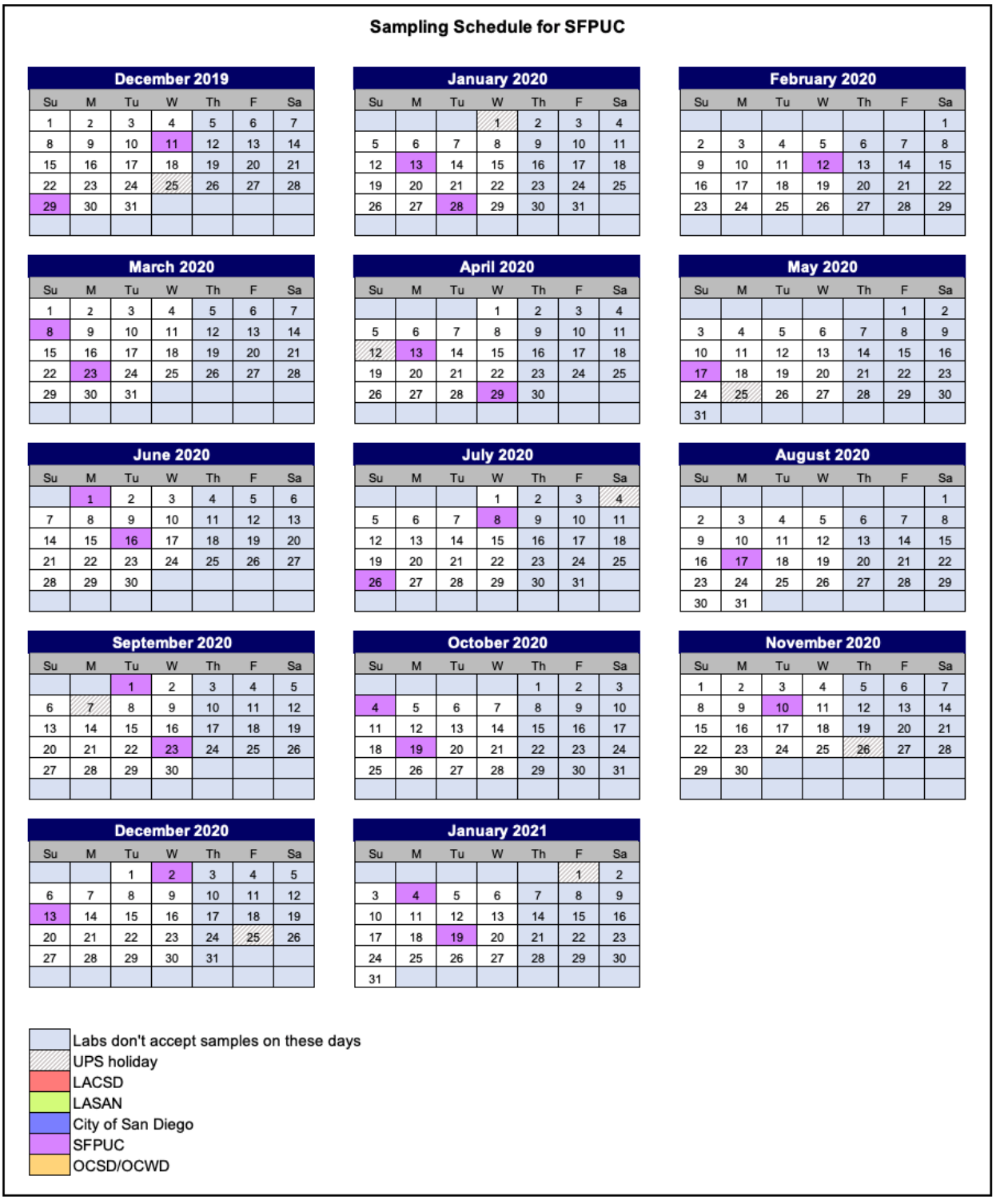

Figure 4: SFPUC Sampling Schedule Overview

Table 5: SFPUC Sampling Schedule Details

Sample #	Date	Time of Day	Shipping Destination (lab)
1	Wednesday, December 11, 2019	11	Scientific Methods
2	Sunday, December 29, 2019	8	Cel Analytical
3	Monday, January 13, 2020	13	BCS Labs
4	Tuesday, January 28, 2020	10	Scientific Methods
5	Wednesday, February 12, 2020	15	Cel Analytical
6	Monday, March 9, 2020	15	BCS Labs
7	Monday, March 23, 2020	11	Cel Analytical
8	Monday, April 13, 2020	9	Cel Analytical
9	Wednesday, April 29, 2020	13	BCS Labs
10	Sunday, May 17, 2020	10	Cel Analytical
11	Monday, June 1, 2020	15	Cel Analytical
12	Tuesday, June 16, 2020	12	BCS Labs
13	Wednesday, July 8, 2020	9	BCS Labs
14	Sunday, July 26, 2020	15	Cel Analytical
15	Monday, August 17, 2020	12	BCS Labs
16	Tuesday, September 1, 2020	8	BCS Labs
17	Wednesday, September 23, 2020	13	Cel Analytical
18	Sunday, October 4, 2020	9	BCS Labs
19	Monday, October 19, 2020	13	Cel Analytical
20	Tuesday, November 10, 2020	11	Cel Analytical
21	Wednesday, December 2, 2020	8	BCS Labs
22	Sunday, December 13, 2020	13	BCS Labs
23	Monday, January 4, 2021	9	Cel Analytical
24	Tuesday, January 19, 2021	12	BCS Labs

