

# Comparative evaluation of boil-based DNA extraction methods paired with multiplex qPCR for detection of *Vibrio cholerae* across multiple sample types.

## Authors:

Ayazika KT<sup>1</sup>, Baumgartner ET<sup>1</sup>, Safir A<sup>1</sup>, Islam MO<sup>2</sup>, Murt KN<sup>1</sup>, Luo W<sup>1</sup>, Sack DA<sup>1</sup>, Liu J<sup>3</sup>, Taniuchi M<sup>4</sup>, Debes AK<sup>1</sup>

<sup>1</sup>Department of International Health, Johns Hopkins University Bloomberg School of Public Health, Baltimore, United States

<sup>2</sup>Department of Civil and Environmental Engineering, University of Virginia, Charlottesville, United States

<sup>3</sup>School of Public Health, Qingdao University, Qingdao, Shandong, China

<sup>4</sup>Division of Infectious Diseases & International Health, University of Virginia, Charlottesville, United States

## Abstract:

This protocol describes laboratory-based procedures for the comparative evaluation of boil-based DNA extraction methods paired with multiplex quantitative polymerase chain reaction (qPCR) assays for the detection of *Vibrio cholerae* across multiple enteric specimen types. The protocol was designed to support optimization and standardization of molecular workflows for cholera surveillance by systematically assessing DNA extraction approaches, specimen matrices, and qPCR assay performance under controlled conditions.

All parameters were optimized in a laboratory setting using independently prepared, spiked stool and alternative specimen types. These preparations do not reflect the chronological or logistical workflow of specimen collection and processing in a cholera treatment center. In field applications, a single patient specimen type would be collected, preserved according to standard practice, and extracted using the corresponding protocol described for that specimen. This comparative laboratory evaluation was conducted to inform selection of appropriate specimen types, DNA extraction methods, and qPCR assays for setting-specific surveillance needs.

The protocol was developed in response to the recent expanded use of qPCR-based molecular diagnostics in low-resource settings. By evaluating alternative specimen types in combination with simplified extraction methods and multiplex qPCR assays, this protocol aims to support informed methodological choices that maximize sensitivity and specificity for *V. cholerae* detection in enteric disease surveillance.

**COMPARATIVE EVALUATION OF BOIL-BASED DNA EXTRACTION METHODS PAIRED WITH MULTIPLEX QPCR FOR DETECTION OF *VIBRIO CHOLERAE* ACROSS MULTIPLE SAMPLE TYPES..... 1**

AUTHORS: ..... 1  
 ABSTRACT:..... 1

**1. PREPARATION OF SPIKED STOOL..... 5**

A. *V. CHOLERA*E CULTURE ..... 5  
*Materials*..... 5  
*Protocol*..... 5

B. STOOL PREPARATION..... 5  
*Materials*..... 5  
*Protocol*..... 6

C. SERIAL DILUTION PREPARATION..... 6  
*Materials*..... 6  
 Preliminary steps (check when completed): ..... 6  
*Protocol*..... 7  
 Table 1. Serial Dilution Table from Broth Culture. .... 7  
 Table 2. Code for dilution factor. .... 8

D. SPIKING STOOL ..... 8  
*Materials*..... 8  
 Preliminary steps (check when completed): ..... 8  
*Protocol*..... 8

**2. PRESERVATION OF ALTERNATIVE SAMPLE TYPES..... 9**

Table 3. Sample type overview. .... 9

A. FILTER PAPER PRESERVATION: DRIED STOOL SPOT (DSS) PROTOCOL..... 9  
*Materials*..... 9  
 Preliminary steps (check when completed): ..... 9  
*Protocol*..... 9

B. RDT SPECIMEN PRESERVATION..... 10  
*Materials*..... 10  
 Preliminary steps (check when completed): ..... 10  
*Protocol*..... 10  
 Figure 1. Interpretation of RDT results. .... 11

C. SWAB PREPARATION PROTOCOL ..... 11  
*Materials*..... 11  
 Preliminary steps (check when completed): ..... 11  
*Protocol*..... 12

**3. SAMPLE EXTRACTION..... 12**

A. BOIL EXTRACTION PROTOCOL A..... 12  
*Materials*..... 12  
*Protocol*..... 12

B. BOIL EXTRACTION PROTOCOL B..... 12  
*Materials*..... 12  
*Protocol*..... 13

C. BOIL EXTRACTION PROTOCOL C..... 13  
*Materials*..... 13  
*Protocol*..... 13

D. FILTER PAPER BOIL EXTRACTION..... 14  
*Materials*..... 14  
*Protocol*..... 14

E. SPIKED WHOLE STOOL- , COLUMN-BASED EXTRACTION ..... 15  
*Materials*..... 15  
 Preliminary steps (check when completed) ..... 15

<i>Protocol</i> .....	16
F.    SPIKED SWAB-, COLUMN-BASED EXTRACTION.....	17
<i>Materials</i> .....	17
Preliminary steps (check when completed) .....	17
<i>Protocol</i> .....	17
G.    RDT- CHELEX DNA EXTRACTION (HALF CIRCLE EXTRACTION).....	18
<i>Materials</i> .....	18
Preliminary steps (check when completed) .....	18
<i>Protocol</i> .....	19
<b>4. QUANTITATIVE PCR.....</b>	<b>20</b>
Table 4. Overview of qPCR assays.....	20
A.    STANDARD CURVE.....	20
<i>Materials</i> .....	20
Preliminary steps (check when completed) .....	20
<i>Protocol</i> .....	21
Table 5. Note: if preparing two plates in one day, adjust the volumes as follows:.....	21
A.    QPCR PROTOCOL A.....	21
<i>Materials</i> .....	21
Preliminary steps (check when completed) .....	22
<i>Protocol</i> .....	22
Table 6. Protocol A Master mix preparation.....	22
Protocol A qPCR Setup: .....	23
Table 7. Protocol A qPCR thermocycler conditions.....	23
Protocol A Data analysis and interpretation: .....	23
Table 8. Protocol A results interpretation.....	23
B.    QPCR PROTOCOL B.....	23
Table 9. Bioperfectus kit components.....	24
<i>Materials</i> .....	24
Preliminary steps (check when completed) .....	24
<i>Protocol</i> .....	24
Table 10. Protocol B Master mix preparation.....	25
Protocol B qPCR machine set up.....	25
Table 11. Protocol B thermocycler conditions.....	25
Protocol B data analysis and interpretation.....	26
Table 12. Protocol B, Bioperfectus kit 1 results interpretation.....	26
Table 13. Protocol B, Bioperfectus kit 2 results interpretation.....	26
C.    QPCR PROTOCOL C.....	26
<i>Materials</i> .....	26
Preliminary steps (check when completed) .....	27
<i>Protocol</i> .....	27
Table 14. Protocol C master mix preparation.....	27
Protocol C qPCR Machine Set Up.....	28
Table 15. Protocol C thermocycler conditions.....	28
Protocol C Data Analysis and Interpretation .....	28
Table 16. Protocol C PCR results interpretation.....	28
<b>APPENDIX A –STANDARDIZED POSITIVE CONTROL.....</b>	<b>30</b>
<i>Materials</i> .....	30
<i>Procedure</i> .....	30
<b>APPENDIX B—LYOPHILIZED PRIMER RECONSTITUTION .....</b>	<b>31</b>
<i>Materials</i> .....	31
<i>Protocol</i> .....	31
<b>APPENDIX C—QPCR PROTOCOL A PRIMER/PROBE MIXES.....</b>	<b>32</b>
Table 17. Protocol A primer and probe sequences.....	32
VIBRIO PRIMER/PROBE MIX PREPARATION: .....	32

<i>Materials</i> .....	32
<i>Protocol</i> .....	32
Table 18. Protocol A primer/probe (P/P) mixture components.....	32
INTERNAL POSITIVE CONTROL (IPC) MIX PREPARATION: .....	33
<i>Materials</i> .....	33
<i>Protocol</i> .....	33
Table 19. Protocol A qPCR internal positive control (IPC) mixture components.....	33
<b>APPENDIX D—QPCR PROTOCOL C PRIMER/PROBE MIX.....</b>	<b>34</b>
Table 20. Protocol C primer and probe sequences.....	34
PREPARATION OF THE PRIMER/PROBE MIX.....	34
<i>Materials</i> .....	34
<i>Protocol</i> .....	34
Table 21. Protocol C primer/probe (P/P) mix components.....	35
<b>REFERENCES.....</b>	<b>36</b>

# 1. Preparation of spiked stool.

## DAY 1

### a. *V. cholerae* culture

Plate pure *V. cholerae* X25049 on Luria Agar and incubate overnight at 37°C for spiking experiments.

