Creation and processing of different sample types for Whole Genome Sequencing of *Vibrio cholerae*

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Abstract

This protocol describes laboratory procedures for the creation and preparation of alternate sample types for collection during a cholera outbreak for whole genome sequencing. The purpose of this protocol is to evaluate the implementation of low cost and easy-to-use methods of sample collection and preservation to generate usable sequences in cholera surveillance.

Whole stool and samples enriched by alkaline peptone water were collected in an outbreak setting according to standard operating procedures and transported to our laboratory at Johns Hopkins University. Additional samples were created from whole stool in a controlled laboratory setting. DNA is extracted from the matched samples in parallel according to the procedures described in this protocol.

This protocol was developed to address the high costs associated with the gold standard sample method for whole genome sequencing, which is isolation of *V. cholerae* through culture. Laboratory resources for culture and cold chain storage are limited in the countries that experience cholera epidemics. Evaluation of culture-independent methods of sample collection and transportation could provide more cost-effective ways of specimen handling.

CREATION AND PROCESSING OF DIFFERENT SAMPLE TYPES FOR WHOLE GENOME SEQUENCING OF VIBRIO CHOLERAE

Handling thick stool

On arrival in the laboratory, stool samples are given IDs and stored at -80°C.

- Samples should only be taken out of the -80°C freezer to be processed. Avoid multiple freeze-thaw cycles.
- Samples are thawed on ice, weighed, and recorded.
- If the sample is too thick, it will be suspended in sterile 1X PBS per procedure listed below. We do not anticipate stool to be thick because of the pathology of *V. cholerae.*

Materials needed

- Whole stool samples
- Tabletop vortex
- P1000 pipette
- Cut 1000µL pipette tips.
- Foam container with ice
- 15mL pre-labeled falcon tub

Preliminary steps

- Prepare cut tips: Cut ~2mm off the tips of a full box of 200µL pipette tips. Use clean, autoclaved scissors to cut.
- Prepare sterile 1X PBS

Procedure

1. Weigh the stool and record the weight
2. The stool will be mixed with sterile 1X PBS in a ratio of 600µl of PBS for 1g of feces, pipette multiple times then vortex until fully homogenized.
3. The sample will then be ready for use.

Note: Stool collected from patients with cholera is unlikely to require dilution because of the extremely watery nature of diarrhea.

We will use this protocol to prepare different sample types from whole stool in the laboratory. The sample types are Rapid diagnostic test (RDT) strip, swabs, filter paper and stool extracted using a column-based kit.

Qiagen extraction from whole stool

The procedure detailed below is extracted from the manufacturer's website and manual.(1)

Materials needed

- QIAamp Fast Stool Mini Kit (Qiagen cat#51604)
- Prepared InhibitEX
- 96%-100% Ethanol
- DEPC treated H20
- 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 2.0mL centrifuge tubes, 1 tube with spin column, 1 x 1.5mL centrifuge tube

Preliminary steps

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.
    a. 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):
    a. 2 x 2.0mL labeled centrifuge tubes for sample processing.
    b. 1 x tube with spin column labeled (included with QIAamp kit) for sample processing.
    c. 1 x 1.5mL centrifuge tube, labeled for long-term storage.
4. Prepare InhibitEX in a 15mL falcon tube

Procedure

1. Aliquot 200ul of stool into a 2ml centrifuge tube for extraction.
2. Line waste container with a plastic bag. Set up two heat blocks at 95°C and 70°C respectively.