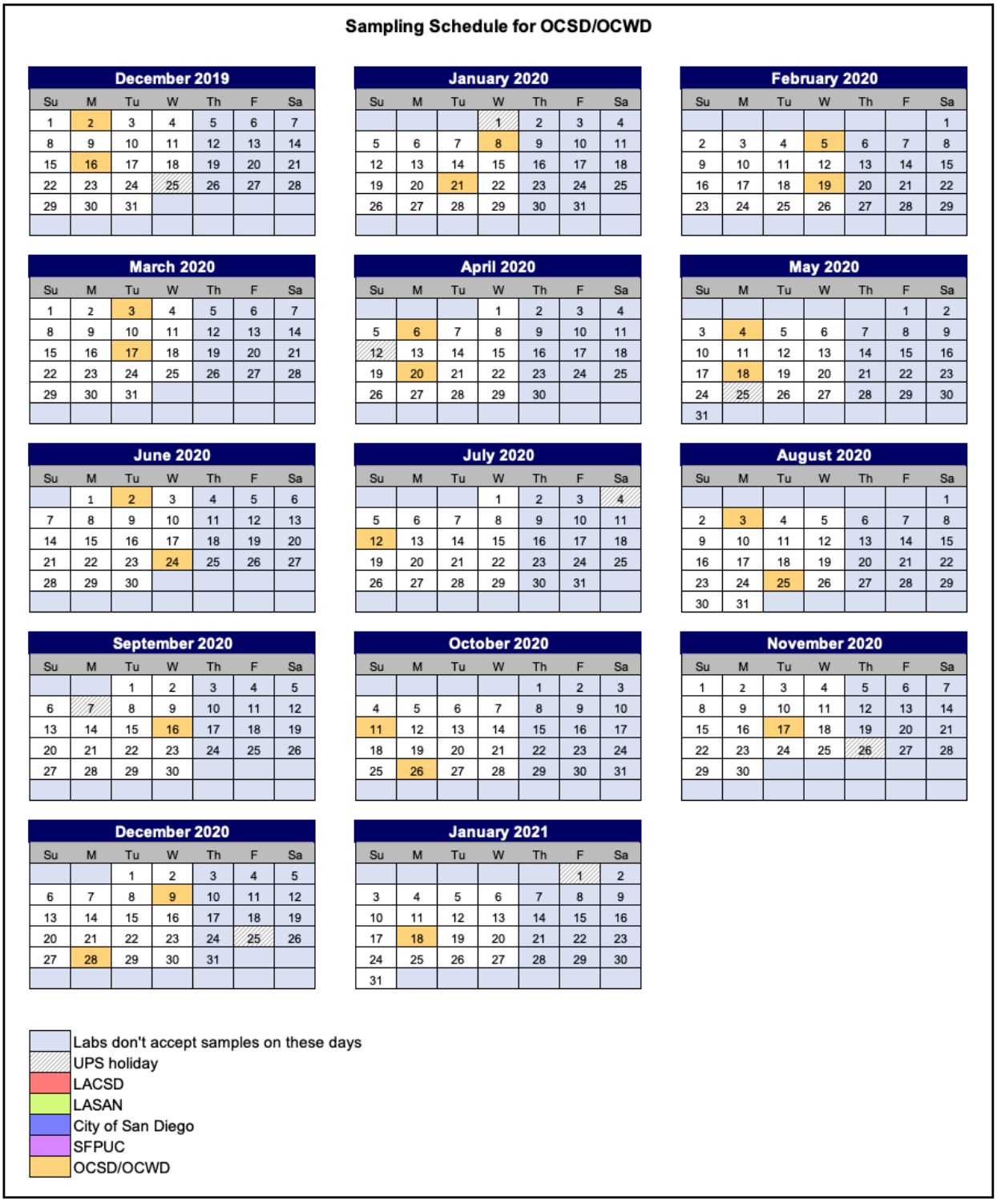

Figure 5: OCSD Sampling Schedule Overview

Table 6: OCSD/OCWD Sampling Schedule Details

Sample #	Date	Time of Day	Shipping Destination (lab)	OCWD/ OCWD
1	Monday, December 2, 2019	12	BCS Labs	OCSD
2	Monday, December 16, 2019	14:30	Scientific Methods	**OCWD**
3	Wednesday, January 8, 2020	11	Cel Analytical	OCSD
4	Tuesday, January 21, 2020	8	BCS Labs	OCSD
5	Wednesday, February 5, 2020	13	Scientific Methods	**OCWD**
6	Wednesday, February 19, 2020	9	Cel Analytical	OCSD
7	Tuesday, March 3, 2020	13	BCS Labs	**OCWD**
8	Tuesday, March 17, 2020	10	BCS Labs	OCSD
9	Tuesday, March 17, 2020	14:30	Cel Analytical	**OCWD**
10	Monday, April 20, 2020	11	BCS Labs	OCSD
11	Monday, May 4, 2020	15	BCS Labs	**OCWD**
12	Monday, May 18, 2020	11	Cel Analytical	OCSD
13	Tuesday, June 2, 2020	8	BCS Labs	OCSD
14	Wednesday, June 24, 2020	13	Cel Analytical	OCSD
15	Sunday, July 12, 2020	8	Cel Analytical	OCSD
16	Monday, August 3, 2020	9	BCS Labs	OCSD
17	Tuesday, August 25, 2020	14	Cel Analytical	OCSD
18	Wednesday, September 16, 2020	12	Cel Analytical	OCSD
19	Sunday, October 11, 2020	8	BCS Labs	OCSD
20	Monday, October 26, 2020	14:30	BCS Labs	OCSD
21	Tuesday, November 17, 2020	13	Cel Analytical	OCSD
22	Wednesday, December 9, 2020	10	Cel Analytical	OCSD
23	Monday, December 28, 2020	14	BCS Labs	OCSD
24	Monday, January 18, 2021	13	Cel Analytical	OCSD

As indicated in the schedule above (Table 6), some of the samples from the OCSD Plant 1 will be collected by the OCSD staff and some of the samples will be collected by the OCWD staff. The labs will ship the supplies to the corresponding address.