#### Materials

- *V. cholerae* glycerol stock
- Sterile Luria agar (LA) culture plate
  - Luria agar catalog no. DF0445-17-4
  - Petri dish catalog no. FB0875712
- Sterile inoculating loop (catalog no. 22-363-599)
- Ice container

#### Protocol

1. Remove glycerol stock from the -80°C freezer.
2. Take a 10uL loop, scrape top of glycerol stock.
3. Streak LA plate in four quadrants.
4. Immediately return glycerol stock to freezer.
5. Incubate overnight at 37°C.

Note: if not using a pure bacterial glycerol stock, you will need to isolate and identify *V. cholerae* from a suspected sample before proceeding. Use a sterile inoculating loop and quadrant streak the suspected *V. cholerae* source on a Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) agar plate and identify bacterial colonies with the appropriate biochemical and serological tests. Incubate overnight, and in the morning select 1-2 *V. cholerae* colonies to plate on LA in four quadrants using a sterile inoculating loop.

### b. Stool preparation

#### Materials

- Weighing scale
- Falcon™ 50 mL High Clarity Conical Centrifuge Tubes (catalog no. 14-959-49A)
- Sterile 1X Phosphate buffered saline (PBS) (catalog no. P3813-1PAK)
- Serological pipettes 10mL, 25mL (catalog no's 170356N, 170368N)
- Parafilm® M Lab Film (catalog no. 1222J99)
- Stool\*

*\*Stool from a source without prior exposure to *V. cholerae* and without an active enteric infection. The source used in this study was stool from a canine without an active enteric infection or prior exposure to *V. cholerae*.*

Note: Stool from patients infected with *V. cholerae* is liquid, described as ‘rice water’. Therefore, the goal is to achieve similar consistency with the stool used for spiking experiments in the laboratory.

## Protocol

1. Weigh the falcon tube and record the mass.
2. Weigh the stool in the falcon tube and record the value.
3. Subtract the mass of stool from that of the combined stool and falcon tube. This will be the weight of the stool.
4. Add PBS to the falcon tube, 2ml of PBS for every gram of solid stool. Shake the tube to break up the larger particles and use a vortex to mix for a liquid consistency that can be pipetted.
5. Seal the falcon tubes with parafilm and store at 4°-8° C refrigerator.

## DAY 2

### c. Serial dilution preparation

#### Materials

- Pure growth of *Vibrio cholerae* on agar
- Sterile Luria Broth (LB) (catalog no. DF0446-17-3)
- Sterile inoculating loops (catalog no. 22-363-599)
- Falcon™ Round-Bottom Polypropylene Test Tube (catalog no. 14-959-10B)
- Fisherbrand™ Premium 1.5mL Microcentrifuge Tubes (catalog no. 05-408-130)
- Tube rack
- 10% Bleach
- Tabletop vortex
- Spectrophotometer

*Preliminary steps (check when completed):*

1. Prepare solution of 10% bleach
2. Prepare sterile LB plates
3. Label centrifuge tubes for the serial dilution of the liquid culture into LB (step 3).

Note: Discard all pipette tips, loops and used centrifuge tubes in the 10% bleach solution during the workflow.

## Protocol

1. Using a sterile loop, pick 2 colonies of *V. cholerae* from the pure growth on agar. Inoculate the LB broth in a sterile culture tube with the colonies. Incubate this preparation at 37°C for 2 hours.
2. After 2-4 hours, measure the Optical Density (OD<sub>600</sub>) of broth culture to estimate colony forming units per milliliter (cfu/mL).
  - a. Note: The OD<sub>600</sub> conversion to cfu/mL depends on the calibration of the spectrophotometer. If this conversion is unknown, use the colony count technique (see steps 4-5) to estimate the starting concentration of the broth culture.
3. Dilute the broth culture accordingly to a concentration of 1x10<sup>9</sup> cfu/mL and make 10-fold serial dilutions in broth until a concentration of 1x10<sup>0</sup> cfu/mL is achieved.
  - b. Aliquot 8x 900uL of LB into nine labeled 1.5mL Eppendorf tubes.
  - c. Dilute the starting bacteria culture 10-fold by adding 100uL to 900uL aliquoted LB, mixing well, then repeating by taking 100uL of the dilution just created and adding to the next aliquot of 900uL LB. Repeat until there are 8 tubes of the diluted bacteria.

Table 1. Serial Dilution Table from Broth Culture.

	Starting culture	Dilution 1	Dilution 2	Dilution 3	Dilution 4	Dilution 5	Dilution 6	Dilution 7	Dilution 8
CFU/mL	Y	Y x10 <sup>-1</sup>	Y x10 <sup>-2</sup>	Y x10 <sup>-3</sup>	Y x10 <sup>-4</sup>	Y x10 <sup>-5</sup>	Y x10 <sup>-6</sup>	Y x10 <sup>-7</sup>	Y x10 <sup>-8</sup>

4. If using colony counts to determine starting concentration of broth culture, prepare two Luria agar plates for dilutions 5, 6, 7, and 8 (i.e. 4x2 Luria agar plates total). On each of these plates, spread 100 uL of respective dilution and incubate at 37°C overnight.
  - a. Note: Dilutions 5-8 were chosen based on prior experience with this broth culture preparation. However, if researcher aims to be more conservative with estimates, plating the entire dilution scale in duplicate is recommended.
5. On Day 3, count and record the number of colonies present on each of the plates.
  - a. Calculate the average colonies present for each dilution.
    - i. For each plate with discrete colonies, mark each with a marker on the reverse side of the petri dish. Record the count for the two duplicate plates of corresponding concentration.
    - ii. For each dilution there are two LA plates; take the average by adding the total number of colonies between the two plates and divide by two.
  - b. For the set of plates with an average that is within 30-300 colonies, scale to determine initial cfu/mL (variable Y in Table 1).
    - i. Use the formula: CFU/mL = (Dilution Factor × Avg number of colonies counted)/volume plated
      1. *Dilution factor is the original concentration / final concentration.*

Table 2. Code for dilution factor.

Broth serial dilution	Dilution factor
$10^{-1}$	10
$10^{-2}$	100
$10^{-3}$	1000
$10^{-4}$	10000
$10^{-5}$	100000
$10^{-6}$	1000000
$10^{-7}$	10000000
$10^{-8}$	100000000
$10^{-9}$	1000000000

ii. For example: if the  $1 \times 10^{-7}$  cfu/mL dilution (dilution factor =  $10^7$ ) produced two LA plates with an average colony count of 200 colonies, then the equation becomes  $[10^7 \times 200/0.1] = 2 \times 10^{10}$  cfu/mL

6. Based on calculated Y, it may be necessary to adjust the estimated dilution concentrations in Table 1. Redo calculation for serial dilutions using  $C_1V_1 = C_2V_2$  formula.

## d. Spiking stool

### Materials

- Stool for spiking
  - prepared day prior, Section 1b
- Serial dilution in LB
  - prepared in Section 1c
- Fisherbrand™ Premium 1.5mL Microcentrifuge Tubes (catalog no. 05-408-130)
- Tube rack
- 10% Bleach
- Tabletop vortex

*Preliminary steps (check when completed):*

- \_\_\_ Prepare solution of 10% bleach
- \_\_\_ Prepare sterile LB plates
- \_\_\_ Label Eppendorf tubes for the serial dilution of the liquid culture into LB (step 3).

### Protocol

1. Using the stool prepared the day prior to create the spiked samples. Prepare 8 aliquots of the stool sample, each with 1530  $\mu$ l.

2. Add 170  $\mu\text{l}$  of the *V. cholerae* dilution  $1 \times 10^9$  cfu/mL to one 1530  $\mu\text{L}$  aliquot of stool. Vortex to mix thoroughly. This sample now contains  $1 \times 10^8$  cfu/mL, label tube accordingly.
3. Add 170  $\mu\text{l}$  of the *V. cholerae* dilution  $1 \times 10^8$  cfu/mL to one 1530  $\mu\text{L}$  aliquot of stool. Vortex to mix thoroughly. This sample now contains  $1 \times 10^7$  cfu/mL, label tube accordingly.
4. Repeat process for the remaining dilutions until there is a range of spiked stool samples from  $1 \times 10^8$  cfu/mL to  $1 \times 10^0$  cfu/mL.
5. Samples should be stored on ice or at  $4^\circ\text{C}$  until ready for same-day use. For longer term storage, keep samples at or below  $-20^\circ\text{C}$ .

## 2. Preservation of alternative sample types.

From 1700  $\mu\text{L}$  of spiked stool prepared in Section 1d, make the following sets of samples outlined in Table 3 in preparation for extraction.

Table 3. Sample type overview.

Sample type	Volume spiked stool required per sample ( $\mu\text{l}$ )	Sample concentrations
Whole stool	200	$10^8$ to $10^0$
DSS	20	$10^8$ to $10^0$
RDT	50	$10^8$ to $10^0$
Swab	150	$10^8$ to $10^0$

### a. Filter paper preservation: dried stool spot (DSS) protocol

#### Materials

- Whatman™ 903 Protein Saver Card (catalog no. 05-715-121)<sup>1</sup>
- Cut 200  $\mu\text{l}$  pipette tips
- Pipette (P200)
- Tabletop vortex
- Spiked stool sample(s)
  - prepared in Section 1d

*Preliminary steps (check when completed):*

\_\_\_\_ Prepare cut tips: Cut  $\sim 2$ mm off the tips of a full box of 200  $\mu\text{L}$  pipette tips. Use clean, autoclaved scissors to cut.