3. Aliquot 200uL of whole stool to the first set (of two) labeled 2mL centrifuge tubes for sample processing.
4. Aliquot 200uL of RNase-free water to one of first set of 2mL centrifuge tube labeled Qiagen Kit Blank.
5. Add 1mL InhibitEX to each tube with sample. Place tube sealers on each tube and incubate at 95C for 5 minutes.
6. Centrifuge samples for 1 minute at 13,300 rpm.
7. Add 25uL Proteinase K to the second set of labeled 2.0mL centrifuge tubes for sample processing.
8. Transfer 600uL 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.)
9. Add 600uL buffer AL to each sample tube and briefly vortex.
10. Incubate samples at 70C for 10 minutes. (Open the spin columns from the kit and label them on the top)
11. add 600uL ethanol to each sample and vortex thoroughly.
12. Transfer 600uL 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.
13. Repeat step 13 until all the lysate is put through the spin column.
14. Add 500uL 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.
15. Add 500uL 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.
16. Centrifuge for an additional 3 minutes at 13,300 rpm to ensure all liquid has passed through the spin column.
17. Transfer spin columns to the correspondingly labeled, "final" 1.5mL centrifuge tubes. Add 200uL 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.
18. Store eluted nucleic acid in a covered container of ice until qPCR, then store at -20C or -80C.

Direct stool RDT

Materials needed

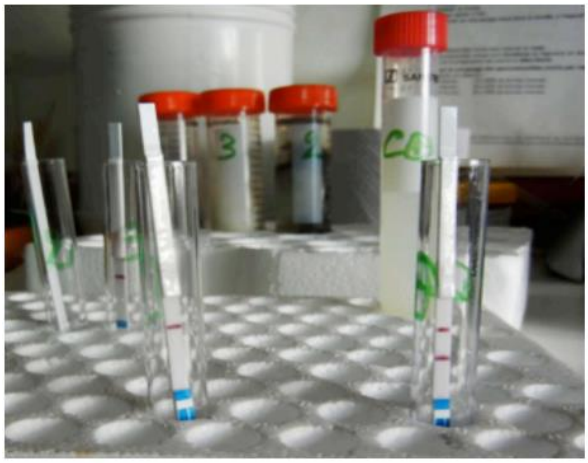
- Whole stool samples
- P200 pipette
- Cut 200uL pipette tips.
- Foam container with ice

Procedure

2 drops of stool will be aliquoted for confirmation testing using the Pasteur pipette provided by the manufacturer in the Crystal VC-O1 detection kit (Arkray Healthcare Pvt. Ltd. Ref 16IC211-10). Ensure that you open a new sachet for each test. Do not discard the strip after testing; it will be used for DNA extraction. These instructions are extracted from the manufacturer's kit insert.(2)

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 cut pipette tips, dispense 2 drops of liquid stool sample into sample processing vial with the help of disposable plastic droppers provided in the kit.
3. Recap the sample processing vial tightly and shake it to mix the content properly (Avoid any spillage while doing this)
4. Remove the transparent cap of the sample processing vial and break the outer end of the blue colored 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 of processed sample from sample processing vial into the test tube provided in the kit, as described in step 1
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. **Reading too late can give false results.**
10. The used RDT will be dried and saved in its foil packaging labelled with the sample ID.

Interpretation of results



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

A positive sample

(2)

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.

DNA Extraction from RDT

Materials

- 70% ethanol in spray bottle
- 10% Bleach
- 1.5ml tubes
- Pipettes and pipette tips (P200, P1000)
- Autoclaved 1x PBS
- Nuclease-free water
- Chelex100 Resin (BIO RAD Cat. # 142-1253)
- Fine tipped marker
- Microcentrifuge
- Heat block
- Gloves

Preliminary steps

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 H20 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. Add 1ml of the 1x PBS to tube #1 (tube with RDT)
2. Incubate the tubes at room temp for 10 minutes
3. Centrifuge the samples for 2 minutes at 13300 rpm
4. Discard the supernatant
5. Add 1ml of 1X PBS to each tube
6. Centrifuge the samples for 2 minutes at 13300 rpm
7. Discard the supernatant
8. Add 100ul of the 1.5% solution of chelex in nuclease-free water to each tube. Make sure chelex solution is thoroughly mixed before adding.
9. Close the tubes and place sealing cap to prevent tube from popping off
10. Incubate the samples at 100 °C for 8 minutes
11. Centrifuge the samples at 13300 rpm for 1 minute
12. 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.
13. Store DNA samples at -20 °C