Table 7: Cel Analytical Schedule

Lab #	WWTP	WWTP Sample #	Date of Sample Collection	Time of Day
1	LACSD	1	Sunday, December 1, 2019	8
2	San Diego	2	Wednesday, December 18, 2019	14
3	SFPUC	2	Sunday, December 29, 2019	8
4	OCSD	3	Wednesday, January 8, 2020	11
5	LASAN	3	Wednesday, January 22, 2020	14
6	San Diego	5	Tuesday, February 4, 2020	12
7	LACSD	5	Sunday, February 9, 2020	14
8	SFPUC	5	Wednesday, February 12, 2020	15
9	OCSD	6	Wednesday, February 19, 2020	9
10	LASAN	6	Wednesday, March 4, 2020	12
11	San Diego	7	Monday, March 9, 2020	14
12	LACSD	7	Tuesday, March 17, 2020	8
13	OCWD	9	Tuesday, March 17, 2020	14:30
14	SFPUC	7	Monday, March 23, 2020	11
15	San Diego	8	Monday, March 30, 2020	13
16	SFPUC	8	Monday, April 13, 2020	9
17	LASAN	9	Monday, April 20, 2020	10
18	LACSD	9	Sunday, April 26, 2020	12
19	LACSD	10	Monday, May 11, 2020	8
20	SFPUC	10	Sunday, May 17, 2020	10
21	OCSD	12	Monday, May 18, 2020	11
22	San Diego	11	Wednesday, May 27, 2020	14
23	SFPUC	11	Monday, June 1, 2020	15
24	LACSD	12	Wednesday, June 10, 2020	9
25	LASAN	12	Sunday, June 14, 2020	10
26	OCSD	14	Wednesday, June 24, 2020	13
27	LACSD	13	Sunday, June 28, 2020	14
28	OCSD	15	Sunday, July 12, 2020	8
29	LASAN	14	Tuesday, July 14, 2020	10
30	San Diego	14	Wednesday, July 15, 2020	11
31	SFPUC	14	Sunday, July 26, 2020	15
32	LASAN	15	Wednesday, August 5, 2020	8
33	San Diego	16	Monday, August 17, 2020	11
34	OCSD	17	Tuesday, August 25, 2020	14
35	LACSD	16	Wednesday, September 2, 2020	15
36	San Diego	17	Tuesday, September 8, 2020	9
37	OCSD	18	Wednesday, September 16, 2020	12
38	SFPUC	17	Wednesday, September 23, 2020	13
39	LASAN	18	Tuesday, September 29, 2020	14
40	San Diego	19	Sunday, October 11, 2020	10
41	SFPUC	19	Monday, October 19, 2020	13
42	LACSD	19	Tuesday, October 27, 2020	14
43	LASAN	20	Sunday, November 1, 2020	8
44	San Diego	20	Monday, November 2, 2020	9
45	SFPUC	20	Tuesday, November 10, 2020	11

Lab #	WWTP	WWTP Sample #	Date of Sample Collection	Time of Day
46	OCSD	21	Tuesday, November 17, 2020	13
47	LASAN	21	Monday, November 30, 2020	15
48	San Diego	22	Wednesday, December 9, 2020	9
49	OCSD	22	Wednesday, December 9, 2020	10
50	LACSD	22	Monday, December 14, 2020	11
51	SFPUC	23	Monday, January 4, 2021	9
52	LASAN	23	Wednesday, January 6, 2021	8
53	San Diego	23	Sunday, January 10, 2021	10
54	OCSD	24	Monday, January 18, 2021	13
55	LASAN	24	Sunday, January 24, 2021	14