<sup>1</sup> <https://www.cytivalifesciences.com/en/us/products/items/903-proteinsaver-card-p-01011>

## Protocol

1. Vortex to ensure sample is well mixed.
  - a. If sample is frozen, remove from freezer and allow to thaw on ice approximately halfway before vortexing.
2. Using a P200 and cut 200µl tips, pipet 20µl of stool into the center of each circle on the correspondingly labeled Whatman card. If stool is not completely liquid, spread evenly within the circle using the pipet tip. Set aside at room temperature, leaving the card open.
3. Repeat for remaining samples.
4. Return original sample vials to -80 immediately after spotting and aliquoting is complete.
5. Allow each spotted sample to dry for a minimum of 90 minutes before closing card. Store at room temperature until extraction.

## b. RDT specimen preservation

### Materials

- Spiked stool sample(s)
  - prepared in Section 1d
- Foam container with ice
- Arkray Crystal VC-O1 Rapid Diagnostic Test (RDT) Kit (S0003373)

### *Preliminary steps (check when completed):*

\_\_\_ Prepare cut tips: Cut ~2mm off the tips of a full box of 200uL pipette tips. Use clean, autoclaved scissors to cut.

*Note: The Crystal VC-O1 is an Immunochromatographic One Step Rapid Visual Test for Vibrio cholerae O1 dipstick that will be used for this procedure.<sup>2</sup> Ensure that you open a new sachet for each test. Do not discard the strip after testing, it will be used for DNA extraction.*

*Note: 50µl of stool will be aliquoted for confirmation testing using the V. cholerae RDT-protocol according to the manufacturer's instructions. Please reference the manufacturer kit insert.*

## Protocol

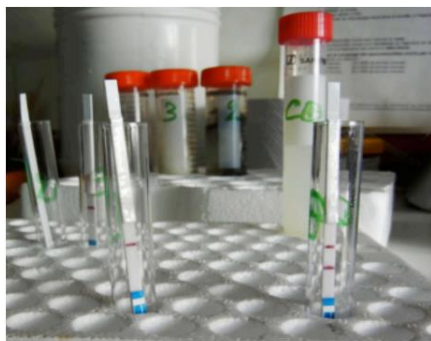
1. Take the sample processing vial containing Reagent 2 and unscrew the cap with the sampling stick attached to it (keep the sample processing vial in vertical position to avoid loss of solution).
2. Using the Pasteur pipette provided in the RDT kit, dispense two drops of liquid stool sample into sample processing vial with the help of disposable plastic droppers provided in the kit.
  - a. If sample is frozen, remove from freezer and allow to thaw on ice approximately halfway before vortexing to ensure sample is thoroughly mixed.
3. Recap the sample processing vial tightly and shake it to mix the content properly.

---

<sup>2</sup> <https://www.cdc.gov/cholera/pdf/crystal-vc-eng-p.pdf>

4. Remove the transparent cap of the sample processing vial and break the tip of the blue colored cap/sampling stick to enable addition of stool suspension to the test tube. Broken tip should be dropped in waste container and discarded as biohazard waste.
5. Dispense 4 drops from the sample processing vial into the test tube provided in the kit.
6. Recap the vial with the transparent cap after taking the sample for testing.
7. Remove the Test Dipstick (Reagent 1) from the pouch and label it with the sample identification number.
8. Place the Test Dipstick vertically into the test tube in such a way that the arrows point towards the bottom.
9. Read the results from the Test Dipstick in the test tube between 15-30 minutes. Do not read /interpret the results after 30 minutes.
  - a. Note: reading the dipstick past 30 minutes is likely to give false results!
10. The used RDT will be saved in its foil packaging labelled with the sample ID.

Figure 1. Interpretation of RDT results.



Negative sample is on the left. The positive sample is on the right.



A positive sample

*The upper line on each strip is the control. It must be present for all samples, positive or negative. Its absence means the test is invalid.*

### c. Swab preparation protocol

#### Materials

- Fisherbrand™ Wood Handled Cotton Swabs (catalog no. 22-363-172)
- Fisherbrand™ Premium 2.0mL Microcentrifuge Tubes (catalog no. 05-408-138)
- Cut 200uL pipette tips
- Pipettor (P200)
- Spiked stool sample(s)
  - *prepared in Section 1d*

*Preliminary steps (check when completed):*

\_\_\_ Prepare cut tips: Cut ~2mm off the tips of a full box of 200uL pipette tips. Use clean, autoclaved scissors to cut.

## Protocol

1. Aliquot 150µl of stool in a sterile 2ml centrifuge tube using cut 200uL tip.
  - a. If sample is frozen, remove from freezer and allow to thaw on ice approximately halfway before vortexing to ensure sample is thoroughly mixed.
2. A sterile swab is inserted and swirled to absorb all the stool. The tip is then broken off and placed in a 2ml centrifuge tube for immediate extraction or can be re-sheathed and stored at room temperature until DNA extraction.

## 3. Sample extraction

### a. Boil extraction protocol A

This procedure is a modification of the method described by Peng et al. (1).

## Materials

- Spiked whole stool sample(s)
  - *Prepared in Section 1d*
- Fisherbrand™ Premium 1.5mL Microcentrifuge Tubes (catalog no. 05-408-130)
- Ice container
- Heat block
- Cap locks for microcentrifuge tubes
- Tabletop centrifuge
- P1000 and 1000uL tips

## Protocol

1. Place 200µl of stool suspension in 1.5ml Eppendorf tube.
  - a. If sample is frozen, remove from freezer and allow to thaw on ice approximately halfway before vortexing to ensure sample is thoroughly mixed.
2. Incubate at 100°C for 15 minutes.
  - a. Note: use cap locks to ensure the tubes do not open during the heating process.
3. Immediately place samples on ice or freeze at 0°C for at least 5 minutes.
4. Centrifuge samples at 13.3k rpm for 15 minutes.
5. Save the supernatant at -20°C or use immediately.

## b. Boil extraction protocol B

### Materials

- Spiked whole stool sample(s)
  - *Prepared in Section 1d*
- Fisherbrand™ Premium 1.5mL Microcentrifuge Tubes (catalog no. 05-408-130)
- Tube rack
- Chelex 100 Resin (catalog no. 1421253)
- UltraPure™ DNase/RNase-Free Distilled Water
- Heat block
- Cap locks for microcentrifuge tubes
- Tabletop centrifuge

### Protocol

1. Aliquot 200µl of stool into a 1.5ml Eppendorf tube.
  - a. If sample is frozen, remove from freezer and allow to thaw on ice approximately halfway before vortexing to ensure sample is thoroughly mixed.
2. Centrifuge stool at 13300 rpm for 5 minutes, discard the supernatant.
3. Re-suspend the pellet in 200µl of 1.5% solution of Chelex-100 resin in nuclease-free water.
4. Boil at 100°C for 10 minutes.
  - a. Note: use cap locks to ensure the tubes do not open during the heating process.
5. Centrifuge at 13300 rpm for 2 minutes and use supernatant or save at -20C.

## c. Boil extraction protocol C

### Materials

- Spiked whole stool sample(s)
  - *Prepared in Section 1d*
- Fisherbrand™ Premium 1.5mL Microcentrifuge Tubes (catalog no. 05-408-130)
- Chelex 100 Resin (catalog no. 1421253)
- UltraPure™ DNase/RNase-Free Distilled Water
- Heat block, set at 100C
- Tabletop centrifuge
- Cap locks for microcentrifuge tubes
- Tube rack(s)
- Pipettes (P200, P1000)
- Pipette tips (200µl, 1000µl)
- Sterile 1X Phosphate buffered saline (PBS) (catalog no. P3813-1PAK)

## Protocol

1. Place 200µl of stool suspension in a 1.5ml Eppendorf tube.
  - a. If sample is frozen, remove from freezer and allow to thaw on ice approximately halfway before vortexing to ensure sample is thoroughly mixed.
2. Add 1ml sterile PBS and centrifuge at 13,300 rpm for 5 minutes and discard supernatant.
3. Centrifuge at 13,300 rpm for 5 minutes to precipitate the bacteria. Discard supernatants completely.
4. Add 200µl Chelex solution and vortex to resuspend the pellet.
5. Boil at 100°C for 10 minutes.
  - a. Note: use cap locks to ensure the tubes do not open during the heating process.
6. Centrifuge at 13300rpm for 2 minutes and use supernatant or save at -20°C.