Direct stool swab DNA extraction

Materials needed

- QIAamp Fast Stool Mini Kit (Qiagen cat#51604)
- 96%-100% Ethanol
- DEPC treated H20
- Heating block (2 preferred, 1 minimum)
- Tabletop centrifuge
- Tabletop vortex
- Tube rack(s)
- Tube sealers
- Pipettes (P10, P20, P200, P1000)
- Pipette tips (200uL, 1000uL)
- Serological pipettes (10mL, 25mL)
- 15mL falcon tube
- For each sample (+ blank): 2 x 2.0mL centrifuge tubes, 1 tube with spin column, 1 x 1.5mL centrifuge tube

Preliminary steps

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. If only one heating block is available, set it to 95C 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 long-term storage.
4. Prepare InhibitEX + control solution in a 15mL falcon tube as calculated below.
5. Write down lot number of QIAamp kit + the date that EtOH

Creation of stool swabs

1. 150ul of stool is aliquoted into a sterile 2ml centrifuge tube.
2. A sterile swab is inserted, left to absorb the stool and then swirled to ensure it absorbs all of it, dried then re-sheathed and stored at room temperature or used immediately for DNA extraction. DNA will be extracted from the swab using the process outlined below

Procedure

1. In a sterile 2ml centrifuge tube, cut the tip of the swab using sterile scissors.
2. Add 1ml of inhibitEX to the centrifuge tube with the swab.
3. Cover the tube and vortex for two minutes or longer until the stool has been completely disengaged from the swab.
4. Continue extraction with Qiagen stool extraction protocol

Filter paper spotting

Materials needed

- Whole stool samples
- Tabletop vortex
- Pasteur pipette
- Whatman 903 Protein Saver Filter Paper cards
- Foam container with ice
- 1.5mL pre-labeled centrifuge tubes

Preliminary steps

1. Label one FP card spot cut out per sample and for the FP blank
2. Label one 1.5mL centrifuge tube per sample and for the Qiagen Kit Blank
3. Prepare cut tips: Cut ~2mm off the tips of a full box of 200uL pipette tips. Use clean, autoclaved scissors to cut.

Procedure

1. Fill a foam container with ice.
2. Move stool samples from -80 into the prefilled ice container and cover with lid.
    a. Once stool samples are half thawed, you may vortex until homogenous.
    b. Keep stool samples on ice for the duration of the protocol.
3. Using a Pasteur pipette, add 2 drops of stool into the center of each circle on the correspondingly labeled Whatman card. If the stool is not completely liquid, spread evenly within the circle using the pipet tip. Set aside at room temperature.
4. Repeat steps 1-3 for the remaining samples.
5. Start a timer for 90 minutes (drying time for Filter Paper card samples).
6. Place whole stool samples immediately back in -80C.

Filter paper extraction

Materials needed

- Heat block, set at 100C.
- Tabletop centrifuge
- Tabletop vortex
- Tube rack(s)
- Tube sealers
- Clean, autoclaved surgical scissors (1 per sample plus 2-3 extra)
- Pipettors (P10, P20, P200, P1000)
- Pipette tips (10µL,20µL, 200µL, 1000µL)
- 1X RNase-free PBS, prepared using RNase-free H2O and RNase-free 10x PBS (at least 2mL needed per extraction)
- Prepared Chelex solution

Preliminary steps

1. Set heat block to 100C
2. Prepare 1X PBS (use 10X RNase-free PBS and RNase-free water to dilute)
3. Prepare the following tubes for each sample being extracted (+1 blank):
    - 1 x 1.5mL centrifuge tube, “roughly” labeled.
    - 1 x 1.5mL centrifuge tube, labeled for long-term RNA storage.
    - 1.5% Chelex solution
4. Prepare the Chelex solution according to the calculations below:
    a. *Chelex solution calculation:*

[Total samples (+ FP Blank)] x 200uL*[1.10% loss] x 1.5% = Total mg Chelex

Note: This calculation will most likely be too small to accurately weigh. Add sufficient amount of Chelex into the 15mL conical tube and re-calculate how many uL of RNase free water is required for the 1.5% solution.