Table 8: BCS Lab Schedule

Lab #	WWTP	WWTP Sample #	Date of Sample Collection	Time of Day
1	OCSD	1	Monday, December 2, 2019	12
2	LASAN	1	Monday, December 9, 2019	9
3	LACSD	2	Monday, December 30, 2019	13
4	San Diego	3	Sunday, January 5, 2020	10
5	SFPUC	3	Monday, January 13, 2020	13
6	OCSD	4	Tuesday, January 21, 2020	8
7	LACSD	4	Wednesday, January 29, 2020	9
8	LASAN	4	Sunday, February 2, 2020	11
9	San Diego	6	Wednesday, February 26, 2020	10
10	OCWD	7	Tuesday, March 3, 2020	13
11	SFPUC	6	Monday, March 9, 2020	15
12	OCSD	8	Tuesday, March 17, 2020	10
13	LASAN	7	Wednesday, March 18, 2020	9
14	LASAN	8	Sunday, March 29, 2020	12
15	LACSD	8	Wednesday, April 8, 2020	14
16	San Diego	9	Tuesday, April 14, 2020	8
17	OCSD	10	Monday, April 20, 2020	11
18	SFPUC	9	Wednesday, April 29, 2020	13
19	OCWD	11	Monday, May 4, 2020	15
20	LASAN	10	Tuesday, May 5, 2020	14
21	San Diego	10	Wednesday, May 13, 2020	9
22	LACSD	11	Tuesday, May 26, 2020	12
23	OCSD	13	Tuesday, June 2, 2020	8
24	LASAN	11	Wednesday, June 3, 2020	13
25	San Diego	12	Monday, June 8, 2020	11
26	SFPUC	12	Tuesday, June 16, 2020	12
27	LASAN	13	Monday, June 29, 2020	15
28	San Diego	13	Tuesday, June 30, 2020	8
29	SFPUC	13	Wednesday, July 8, 2020	9
30	LACSD	14	Monday, July 20, 2020	13
31	San Diego	15	Sunday, July 26, 2020	14
32	OCSD	16	Monday, August 3, 2020	9
33	LACSD	15	Tuesday, August 11, 2020	10
34	SFPUC	15	Monday, August 17, 2020	12
35	LASAN	16	Sunday, August 23, 2020	13
36	SFPUC	16	Tuesday, September 1, 2020	8
37	LACSD	17	Sunday, September 13, 2020	10
38	LASAN	17	Monday, September 14, 2020	11
39	San Diego	18	Wednesday, September 30, 2020	15
40	SFPUC	18	Sunday, October 4, 2020	9
41	LACSD	18	Monday, October 5, 2020	8
42	OCSD	19	Sunday, October 11, 2020	8
43	LASAN	19	Wednesday, October 21, 2020	12
44	OCSD	20	Monday, October 26, 2020	14:30

Lab #	WWTP	WWTP Sample #	Date of Sample Collection	Time of Day
45	LACSD	20	Wednesday, November 11, 2020	10
46	San Diego	21	Tuesday, November 17, 2020	12
47	LACSD	21	Sunday, November 29, 2020	14
48	SFPUC	21	Wednesday, December 2, 2020	8
49	SFPUC	22	Sunday, December 13, 2020	13
50	LASAN	22	Tuesday, December 15, 2020	12
51	OCSD	23	Monday, December 28, 2020	14
52	LACSD	23	Tuesday, January 5, 2021	15
53	SFPUC	24	Tuesday, January 19, 2021	12
54	LACSD	24	Wednesday, January 20, 2021	11
55	San Diego	24	Monday, January 25, 2021	15

Table 9: Scientific Methods Schedule

	WWTP	WWTP Sample #	Date of Sample Collection	Time of Day
1	San Diego	1	Tuesday, December 3, 2019	10
2	SFPUC	1	Wednesday, December 11, 2019	11
3	OCWD	2	Monday, December 16, 2019	14:30
4	LASAN	2	Tuesday, January 7, 2020	9
5	San Diego	4	Monday, January 20, 2020	15
6	LACSD	3	Tuesday, January 21, 2020	12
7	SFPUC	4	Tuesday, January 28, 2020	10
8	OCWD	5	Wednesday, February 5, 2020	13
9	LASAN	5	Monday, February 17, 2020	8
10	LACSD	6	Monday, March 2, 2020	11

Appendix 4

Laboratories Sample Handling Procedures & Chain of Custody (COC)