## d. Filter paper boil extraction

### Materials

- Dried stool spot (DSS) on Whatman filter paper (FP)
  - *Prepared in Section 2a*
- 10% Bleach
- Deionized (DI) H<sub>2</sub>O
- Heat block, set at 100°C
- Cap locks for microcentrifuge tubes
- Fisherbrand™ Premium 1.5mL Microcentrifuge Tubes (catalog no. 05-408-130)
- Tabletop centrifuge
- Tabletop vortex
- Tube racks
- Clean, autoclaved surgical scissors (1 per sample plus 2-3 extra)
- Pipettes (P10, P20, P200, P1000)
- Pipette tips (10µl, 20µl, 200µl, 1000µl)
- Sterile 1X Phosphate buffered saline (PBS) (catalog no. P3813-1PAK)
- Chelex 100 Resin (catalog no. 1421253)
- UltraPure™ DNase/RNase-Free Distilled Water

### Protocol

1. Carefully cut out the circle of each filter paper spot and place in the correspondingly labeled 1.5mL centrifuge tube.
  - a. For the blank, cut out one spot from an unused filter paper card and place it in the correspondingly labeled 1.5mL centrifuge tube.
  - b. Use a new pair of scissors for each sample, and place scissors in 10% bleach solution after use, then in DI water, and lay open to dry on an absorbent pad. Once dry, autoclave scissors before using for a future extraction.

- c. Note: If bleach is unavailable, spray all scissors with 70% ethanol. Once dry, flip the scissors and spray the other side.
2. Add 1mL of autoclaved 1X PBS to each tube containing FP spots. Ensure the FP is fully submerged in the PBS. Incubate at room temperature for 10 minutes.
  - a. Once 10 minutes have elapsed, centrifuge the samples at 13,300 rpm for 2 minutes. Discard the supernatant from each tube.
3. Add 1mL of autoclaved 1X PBS to each tube.
4. Centrifuge the samples at 13,300 rpm for 2 minutes and discard the supernatant (push down the filter paper to the bottom of the tube and discard PBS as much as possible).
5. Vortex the 1.5% Chelex and add 200µl to each sample.
  - a. Note: The Chelex will separate easily. To ensure an even, homogenous amount is added to each sample, vortex the solution after every 4 samples.
6. Incubate samples at 100C for 8 minutes.
  - a. Note: use cap locks to ensure the tubes do not open during the heating process.
7. Once 8 minutes have elapsed, centrifuge samples at 13,300 rpm for 2 minutes.
8. Transfer the supernatant from the FP spot-containing tubes to the correspondingly labeled “final” 1.5 mL tubes. Chelex beads should have sedimented at the bottom of the tube after centrifugation but carefully transfer the supernatant to ensure no beads are included.
9. Store the extracted nucleic acid in a covered container of ice until qPCR, then store in -20C.

## e. Spiked whole stool- Qiagen<sup>3</sup>, column-based extraction

### Materials

- Spiked stool sample(s)
  - *Prepared in Section 1d*
- QIAamp Fast Stool Mini Kit (Qiagen catalog no. 51604)
- Prepared InhibitEX + Controls solution (see preliminary steps)
- 96%-100% Ethanol
- DEPC treated H2O
- Heating block (2 preferred, 1 minimum)
- Tabletop centrifuge
- Tabletop vortex
- Tube rack(s)
- Tube sealers
- Pipettes (P10, P20, P200, P1000)
- Pipette tips (10µl, 20µl, 200µl, 1000µl)
- Serological pipettes (10mL, 25mL)
- 15mL falcon tube
- For each sample (+ blank):
  - 2 x Fisherbrand™ Premium 2.0mL Microcentrifuge Tubes (catalog no. 05-408-138)

---

<sup>3</sup> <https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/genomic-dna/qiaamp-powerfecal-pro-dna-kit>

- 1 tube with spin column,
- 1 x Fisherbrand™ Premium 1.5mL Microcentrifuge Tubes (catalog no. 05-408-130)

*Preliminary steps (check when completed)*

1. Prepare all reagents in the QIAamp kit according to the kit's box directions (only when opening a new Qiagen Kit)
2. Preheat two heating blocks: one at 70C and one at 95C.
3. If only one heating block is available, set it to 95C to start.
4. Prepare the following tubes for each sample being extracted (+1 blank):
5. 2 x 2.0mL labeled centrifuge tubes for sample processing.
6. 1 x tube with spin column labeled (included with QIAamp kit) for sample processing.
7. 1 x 1.5mL centrifuge tube, labeled for long-term storage.
8. Prepare InhibitEX + extrinsic control in a 15mL falcon tube.

Write down lot number of QIAamp kit + the date that EtOH was added to kit reagents:

Lot Number: \_\_\_\_\_

Date EtOH Added: \_\_\_\_\_

## Protocol

1. Aliquot 200µl of whole stool to the first set (of two) labeled 2mL centrifuge tubes for sample processing.
  - a. If sample is frozen, remove from freezer and allow to thaw on ice approximately halfway before vortexing to ensure sample is thoroughly mixed.
2. Aliquot 200µl of nuclease-free water to one of first set of 2mL centrifuge tube labeled Qiagen Kit Blank.
3. Add 1mL InhibitEX with PhHV extrinsic control to each tube with sample. Incubate at 95C for 5 minutes.
  - a. Note: use cap locks to ensure the tubes do not open during the heating process.
4. Centrifuge samples for 1 minute at 13,300 rpm.
5. Add 25µl Proteinase K to the second set of labeled 2.0mL centrifuge tubes for sample processing.
6. Transfer 600µl of lysate from each sample tube and place it in the corresponding new 2.0mL labeled tube containing the Proteinase K. (**Discard** the first set 2.0mL tube containing the remaining lysate.)
7. Add 600µl buffer AL to each sample tube and briefly vortex.
8. Incubate samples at 70C for 10 minutes.
  - a. Note: use cap locks to ensure the tubes do not open during the heating process.
  - b. During this time, open the spin columns from the kit and label them on the top.
9. add 600µl ethanol to each sample and vortex thoroughly.
10. Transfer 600µl lysate to each sample's corresponding spin column (Do not discard the tube containing the lysate). Centrifuge the spin columns for 1 minute at 13,300 rpm. Discard the waste from the collection tube and reuse the collection tube.
11. Repeat step 13 until all the lysate is put through the spin column.
12. Add 500µl buffer AW1 to the spin column & centrifuge for 1 minute at 13,300 rpm. Discard the waste from the collection tube and reuse the collection tube.
13. Add 500µl buffer AW2 to the spin column & centrifuge for 3 minutes at 13,300 rpm. Discard collection tube and transfer spin column to a fresh collection tube.

14. Centrifuge for an additional 3 minutes at 13,300 rpm to ensure all liquid has passed through the spin column.
15. Transfer spin columns to the correspondingly labeled, “final” 1.5mL centrifuge tubes. Add 200µl of buffer ATE directly onto the membrane and let incubate at room temperature for 1 minute. Centrifuge for 1 minute at 13,300 rpm to fully elute nucleic acid from the spin column into the 1.5mL centrifuge tube. Discard the filter tubes.
16. Store eluted nucleic acid in a covered container of ice until qPCR, then store at -20°C or -80°C.

## f. Spiked swab-Qiagen<sup>4</sup>, column-based extraction

### Materials

- Spiked swabs(s)
  - Prepared in Section 2c
- QIAamp Fast Stool Mini Kit (catalog no. 51604)
- Prepared InhibitEX + Controls solution (see preliminary steps)
- 96%-100% Ethanol
- DEPC treated H<sub>2</sub>O
- Heating block (2 preferred, 1 minimum)
- Tabletop centrifuge
- Tabletop vortex
- Tube rack(s)
- Cap lock for microcentrifuge tubes
- Pipettes (P10, P20, P200, P1000)
- Pipette tips (10µl, 20µl, 200µl, 1000µl)
- Serological pipettes (10mL, 25mL)
- 15mL falcon tube
- For each sample (+ blank):
  - 2 x Fisherbrand™ Premium 2.0mL Microcentrifuge Tubes (catalog no. 05-408-138)
  - 1 tube with spin column,
  - 1 x Fisherbrand™ Premium 1.5mL Microcentrifuge Tubes (catalog no. 05-408-130)

### *Preliminary steps (check when completed)*

1. Prepare all reagents in the QIAamp kit according to the kit’s box directions (only when opening a new Qiagen Kit)
2. Preheat two heating blocks: one at 70°C and one at 95°C. If only one heating block is available, set it to 95°C to start.
3. Prepare the following tubes for each sample being extracted (+1 blank):
  - 2 x 2.0mL labeled centrifuge tubes for sample processing.
  - 1 x tube with spin column labeled (included with QIAamp kit) for sample processing.
  - 1 x 1.5mL centrifuge tube, labeled for final DNA for long-term storage.