Total mg Chelex (weighed)/ 1.5% = Total uL RNase free water

Procedure

1. After the 90” drying time has elapsed, carefully cut out the circle of each filter paper spot and place in the correspondingly labeled 1.5mL centrifuge tube. For the blank, cut out one spot from an unused filter paper card and place it in the correspondingly labeled 1.5mL centrifuge tube. Use a new pair of scissors for each sample, and lay the scissors open on an absorbent under pad after use.
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. At this time, spray all scissors with 70% ethanol. Once dry, flip the scissors and do the same to the other side.
3. Once 10 minutes have elapsed, centrifuge the samples at 13,300 rpm for 2 minutes. Discard the supernatant from each tube.
4. Add 1mL of autoclaved 1X PBS to each tube.
5. 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).
6. Vortex the 1.5% Chelex and add 200uL 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.
7. Incubate samples at 100°C for 8 minutes.
8. Once 8 minutes has elapsed, centrifuge samples at 13,300 rpm for 2 minutes.
9. 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.
10. Store the extracted nucleic acid in a covered container of ice until qPCR, then store in -20C.

Creation of samples from bacterial cultures

Vibrio cholerae was cultured in Cameroon by inoculating the stool collected from patients in APW and pure colonies isolated from growth on TCBS agar. The V. cholerae was confirmed by

RDT and stab cultures were created on nutrient agar and T1N1 media. The stabs were shipped to Johns Hopkins University for further processing.

Handling stab cultures

Materials needed

- Luria Bertani agar (LB)plates
- Sterile inoculating loops
- Incubator (35-37°C)

Procedure

1. Inspect the stab to ensure the culture is not dried out or contaminated. Look for shrinking and significant cracking.
2. Pick a sterile inoculating loop, careful not to touch the end that comes into contact with the culture medium.
3. Insert the loop into the stab culture and scrape a bit of growth from the stab line.
4. Streak the loop on LB agar
5. Incubate the plates in the incubator overnight for 18-24 hours.
6. Check pure growth of bacterial colonies the next day.
7. Pick a colony and inoculate it into LB broth, incubate overnight at 35-37C
8. Test the broth using V. cholerae RDT using the earlier detailed procedure using 100µl of broth.
9. Proceed only with samples that are proven to be V. cholerae.

Boiled Isolates

Materials needed

- Heat block, set at 100C.
- Tabletop centrifuge
- Tabletop vortex
- Tube rack(s)
- Tube sealers
- Clean, autoclaved surgical scissors (1 per sample plus 2-3 extra)

- Pipettors (P10, P20, P200, P1000)
- Pipette tips (10µL,20µL, 200µL, 1000µL)
- 1X RNase-free PBS, prepared using RNase-free H2O and RNase-free 10x PBS (at least 2mL needed per extraction)
- Prepared Chelex solution

Procedure

1. Aliquot 200ul of the overnight cultures of Vibrio cholerae in LB broth into 1.5ul centriguge tubes
2. Centrifuge 200ul culture broth at 13300 rpm for 5 minutes, discard the supernatant.
3. Resuspend pellet in 200ul of 1.5% solution of Chelex in nuclease-free water
4. Boil at 100°C for 10 minutes
5. Centrifuge at 13300 rpm for 2 minutes and use supernatant or save at –20°C

Qiagen extraction of broth cultures

Materials needed

- QIAamp Fast Stool Mini Kit (Qiagen cat#51604)
- Prepared InhibitEX
- 96%-100% Ethanol
- DEPC treated H20
- 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 2.0mL centrifuge tubes, 1 tube with spin column, 1 x 1.5mL centrifuge tube

Procedure

Follow the same method of extraction detailed earlier for whole stool.