SCIENTIFIC METHODS

12441 Beckley Street • Granger, Indiana 46530
574.277.4078 [Phone] • www.scientificmethods.com

WRF 4952 – Measure Pathogens in Wastewater

(Cryptosporidium *muris*, Giardia *lamblia,* Adenoviruses, Enteroviruses, Norovirus & Coliphages: male specific, MS2 & ΦX174)

Contact Name:	**Contact Phone:**
Location:	**Contact Cell:**
Address:	**Contact Email:**
City/State/ZIP:	

Sample ID:	Sampling Location:	Collection Date	Collection Time	Volume (L)	Matrix:	Analysis Requested:

Sampled by (signature): Printed Name:	Date:	Time & Temperature:
Received by:	Date:	Time & Temperature:

CHAIN OF CUSTODY RECORD

www.microbioservices.com

FL DOH Laboratory #E82924, EPA# FL01147

BCS Laboratories
4609 NW 6th street, Building A
Gainesville, FL 32609
Tel. (352) 377-9272, Fax. (352) 377-5630

Client Name and Address: WRF 4952 "Measure Pathogens in Wastewater"

Compliance Monitoring? ☐ Yes ☐ No

City/State/Zip: _____ **Project ID:** _____

Project Manager: _____ **Facility/Site:** _____

Telephone Number: _____ **Fax No.:** _____ **Report To:** _____

Sampler Name: (Print) _____ **Email:** _____

Sampler Signature: _____ **Invoice To:** _____

									Preservative			Matrix					Analysis																				
Sample ID / Description	Date Sampled	Time Sampled	No. of containers shipped	Grab	Composite	Field Filtered	pH at collection	Temperature at collection	Na Thiosulfate	Ice / frozen Ice packs	Other (specify):	Groundwater	Wastewater	Drinking Water	Biosolid/Sludge/Soil (circle)	Other (specify):	Culture Enteroviruses/Enteric virus	EPA 1615 enterovirus	EPA 1615 Noroviruses	Cryptosporidium & Giardia	E. coli	Enterococcus	Total Coliform	Yeast & Mold	Fecal coliform	Helminth Ova	Plate Count Bacteria (HPC)	Salmonella spp	Legionella (Culture or Genetic)	Pseudomonas	N. fowleri	Other:	**RUSH TAT (Fee Applies)**

Special Instructions:

Laboratory Comments:

Method of Shipment: UPS Postal DHL FEDEX Other:

Temp. @ recp't: _____ IR- _____ Corrected for IR **°C**

Relinquished by:	Date	Time	Received by:	Date	Time
Relinquished by:	Date	Time	Received by BCS Laboratories:	Date	Time

Custody Seal: Intact Broken N/A

Presence of visible ice Y N

Presence of ice packs Y N

Hold Time _____

cel analytical,inc.
water, wastewater & soil laboratory services

82 Mary Street Suite #2
San Francisco, Ca 94103
Tel: (415) 882-1690
Fax: (415) 882-1685

Chain of Custody Record

Lab ID_____

Custody and Sample Information - Print ALL information. Put N/A in blanks not applicable.

Page___of ____

SOP # SP02 Rev.4. 2019

Report to:	**Send Invoice to**	**Project**	**Turn around time**
	same	WRF 4952 "Measure Pathogens in Wastewater"	Standard (7-10 d)

Sampler's Signature/Date & Time

Item No.	Sample Identification	Sampled Date/Time	Matrix: Water	Matrix: other	Total # Containers	Sample Type: Garb=G Composite=C	sample Volume= Liter	Indicate Analysis Requested
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								

Released By	Date	Time	Received By:	Date	Time

Sample condition: Accepted _____ Rejected_____

Temperature at the time of receipt:

Ice pack /Blue Ice/Wet Ice

Holding Time Preserved: Yes No

Comments: Client Comment area

For water samples indicate type:
Drinking water (DW), Source water (SW),
RAW_Wastewater (RW), Recreational Water (RW),
Stormwater (S)