---

<sup>4</sup> <https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/genomic-dna/qiaamp-powerfecal-pro-dna-kit>

## Protocol

1. Cut the tip of the swab into a 2ml centrifuge tube.
2. Add 1ml of Inhibitex and vortex for 2 minutes to loosen stool from the swab.
3. Incubate the samples at 95°C for 5 minutes.
  - a. Note: use cap locks to ensure the tubes do not open during the heating process.
4. Centrifuge samples for 1 minute at 13,300 rpm.
5. Add 25µl Proteinase K to the second set of labeled 2.0mL centrifuge tubes for sample processing.
6. Transfer 600µL of lysate from each sample tube and place it in the corresponding new 2.0mL labeled tube containing the Proteinase K. (Discard the first set 2.0mL tube containing the remaining lysate and swab.)
7. Add 600µl buffer AL to each sample tube and briefly vortex.
8. Incubate samples at 70°C for 10 minutes.
  - a. Note: use cap locks to ensure the tubes do not open during the heating process.
  - b. During this time, open the spin columns from the kit and label them on the top.
9. Add 600µl ethanol to each sample and vortex thoroughly.
10. Transfer 600µl lysate to each sample's corresponding spin column (Do not discard the tube containing the lysate). Centrifuge the spin columns for 1 minute at 13,300 rpm. Discard the waste from the collection tube and reuse the collection tube.
11. Repeat step 13 until all the lysate is put through the spin column.
12. Add 500µl buffer AW1 to the spin column & centrifuge for 1 minute at 13,300 rpm. Discard the waste from the collection tube and reuse the collection tube.
13. Add 500µl buffer AW2 to the spin column & centrifuge for 3 minutes at 13,300 rpm. Discard collection tube and transfer spin column to a fresh collection tube.
14. Centrifuge for an additional 3 minutes at 13,300 rpm to ensure all liquid has passed through the spin column.
15. Transfer spin columns to the correspondingly labeled, "final" 1.5mL centrifuge tubes. Add 200µl of buffer ATE directly onto the membrane and let incubate at room temperature for 1 minute. Centrifuge for 1 minute at 13,300 rpm to fully elute nucleic acid from the spin column into the 1.5mL centrifuge tube. Discard the filter tubes.
16. Store eluted nucleic acid in a covered container of ice until qPCR, then store at -20C or -80C.

## g. RDT- Chelex DNA Extraction (half circle extraction)

### Materials

- RDT dipstick(s)
  - *prepared using spiked stool sample(s) in Section 2b*
- 10% Bleach
- Deionized (DI) water
- 1 x Fisherbrand™ Premium 1.5mL Microcentrifuge Tubes (catalog no. 05-408-130)
- Pipettes and pipette tips
- Distilled dH2O
- UltraPure™ DNase/RNase-Free Distilled Water
- Sterile 1X Phosphate buffered saline (PBS) (catalog no. P3813-1PAK)

- Chelex 100 Resin (catalog no. 1421253)
- Fine tipped marker
- Centrifuge
- Hot plate or water bath
- Gloves

*Preliminary steps (check when completed)*

1. Turn on hot plate to allow time for it to heat up to appropriate temperature (100 °C)
2. Solutions to be made:
  - 1x PBS solution - (autoclaved)
  - 1.5% Chelex solution
    - Example: weigh out 0.122g of Chelex then add 8.1mL of nuclease-free H<sub>2</sub>O to the tube. the remaining Chelex solution cannot be stored so be sure that it is discarded after each set of extractions
3. Label 2 sets of tubes with the appropriate ID (e.g. date of extraction, ID of sample, etc.)
  - Tube #1 is for extraction process
  - Tube #2 is for final DNA (this tube must have proper identification)

## Protocol

1. Carefully cut the vertically, half of the tip of the RDT wick that was placed face down in the test tube. Place in the correspondingly labeled 1.5mL centrifuge tube.
  - a. For the blank, cut half a wick from an unused RDT and place it in the correspondingly labeled 1.5mL centrifuge tube.
  - b. Use a new pair of scissors for each sample, and place scissors in 10% bleach solution after use, then in DI water, and lay open to dry on an absorbent pad. Once dry, autoclave scissors before using for a future extraction.
  - c. Note: If bleach is unavailable, spray all scissors with 70% ethanol. Once dry, flip the scissors and spray the other side.
2. Add 1ml of the 1x PBS to tube #1 (tube with RDT).
3. Incubate the tubes at room temperature for 10 minutes.
4. Centrifuge the samples for 2 minutes at 13,300 rpm.
5. Discard the supernatant.
6. Add 1ml of 1X PBS to each tube.
7. Centrifuge the samples for 2 minutes at 13,300 rpm.
8. Discard the supernatant.
9. Add 100µl of the dH<sub>2</sub>O/Chelex solution to each tube.
 

*\*Make sure Chelex solution is thoroughly mixed before adding*
10. Close the tubes and place sealing cap to prevent tube from popping off
11. Incubate the samples at 100°C for 8 minutes.
  - a. Note: use cap locks to ensure the tubes do not open during the heating process.
12. Centrifuge the samples at 14,000 rpm for 1 minute
13. Transfer the supernatant to the second set of labeled tubes (Tube #2) making sure that the sample from Tube #1 goes into the tube with the corresponding sample ID.
14. Store DNA samples at -20°C.

## 4. Quantitative PCR

Four different qPCR protocols designed to detect cholera were used in this study, outlined in Table 4 below.

Table 4. Overview of qPCR assays.

Assay		Description	
<b>Protocol A</b>	In-house	A version of this multiplex assay was shared by our collaborators at the National Institute for Communicable Diseases (NICD) in South Africa. The reaction volumes of components were halved from the original protocol.	(2)
<b>Protocol B</b>	Commercial	There are two multiplex Bioperfectus Vibrio Cholerae Real Time PCR Kits developed by Bioperfectus, a molecular diagnostics firm in China. They are designated Bioperfectus 1 and Bioperfectus 2.	(3)
<b>Protocol C</b>	In-house	The combination of sequences for this multiplex qPCR assay was assembled by our collaborators at the University of Virginia (UVA) for environmental testing.	(7)

### a. Standard curve

A new standard curve should be prepared for every 1-2 plates using the premade and validated positive control prepared from *V. cholerae* cultures. (Appendix A).

The same standard curve protocol was used across all three assays for the purpose of converting output cycle threshold (CT) values into copies per microliter.

### Materials

- Standardized Positive Control  
*\*See Appendix A for how to create the Standardized Positive Control*
- UltraPure™ DNase/RNase-Free Distilled Water
- Sterile tissue culture plate (Cat # 08-772-54)
- Clean tissue culture hood
- Tabletop centrifuge
- Tabletop vortex
- Tube rack(s)
- Pipettes (P10, P20, P200)
- Pipette tips (10µl, 20µl, 200µl)

*Preliminary steps (check when completed)*

Disinfect the PCR hood and ensure all needed materials are disinfected if needed

## Protocol

1. In a hood, unwrap a sterile 96-well round bottom plate. The plate can be used for preparing multiple standard curves, as long as previously used rows are marked, and 1 row of the plate is kept empty between each dilution series to reduce cross contamination.
2. Aliquot 12µl of nuclease-free water into wells 2 through 9, leaving the first well in the row empty.
3. Add 12µl of the linearized PC into well 1, mixing well before transferring from the stock PC solution to the plate.
4. Transfer 4µl of the contents of well 1 (linearized PC) into well 2. Ensure the contents of well 2 are fully mixed by pipetting up and down with a p100 pipette set to 12µl.
5. Transfer 4µl of the contents of well 2 into well 3. Mix in the same manner described in step 4.
6. Repeat step 5 until well 9 has been added to and mixed.

Table 5. Note: if preparing two plates in one day, adjust the volumes as follows:

	Nuclease-free water (added to wells 2-9 in step 2)	Linearized PC (added to well 1 in step 3)	Volume of linearized PC transferred from well to well in steps 4-6	Pipettor volume used for mixing in steps 4-6
One Plate	12µl	12µl	4µl	12µl
Two Plates	24µl	24µl	8µl	24µl

### a. qPCR Protocol A

Protocol A was developed based on the NICD multiplex assay to detect *V. cholerae* genes *toxR* and *ctxA* (toxin gene). A commercial internal control was purchased from Thermo Fisher.

### Materials

- TaqMan Gene Expression Master Mix
- Vibrio primer/probe (P/P) mix  
*\*See Appendix C for primer/probe mix protocol*
- IPC mix  
*\*See Appendix C for primer/probe mix protocol*
- UltraPure™ DNase/RNase-Free Distilled Water
- DNA template
- 1 x Fisherbrand™ Premium 1.5mL Microcentrifuge Tubes (catalog no. 05-408-130)
- Prepared standard curve  
*\*See previous section 4a, "Standard Curve"*
- MicroAmp™ Optical 96-Well Reaction Plate
- MicroAmp™ Optical Adhesive Film
- UltraPure™ DNase/RNase-Free Distilled Water
- Plate roller/sealer

- PCR plate holder
- Tabletop centrifuge
- Tube rack
- Pipettes (P10, P20, P100, P200, P1000)
- Pipette tips (10µl, 20µl, 200µl, 1000µl)

*Preliminary steps (check when completed)*

1. Clean tissue culture hood and ensure all needed materials are disinfected
2. Briefly centrifuge DNA from extracted samples

**Protocol**

1. Remove TaqMan Mix, primer/probe mix, IPC, and DNA from -20°C. Let them thaw briefly.
  - a. While waiting for reagents to thaw, place a qPCR plate into the PCR plate holder. Label with the experiment name and date
2. Combine the quantities of TaqMan Mix, primer/probe mix, IPC, and RNase-free H<sub>2</sub>O in a 1.5mL centrifuge by multiplying the volume needed per well by the number of wells that will be used on the plate, following Table 6 below. Pipette mix after each reagent is added, and vortex briefly once all reagents are combined.
  - a. *Note: add an additional 5-15% extra volume for each reagent to account for volume loss while pipetting.*

Table 6. Protocol A Master mix preparation.