Quantitative PCR of the extracted DNA samples

The purpose of this quantitative PCR (qPCR) is to confirm the presence of V. cholerae in the samples to be sequenced.

Materials needed

- V. cholerae multiplex linearized positive control (stored in -20C)
- RNase-free H20
- 96-well round bottom plate

- Clean tissue culture hood
- Tabletop centrifuge
- Tabletop vortex
- Tube rack(s)
- Pipettes (P10, P20, P100)
- Pipette tips (10uL, 20uL, 200uL)

Primer sequences

toxR	Forward	TGGCATCGTTAGGGTTAGCAA	(3)
	Reverse	CATTCACAGCCCTGAAGTTTCA	
	Probe	FAM-CGTAAGGTTTGTTTTCC-MGB	
ctxA	Forward	ACTCACTCTGTCCTCTTGGCATAA	
	Reverse	GCAGATTCTAGACCTCCTGATGAAAT	
	Probe	NED-ACCACCTGACTGCTT-MGB	

Primer and Probe Mix

1. 100 µM forward primer A: 180 ul
2. 100 µM Reverse primer A: 180 ul
3. 100 µM probe A: 50 uL
4. 100 µM forward primer B: 180 ul
5. 100 µM Reverse primer B: 180 ul
6. 100 µM probe B: 50 uL
7. Nuclease-free water: 100ul

Preliminary steps

- Clean PCR hood and ensure all needed materials are disinfected with ethanol, then RNase away.

Procedure

1. In a PCR hood, unwrap a sterile 96-well round bottom plate.
2. Aliquot 24uL of RNase-free water into wells 2 through 9, leaving the first well in the row empty.
3. Add 24uL of the standard curve V. cholerae DNA (PC) into well 1, mixing well before transferring from the stock PC solution to the plate.

4. Transfer 8uL of the contents of well 1 (PC) into well 2. Ensure the contents of well 2 are fully mixed by pipetting up and down with a p100 pipette set to 24uL.
5. Transfer 8uL 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.

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

	RNase-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	Pipetter volume used for mixing in steps 4-6
One Plate	24uL	24uL	8uL	24uL
Two Plates	48uL	48uL	16uL	48uL

Plating

Materials needed

- 2x AgPath Buffer
- DNase-free H2O
- primer/probe mix
- Enzyme mix
- DNA template
- Prepared standard curve
- MicroAmp™ Optical 96-Well Reaction Plate
- MicroAmp™ Optical Adhesive Film
- Plate roller/sealer
- PCR plate holder
- Tabletop centrifuge
- Tube rack
- Pipettors (P10, P20,P100, P200, P1000)
- Pipette tips (10uL,20uL, 200uL, 1000uL)

Master mix Preparation

Component	Volume (µL) EACH	Wells Used	subtotal(ul)	extra 10%	total (ul)
AgPath Buffer	5	XX			
Primer/Probe Mix	1	XX			
Enzyme Mix	0.4	XX			
Nuclease-Free H_2O	1.6	XX			
Total	**8**	XX			

Procedure

1. Remove AgPath buffer and primer/probe mixes from -20. 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 H2O, and respective primer/probe mixes as calculated in the plate layout worksheet in a 1.5mL centrifuge tube.
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 8uL 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 2uL 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 2uL of RNase-free water to each of the wells designated as NTC. Mix well.
8. Once extracted DNA has been thawed and briefly centrifuged, add 2uL 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.

DNA Quantification

This part of the protocol describes the process of quantifying DNA from our extracted samples using a Qubit® 4 Fluorometer. The procedure listed below is extracted from the manufacturer's manual.(4)

Materials needed

- Sterile falcon tube for mixing Qubit® working solution
- Qubit® assay tubes (500 tubes, Thermo Fisher, Cat. #Q32856)
- Serological pipette
- Pipette (P200)
- Pipette tips (200µl, 10µl)
- Qubit ds DNA HS Assay Kit (Thermo Fisher, Cat #Q32854)

Preparation

1. The Qubit® dsDNA HS Reagent and Buffer are designed for room temperature storage. The Qubit® dsDNA HS Reagent is supplied in DMSO, which freezes at temperatures lower than room temperature. Store the DNA standards at 4°C.
2. To minimize temperature fluctuations, store the Qubit® dsDNA HS Reagent and Buffer at room temperature and insert all assay tubes into the Qubit® Fluorometer only for as much time as it takes for the instrument to measure the fluorescence; the Qubit® Fluorometer can raise the temperature of the assay solution significantly, even over a period of a few minutes.
3. Do not hold the assay tubes in your hand before reading because this warms the solution and results in a low reading.
4. To allow the Qubit® assay to reach optimal fluorescence, incubate the tubes for the DNA and RNA assays for 2 minutes after mixing the sample or standard with the working solution. The fluorescence signal is stable for 3 hours at room temperature after this incubation.
5. Set up the required number of 0.5-mL tubes for standards and samples. The Qubit® dsDNA HS Assay requires 2 standards.
6. Label the tube lids only. Do not label the side of the tube as this could interfere with the sample read.

7. Label the lid of each standard tube correctly. Calibration of the Qubit® Fluorometer requires the standards to be inserted into the instrument in the right order.

Procedure

1. Prepare the Qubit® working solution by diluting the Qubit® dsDNA HS Reagent 1:200 in Qubit® dsDNA HS Buffer.
2. Use a clean plastic tube each time you prepare Qubit® working solution. Do not mix the working solution in a glass container.
3. The final volume in each tube must be 200 µL. Each standard tube requires 190 µL of Qubit® working solution, and each sample tube requires anywhere from 180–199 µL.
4. Prepare sufficient Qubit® working solution to accommodate all standards and samples.
5. Add 190 µL of Qubit® working solution to each of the tubes used for standards.
6. Add 10 µL of each Qubit® standard to the appropriate tube, then mix by vortexing 2–3 seconds. Be careful not to create bubbles.
7. Add Qubit® working solution to individual assay tubes so that the final volume in each tube after adding sample is 200 µL.
8. Note: Your sample can be anywhere from 1–20 µL. Add a corresponding volume of Qubit® working solution to each assay tube: anywhere from 180–199 µL.
9. Add each sample to the assay tubes containing the correct volume of working solution, then mix by vortexing 2–3 seconds. The final volume in each tube should be 200 µL.
10. Allow all tubes to incubate at room temperature for 2 minutes. Proceed to “Reading standards and samples
11. On the Home screen of the Qubit® Fluorometer, press DNA, then select dsDNA High Sensitivity as the assay type. The “Read standards” screen is displayed. Press Read Standards to proceed.
12. Insert the tube containing Standard #1 into the sample chamber, close the lid, then press Read standard. When the reading is complete (~3 seconds), remove Standard #1.
13. Insert the tube containing Standard #2 into the sample chamber, close the lid, then press Read standard. When the reading is complete, remove Standard #2. The instrument displays the results on the Read standard screen.
14. Press Run samples.

15. On the screen, select the sample volume and units: a. Press the + or – buttons on the wheel to select the sample volume added to the assay tube (from 1–20 μL). From the dropdown menu, select the units for the output sample concentration.
16. Insert a sample tube into the sample chamber, close the lid, then press Read tube. When the reading is complete (~3 seconds), remove the sample tube. The instrument displays the results on the assay screen. The top value (in large font) is the concentration of the original sample. The bottom value is the dilution concentration.
17. Repeat step 16 until all samples have been read.
18. After all samples have been read, press data on the screen and choose between the pdf or .csv options to export the data onto the attached USB stick or to the cloud storage if connected to the internet.