<b>Component</b>	<b>Volume per well (µl)</b>	<b>Wells Used</b>	<b>Subtotal (µl)</b>	<b>extra 15%</b>	<b>total (µl)</b>
<b>TaqMan Mix</b>	12.5	52	650	97.5	747.5
<b>Primer/Probe Mix</b>	1.5	52	78	11.7	89.7
<b>IPC Mix</b>	6	52	312	46.8	358.8
<b>Nuclease-Free H<sub>2</sub>O</b>	3	52	156	23.4	179.4
<b>Total</b>	23				1375.4

3. Return master mix components to -20°C.
4. Using a 200µl pipette, transfer 23µl of master mix into each used well
  - a. NOTE: From this step until the plate is sealed, drape a piece of aluminum foil over the top of the plate any time it is not immediately being added to. This will help to prevent light-aided degradation of the master mix components.
5. Add 2µl of the prepared standard curve to the wells designated in the plating diagram. Mix well after adding and use fresh pipette tips for each addition.
6. Add 2µl of nuclease-free water to each of the wells designated as NTC. Mix well.
7. Once extracted DNA has been thawed and briefly centrifuged, add 2µl into each designated well. Mix well after addition.
8. Once all plate components are added, carefully apply a seal to the surface of the plate. Using a roller or plate sealer, ensure that edges are fully sealed.

### *Protocol A qPCR Setup:*

Plate templates should be prepared in advance using ThermoFisher's Design & Analysis software. Once sealed, briefly vortex plate prior to being centrifuged at 1200 rpm for 1 minute. This step ensures that reaction wells are fully mixed before being placed in the machine.

The reaction was run under the following conditions. Sample reaction volumes were entered as 50 uL and the initial lid temperature was set to 105.0 C. The machine passive reference should be ROX.

Once qPCR was complete, results were exported and analyzed in ThermoFisher's Design & Analysis software.

Table 7. Protocol A qPCR thermocycler conditions.

Step	Temperature (°C)	Time (minutes)	Cycle(s)
Reverse transcription	50	2	1
Initial denaturation and Taq activation	95	10	1
PCR amplification and target detection	95	0.25 (15 sec)	40
	60	1	

### *Protocol A Data analysis and interpretation:*

The following results are possible. Samples are considered 'positive' when the Ct value for that specific target is < 36.

Table 8. Protocol A results interpretation.

toxR	ctxA	IPC	Result Interpretation
+	-	+/-	Positive for <i>V. cholerae</i> but negative for cholera toxin
+	+	+/-	Positive for <i>V. cholerae</i> and positive for cholera toxin
-	-	+	Negative for <i>V. cholerae</i> species
-	-	-	Invalid – troubleshoot

## **b. qPCR Protocol B**

qPCR Protocol B requires the use of commercial kits developed by Bioperfectus. There are two available *V. cholerae* detection kits, Bioperfectus 1 (catalog no. YJB10312NW) which detects targets O1 (FAM), OmpW (ROX), O139(VIC) and an RNase P (CY5), and Bioperfectus 2 (catalog no YJB10401W), which detects O1 (FAM), CTX (ROX), O139 (VIC) and OmpW (CY5). The Bioperfectus kit contains the following components:

Table 9. Bioperfectus kit components.

Components	Vials	Volume
PCR Reaction mix	1	625µl
Detection mix	1	375µl

Positive control	1	50µl
Blank control	1	250µl

*Notes:*

- All reagents are stored at  $-20 \pm 5^\circ\text{C}$
- Check expiry date before use and do not use expired reagent
- Keep detection mix away from light
- Avoid repeated freeze-thaw

## Materials

- Reaction mix (included in Bioperfectus kit)
- Detection mix (included in Bioperfectus kit)
- Blank and Positive controls (included in Bioperfectus kit)
- 1 x Fisherbrand™ Premium 1.5mL Microcentrifuge Tubes (catalog no. 05-408-130)
- DNA template
- Prepared standard curve  
\*See earlier section “Standard Curve”
- MicroAmp™ Optical 96-Well Reaction Plate
- MicroAmp™ Optical Adhesive Film
- UltraPure™ DNase/RNase-Free Distilled Water
- Plate roller/sealer
- PCR plate holder
- Tabletop centrifuge
- Tube rack
- Pipettes (P10, P20, P100, P200, P1000)
- Pipette tips (10µl, 20µl, 200µl, 1000µl)

*Preliminary steps (check when completed)*

1. Clean PCR hood and ensure all needed materials are disinfected
2. Briefly centrifuge DNA from extracted samples

## Protocol

1. Remove Reaction and Detection mixes from  $-20^\circ\text{C}$ . Let them thaw briefly.
  - a. While waiting for reagents to thaw, place a qPCR plate into the PCR plate holder. Label with the experiment name and date
2. Combine the quantities of Reaction and Detection mixes in a 1.5mL centrifuge tube by multiplying the volume needed per well by the number of wells that will be used on the plate, following Table 10 below. Pipette mix after each reagent is added, and vortex briefly once all reagents are combined.
  - a. *Note: add an additional 5-15% extra volume for each reagent to account for volume loss while pipetting.*

Table 10. Protocol B Master mix preparation.

Component	Volume per well (µl)	Wells Used	Subtotal (ul)	extra 15%	total (ul)
Reaction mix	12.5	70	875	131.25	1006.25
Detection mix	7.5	70	525	78.75	603.75
<b>Total</b>	20				1610

3. Return Reaction and Detection mixes to -20°C.
4. Transfer 20uL of master mix from original tube into each well that will contain a sample or SC according to plate layout.
5. Add 5uL of the prepared standard curve to the wells designated in the plating diagram. Mix well after adding and use fresh pipette tips for each addition.
6. Add 5uL of nuclease-free water to each of the wells designated as NTC. Mix well.
7. Add 5µl of Blank and Positive control from Bioperfectus kit to each respective well according to plate layout. Mix well.
8. Once extracted DNA has been thawed and briefly centrifuged, add 5µl into each designated well. Mix well after addition.
9. Once all plate components are added, carefully apply a seal to the surface of the plate. Using a roller or plate sealer, ensure that edges are fully sealed.

#### *Protocol B qPCR machine set up*

Plate templates should be prepared in advance using ThermoFisher’s Design & Analysis software. Once sealed, briefly vortex plate prior to being centrifuged at 1200 rpm for 1 minute. This step ensures that reaction wells are fully mixed before being placed in the machine.

The reaction was run under the following conditions. Sample reaction volumes were entered as 25 uL and the initial lid temperature was set to 105.0 C. The machine passive reference should be ROX.

Once qPCR was complete, results were exported and analyzed in ThermoFisher’s Design & Analysis software.

Table 11. Protocol B thermocycler conditions.

Step	Temperature (°C)	Time (minutes)	Cycle(s)
Initial denaturation	95	5	1
Denaturation	95	0.167 (10 seconds)	40
Annealing, extension and fluorescent detection	58	0.5 (30 seconds)	

#### *Protocol B data analysis and interpretation.*

According to manufacturer instructions, a CT value of 38 should be used to determine if a sample is positive or negative; a sample is positive for a given target if the CT value for that target is below 38, and negative if it is equal to or above 38. Data analysis and interpretation should depend on which commercial kit was used, Bioperfectus kit 1 versus kit 2, as each targets different *V. cholerae* genes.

Table 12. Protocol B, Bioperfectus kit 1 results interpretation.

O1	O139	ompW	Internal Control (IC)	Interpretation
+	-	+	+/-	<i>V. cholerae</i> O1 detected
-	+	+	+/-	<i>V. cholerae</i> O139 Detected
-	-	+	+/-	Non-O1, O139 Detected
-	-	-	+	Not detected
-	-	-	-	Invalid

Table 13. Protocol B, Bioperfectus kit 2 results interpretation.