Library Preparation for Whole Genome Sequencing

This part of the protocol describes the processing of the extracted DNA from the samples created for library preparation. It has been copied from the official Illumina DNA Library Preparation Protocol.(5)

Confirm that all kit contents and consumables are present before handling the samples.

Library prep kit contents

1. **Illumina DNA Prep- Beads and Buffers- Room temperature**
   Cat #20049006
   - IPB- Illumina Purification Beads
   - TSB- Target stop buffer- if precipitates observed, heat at 37°C for 10 minutes, then vortex until precipitates are dissolved
   - TWB- Target wash buffer- Use at Room temperature. Vortex to mix

2. **Illumina DNA Prep- PCR and Buffers (-25 to –15C)**
   Cat #20015829
   - RSB- Resuspension buffer
   - TB1 Tagmentation Buffer 1- bring to room temperature. Vortex to mix
   - EPM Enhanced PCR mix- thaw on ice. Invert to mix, then centrifuge briefly

3. **Illumina DNA Prep- Tagmentation (M) Beads 2-8C**
   - BLT Bead-Linked Transposomes (**Cat #20015880**)–Bring to room temperature. Vortex do not mix. Do not centrifuge without pipetting

4. **IDT for Illumina DNA/RNA UD Indexes**
   - 96 Dual Adapter Index Plate **Cat #20091648**

Consumables

1. 1.7ml microcentrifuge tunes Fisher Brand Cat #05-408-129
2. Pipette tips- 10ul, 20ul, 200ul, 1000ul
3. Single channel pipettes
4. Multichannel pipettes 10ul, 200ul
5. 96 well 0.8ml polypropylene deep-well storage plate (MIDI plate)
6. Nuclease-free water
7. Hard shell 96 well PCR plates (BioRad #HSP9601)
8. 96 well PCR plate
9. Microseal B adhesive seals (Bio Rad Cat #MSB1001)
10. Microseal F foil seals (hts labsCat #CS100)
11. Nuclease-free multichannel reagent reservoirs
12. Ethanol 200 proof (absolute) for molecular biology

13. 8-strip PCR tubes

Laboratory equipment

1. PCR Thermocycler with 96 well plate capacity
2. Centrifuge with 96 well plate adapters
3. 96 well plate side skirted magnet (Invitrogen Cat #12027)

Handling instructions

1. Change tips between each sample when transferring samples or reagents.
2. Change tips between each row or each column when adding index adapters with a multichannel pipette. If using a single channel pipette, change tips between each sample.
3. Remove unused index adapter tubes from the working area.
4. Always seal the 96-well plate with the adhesive seal using a rubber roller or plate scraper to cover the plate before the following steps in the protocol:
    a. Shaking steps
    b. Thermal cycling steps
    c. Centrifuge steps
5. Microseal 'B' adhesive seals are effective at -40°C to 110°C and suitable for skirted or semiskirted PCR plates. Use Microseal 'B' seals for thermal cycling or short-term storage.
6. Microseal ‘F’ foil seals are effective at temperatures down to -70°C and are suitable for storing the 96-well plates containing the final libraries long term.
7. Store the BLT stock tube upright in the refrigerator so that the beads are always submerged in the buffer.
8. Vortex the BLT stock tube thoroughly until the beads are resuspended before use. To avoid resettling the beads, centrifugation before pipetting is not recommended.
9. If beads are adhered to the side or top of a 96-well plate, centrifuge at 280 × g for 3 seconds, and then pipette to resuspend.
10. Use the appropriate magnetic stand for the plate.
11. Keep the plate on the magnetic stand until the instructions specify to remove it
12. Do not agitate the plate while it is on the magnetic stand.
13. Do not disturb the bead pellet.