O1	O139	CTX	ompW	Interpretation
+	-	+	+/-	<i>V. cholerae</i> O1 detected
-	+	+	+/-	<i>V. cholerae</i> O139 Detected
-	-	+	+/-	<i>V. cholerae</i> toxin gene Detected
-	-	-	+	<i>V. cholerae</i> detected
-	-	-	-	All Undetected

### c. qPCR Protocol C

This is a multiplex assay that detects the three *V. cholerae* targets O1 (*wbeO1*), *ctxA*, *ompW* and an extraction control, phocine herpes virus (PhHV) received from our collaborators at the University of Virginia, Division of Infectious Diseases.

#### Materials

- 2x AgPath-ID Buffer (catalog no. 43-873-91)
- 25x RT-PCR Enzyme Mix (catalog no. 43-873-91)
- UltraPure™ DNase/RNase-Free Distilled Water
- Protocol C primer/probe mix  
\*See Appendix D for primer/probe mix protocol
- DNA template
- 1 x Fisherbrand™ Premium 1.5mL Microcentrifuge Tubes (catalog no. 05-408-130)
- Prepared standard curve  
\*See earlier section “Standard Curve”
- MicroAmp™ Optical 96-Well Reaction Plate
- MicroAmp™ Optical Adhesive Film
- Plate roller/sealer
- PCR plate holder
- Tabletop centrifuge

- Tube rack
- Pipettes (P10, P20, P200, P1000)
- Pipette tips (10uL, 20uL, 200uL, 1000uL)

*Preliminary steps (check when completed)*

1. Clean tissue culture hood and ensure all needed materials are disinfected
2. Briefly centrifuge DNA template from extracted samples

**Protocol**

1. Remove AgPath buffer and primer/probe mixes from -20°C. Let them thaw briefly.
  - a. While waiting for reagents to thaw, place a qPCR plate into the PCR plate holder. Label with the experiment name and date.
2. Combine the quantities of AgPath buffer, DNase-free H<sub>2</sub>O, and respective primer/probe mix in a 1.5mL centrifuge tube by multiplying the volume needed per well by the number of wells that will be used on the plate, following Table 13 below. Pipette mix after each reagent is added, and vortex briefly once all reagents are combined.
  - a. *Note: add an additional 5-15% extra volume for each reagent to account for volume loss while pipetting.*

Table 14. Protocol C master mix preparation.

<b>Component Volume (µL) EACH</b>	<b>Volume per well (µl)</b>	Wells Used	subtotal(µl)	extra 15%	total (ul)
AgPath Buffer	<b>5</b>	60	300	45	345
Primer/Probe	<b>1.0</b>	60	72	10.8	82.8
Enzyme Mix	<b>0.4</b>	60	24	3.6	27.6
Nuclease-Free H <sub>2</sub> O	<b>1.6</b>	60	84	12.6	96.6
Total	<b>8</b>				552

3. Return AgPath buffer and primer/probe mixes to -20. Take out enzyme mix.
4. Add calculated quantity of enzyme mix and mix well. Immediately return enzyme mix to -20.
5. Using a 10uL pipette, transfer 8µl of master mix into each well being used according to plate layout.
  - a. NOTE: From this step until the plate is sealed, drape a piece of aluminum foil over the top of the plate any time it is not immediately being added to. This will help to prevent light-aided degradation of the master mix components.
6. Using a 10uL pipette, add 2µl of the prepared standard curve to the wells designated in the plating diagram. Mix well after adding and use fresh pipette tips for each addition.
7. Add 2µl of nuclease-free water to each of the wells designated as NTC. Mix well.
8. Once extracted DNA has been thawed and briefly centrifuged, add 2µl into each designated well. Mix well after addition.
9. Once all plate components are added, carefully apply a seal to the surface of the plate. Using a roller or plate sealer, ensure that edges are fully sealed.

### Protocol C qPCR Machine Set Up

Plate templates should be prepared in advance using Thermo Fisher's Design & Analysis software. Once sealed, briefly vortex plate prior to being centrifuged at 1200 rpm for 1 minute. This step ensures that reaction wells are fully mixed before being placed in the machine.

The reaction was run under the following conditions. Sample reaction volumes were entered as 10 uL and the initial lid temperature was set to 105.0 C. The machine passive reference should be ROX.

Once qPCR was complete, results were exported and analyzed in Thermo Fisher's Design & Analysis software.

Table 15. Protocol C thermocycler conditions.

Step	Temperature (°C)	Time (minutes)	Cycle
Reverse transcription	45	20	1
Initial denaturation and Taq activation	95	10	1
PCR amplification and target detection	95	0.25 (15 seconds)	40
	60	1	

### Protocol C Data Analysis and Interpretation

The following results are possible. Samples are considered 'positive' when the Ct value for that specific target is < 35.

Table 16. Protocol C PCR results interpretation.

O1	PhHV	OmpW	CtxA	Interpretation
+	+/-	+	+	Toxigenic <i>V. cholerae</i> O1
-	+/-	+	+	Non O1 toxigenic <i>V. cholerae</i> detected
-	+/-	+	-	Non O1, non tox <i>V. cholerae</i>
-	+	-	-	<i>V. cholerae</i> not detected
-	-	-	-	Invalid

# Appendix A –Standardized Positive Control

Preparation of *V. cholerae* standard curve from bacterial culture.

## Materials

- LB Broth
- LB Agar plates
- Sterile inoculating loops
- Spectrophotometer
- Heat block
- 70% alcohol
- DNA/RNase Away
- 10ml culture tubes

## Procedure

1. Disinfect workspaces with 70% ethanol and DNA/RNase away prior to starting any tasks and allow them to dry.
2. Set up a fresh culture of *V. cholerae* on LB agar and incubate overnight.
3. Retrieve overnight culture and inspect the plate to ensure that the growth is pure.
4. Dispense 5ml of LB broth in a culture tube, and using a sterile loop, pick a single pure colony of *V. cholerae* and inoculate it in the broth.
5. Culture toxigenic *V. cholerae* to log phase, approximately 2 hours. Determine OD and adjust concentration to  $10^8$  cfu/ml.
6. Aliquot 200 $\mu$ l of culture broth from the  $10^8$  concentration into 10 centrifuge tubes.
7. Centrifuge 200 $\mu$ l culture broth at 13,300 rpm for 5 minutes, discard the supernatant.
8. Resuspend pellet in 200 $\mu$ l of 1.5% solution of Chelex-100 resin in nuclease-free water.
9. Boil at 100°C for 10 minutes
10. Centrifuge at 13300 rpm for 2 minutes and save the supernatant.
11. Combine the supernatants into a 2ml centrifuge tube and quantify the DNA using a Qubit fluorometer.
12. Calculate the copies per nanogram in the DNA sample based on the postulate that the average weight of a base pair is 650Da, and Avogadro's number as shown below.

Copy number = (DNA amount (ng)  $\times$  6.022  $\times$  10<sup>23</sup>number/mol)  $\div$  (genome size (bp)  $\times$  1  $\times$  10<sup>9</sup> ng/g  $\times$  650 g/mol of bp).

Based on this, the quantified the DNA in the sample preparation was 7.2ng/ $\mu$ l

Vibrio genome size: 4.1mb (4)

$(7.2 \times 6.022 \times 10^{23}) / (4100000 \times 1 \times 10^9 \times 650) = 1.63 \times 10^6$  copies/ $\mu$ l. (5)

The standard curve solution was stored as 50 $\mu$ l aliquots at -20°C.

The starting quantity in 2 $\mu$ l is 3260000 and that in 5 $\mu$ l is 8150000 copies. This was the standard curve used for the assays.

## Appendix B—Lyophilized primer reconstitution

### Materials

- UltraPure™ DNase/RNase-Free Distilled Water
- 1 x Fisherbrand™ Premium 1.5mL Microcentrifuge Tubes (catalog no. 05-408-130)
- Lyophilized primer
- P200 pipette and tips
- P1000 pipette and tips
- Tabletop centrifuge
- Tabletop vortex

### Protocol

1. Before opening the tube, spin it down in a centrifuge for at least 1-2 minutes to ensure the pellet is in the bottom of the tube.
2. Add nuclease-free water, or a buffer such as IDTE, to reach a final concentration of 100 ng/μL. Pipette up and down to mix.
  - a.  $100\ \mu\text{M} = X\ \text{nmoles lyophilized primer} + (X \times 10\ \mu\text{l molecular grade H}_2\text{O})$
  - b. For example: To determine the amount of water to add to the lyophilized primer simply multiply the number of nmol of primer in the tube by 10. That will be the amount of water to add to make a 100 μM primer stock. For example, if there are 38.2 nmol of primer a 100 μM primer stock is created by adding 382 μl of water. The original primer tubes are used for this 100 μM stock.
3. Incubate at room temperature for 15–20 min.
4. Vortex and spin down briefly.

*Note: aliquot into smaller quantities to avoid multiple freeze-thaws.*
5. Store at -20C.

## Appendix C—qPCR Protocol A primer/probe mixes

Table 17. Protocol A primer and probe sequences.

toxR	Forward	TGGCATCGTTAGGGTTAGCAA	(2)
	Reverse	CATTCACAGCCCTGAAGTTTCA	
	Probe	FAM-CGTAAGGTTTGTTC-MGB	
ctxA	Forward	ACTCACTCTGTCCTCTTGGCATAA	
	Reverse	GCAGATTCTAGACCTCCTGATGAAAT	
	Probe	NED-ACCACCTGACTGCTT-MGB	

The following mixtures (Vibrio primer/probe mix and IPC mix) should be prepared in advance of running the Protocol A qPCR assay.

### Vibrio primer/probe mix preparation:

#### Materials

- Primer stocks (100uM)
  - ToxR forward
  - ToxR reverse
  - CtxA forward
  - CtxA reverse
- Probe stocks (100uM)
  - ToxR
  - CtxA
- 1M tris
- 0.5M EDTA
- Deionized H<sub>2</sub>O
- 1 x Fisherbrand™ Premium 1.5mL Microcentrifuge Tubes (catalog no. 05-408-130)
- P200 pipette and tips
- P1000 pipette and tips

#### Protocol

1. Prepare a stock solution of primer/probe dilution buffer by adding 100uL of 1M tris (pH 8) and 2uL of 0.5M EDTA (pH 8) to 100mL of deionized water. Adjust pH to 8 with concentrated HCl, autoclave, and store at room temperature.
2. Combine the following in a sterile 1.5mL centrifuge tube to prepare the Primer /Probe Mixture:

Table 18. Protocol A primer/probe (P/P) mixture components.

Primer and Probe Mixture	
Component	Quantity
Primer/probe dilution buffer	306 ul

100uM toxR-F (real time) primer	120 ul
100uM toxR-R (real time) primer	120 ul
100uM toxR probe	24 ul
100uM ctxA-F (real time) primer	150 ul
100uM ctxA-R (real time) primer	150 ul
100uM ctxA probe	30 ul
<i>Total volume:</i>	900 ul

3. Aliquot 100uL of the Vibrio primer/probe mixture into nine labeled 1.5mL centrifuge tubes and store at -20C.

## Internal Positive Control (IPC) mix preparation:

### Materials

- 10x Exo IPC mix
- 50x Exo IPC DNA
- 1 x Fisherbrand™ Premium 1.5mL Microcentrifuge Tubes (catalog no. 05-408-130)
- P200 pipette and tips
- P1000 pipette and tips

### Protocol

1. Combine the following in a sterile 1.5mL centrifuge tube to prepare Internal Positive Control:

Table 19. Protocol A qPCR internal positive control (IPC) mixture components.

Internal Positive Control Mixture	
Component	Quantity
10x Exo IPC mix	1000 ul
50x Exo IPC DNA	200 ul
<i>Total volume:</i>	1200 ul

2. Aliquot 200uL of the IPC mix into six labeled 1.5mL centrifuge tubes and store at -20C.

## Appendix D—qPCR Protocol C primer/probe mix

Table 20. Protocol C primer and probe sequences.

V.cholerae-O1	Forward	GTTGAGAAGGGCGGTCTAATAA	(6)
	Reverse	TGTCTGGTACTTGAGTTGGTAAG	
	Probe	FAM-TGCCTCAGCAATGGA-MGB	
PhHV-mdf	Forward	GGGCGAATCACAGATTGAATC	(7)
	Reverse	GCGGTTCCAAACGTACCAA	
	Probe	VIC-TATGTGTCCGCCACCATCTGGATCAAC-MGB	
V.cholerae-ompW-mdf	Forward	CGCTTGGCTATATGTTTACTGRCA	(8)
	Reverse	CACCACCAGAGGTAGAAATCTTAT	
	Probe	CY5-CATYAGTTTTGAAGTCCTYGCTGCTACGC-IABlack	
V.cholerae-ctxA	Forward	TCCGGAGCATAGAGCTTGGAA	(9)
	Reverse	TCGATGATCTTGGAGCATTCC	
	Probe	NED-CCGTGGATTCATCATGCACCGC-MGB	

### Preparation of the Primer/Probe Mix

#### Materials

- Primer stocks (100uM)
  - *V. cholerae* O1 forward
  - *V. cholerae* O1 reverse
  - *V. cholerae* OmpW forward
  - *V. cholerae* OmpW reverse
  - *V. cholerae* CtxA forward
  - *V. cholerae* CtxA reverse
- Probe stocks (100uM)
  - *V. cholerae* O1
  - *V. cholerae* OmpW
  - *V. cholerae* CtxA
- 1M tris
- 0.5M EDTA
- Deionized H<sub>2</sub>O
- 1 x Fisherbrand™ Premium 1.5mL Microcentrifuge Tubes (catalog no. 05-408-130)
- P200 pipette and tips
- P1000 pipette and tips

#### Protocol

1. To make 500uL of P/P mix, add 20uL of each primer (F and R) and corresponding probe to a labeled 1.5mL Eppendorf tube, following the Table 20 below.
  1. Total volume in tube after this step is 240uL.

- Mix by pipetting up and down after each addition.

Table 21. Protocol C primer/probe (P/P) mix components.

Targets	Primers-probes (stock concentration)	Volume (µl)	Half volume (uL)	Concentration (10x)	Final concentration (1x)
V.cholerae-O1	Forward (100 µM)	40	20	4 µM	0.4µM
	Reverse (100 µM)	40	20	4 µM	0.4µM
	Probe (100 µM)	40	20	4 µM	0.4 µM
V.cholerae-PhHV	Forward (100 µM)	40	20	4 µM	0.4 µM
	Reverse (100 µM)	40	20	4 µM	0.4 µM
	Probe (100 µM)	40	20	4 µM	0.4 µM
V.cholerae_ompW-mdf	Forward (100 µM)	40	20	4 µM	0.4 µM
	Reverse (100 µM)	40	20	4 µM	0.4 µM
	Probe (100 µM)	40	20	4 µM	0.4 µM
V.cholerae_ctxA	Forward (100 µM)	40	20	4 µM	0.4 µM
	Reverse (100 µM)	40	20	4 µM	0.4 µM
	Probe (100 µM)	40	20	4 µM	0.4 µM
	TE buffer	520	260		
	Total	1000	500		

- Add 260uL of TE Buffer (or DNase free H<sub>2</sub>O) to P/P mixture. Mix thoroughly.
- Aliquot final P/P mix into labeled tubes, 100uL in each (or the equivalent volume needed per 1-2 plates), to avoid excessive freeze-thaws.
- Store at -20°C.

## References

1. Peng X, Yu KQ, Deng GH, Jiang YX, Wang Y, Zhang GX, et al. Comparison of direct boiling method with commercial kits for extracting fecal microbiome DNA by Illumina sequencing of 16S rRNA tags. *J Microbiol Methods*. 2013 Dec 1;95(3):455–62.
2. Koskela KA, Matero P, Blatny JM, Fykse EM, Olsen JS, Nuotio LO, et al. A multiplatform real-time polymerase chain reaction detection assay for *Vibrio cholerae*. *Diagn Microbiol Infect Dis*. 2009 Nov 1;65(3):339–44.
3. BioPerfectus - Vibrio Cholerae Real Time PCR Kit [Internet]. [cited 2026 Jan 27]. Available from: <https://www.bioperfectus.com/ProductDetail/VibrioCholeraeRealTimePCRKit>
4. NCBI [Internet]. [cited 2025 Jul 16]. *Vibrio cholerae* genome assembly ASM836960v1. Available from: [https://www.ncbi.nlm.nih.gov/datasets/genome/GCF\\_008369605.1/](https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_008369605.1/)
5. Gosselin-Théberge M, Taboada E, Guy RA. Evaluation of real-time PCR assays and standard curve optimisation for enhanced accuracy in quantification of *Campylobacter* environmental water isolates. *J Microbiol Methods*. 2016 Oct 1;129:70–7.
6. Greig DR, Hickey TJ, Boxall MD, Begum H, Gentle A, Jenkins C, et al. A real-time multiplex PCR for the identification and typing of *Vibrio cholerae*. *Diagn Microbiol Infect Dis*. 2018 Mar 1;90(3):171–6.
7. Liu J, Gratz J, Amour C, Kibiki G, Becker S, Janaki L, et al. A Laboratory-Developed TaqMan Array Card for Simultaneous Detection of 19 Enteropathogens. *J Clin Microbiol*. 2013 Feb;51(2):472–80.
8. Kahler AM, Haley BJ, Chen A, Mull BJ, Tarr CL, Turnsek M, et al. Environmental Surveillance for Toxigenic *Vibrio cholerae* in Surface Waters of Haiti. *Am J Trop Med Hyg*. 2015 Jan 7;92(1):118–25.
9. Huang J, Zhu Y, Wen H, Zhang J, Huang S, Niu J, et al. Quadruplex Real-Time PCR Assay for Detection and Identification of *Vibrio cholerae* O1 and O139 Strains and Determination of Their Toxigenic Potential. *Appl Environ Microbiol*. 2009 Nov 15;75(22):6981–5.