14. If beads are aspirated into pipette tips, dispense back into the plate on the magnetic stand and wait until the liquid is clear (~2 minutes).
15. If liquid becomes adhered to the side or top of the tube or well, centrifuge at 280 × g for 3 seconds to pull volume into liquid.

Tagment DNA

This step uses the Bead-Linked Transposomes (BLT) to tagment DNA, which is a process that fragments and tags the DNA with adapter sequences. **BLT m**ust be stored upright so that the beads are always submerged in the buffer. Do not use BLT that has been stored below 2°C.

Preparation

1. Prepare the following consumables:
    - BLT-Vortex to mix. Do not centrifuge before pipetting.
    - TB1-Vortex to mix.
    - These reagents can be left at room temperature
2. Save the following TAG program on the thermal cycler
    - Choose the preheat lid option and set to 100°C Set the reaction volume to 50 µl

Temp(°C)	Time (minutes)
55	15
10	∞

Procedure

1. Add 30 µl DNA to each well of a 96-well PCR plate so that the total input amount is 1-500 ng
2. If DNA volume is < 30 µl, add nuclease-free water to the DNA samples to bring the total volume to 30 µl.
3. Vortex BLT for 10 seconds to resuspend. Repeat as necessary.

- For each sample, combine the following volumes to prepare the Tagmentation Master Mix.

Component	**Volume (µl)**	**No. samples**	**Total**
BLT	11	XX	
TB1	11	XX	

These volumes produce 22 µl Tagmentation Master Mix per sample, which includes extra volume to ensure accurate pipetting

4. Vortex the master mix for 10 seconds to resuspend.
5. Divide the Tagmentation Master Mix volume equally into an 8-tube strip.

6. Using a multichannel pipette, transfer 20 µl Tagmentation Master Mix from the 8-tube strip to each well of the plate containing a sample. Use fresh tips for each sample column.
7. Discard the 8-tube strip after the Tagmentation Master Mix has been dispensed
8. Pipette each sample 10 times to resuspend.
9. Place on the preprogrammed thermal cycler and run the TAG program.

Post Tagmentation Clean Up

This step washes the adapter-tagged DNA on the BLT before PCR amplification.

Preparation

2. Prepare the following consumables:
- TSB-If precipitates are observed, heat at 37°C for 10 minutes, and then vortex until precipitates are dissolved.
- TWB-Use at room temperature. Vortex to mix. Pipette TWB slowly to minimize foaming.
3. Save the following PTC program on the thermal cycler:
- Choose the preheat lid option and set to 100°C

Temp (°C)	Time (minutes)
37	15
10	∞

Procedure

1. Add 10 µl TSB to the plate.
2. Slowly pipette each well 10 times to resuspend the beads and then seal.
3. Place on the preprogrammed thermal cycler and run the PTC program.
4. Place the plate on the magnetic stand and wait until liquid is clear (~3 minutes).
5. Using a multichannel pipette, remove and discard supernatant.
6. Wash beads as follows:
    - Remove the sample plate from the magnetic stand.
    - Use a deliberately slow pipetting technique to add 100 µl TWB directly onto the beads.
    - Pipette slowly until beads are fully resuspended.
    - Place the plate on the magnetic stand and wait until the liquid is clear (-3 minutes).
    - Using a multichannel pipette, remove and discard supernatant.
7. Repeat step 6 to wash beads the second time.
8. Remove the plate from the magnetic stand and use a deliberately slow pipetting technique to add 100 µl TWB directly onto the beads.
9. Pipette each well slowly to resuspend the beads.
10. Seal the plate and place on the magnetic stand until the liquid is clear (~3 minutes).
11. Keep on the magnetic stand until step 4 of the Procedure in Amplify Tagmented DNA The TWB remains in the wells to prevent over drying of the beads.

Amplify Tagmented DNA

This step amplifies the tagmented DNA using a limited-cycle PCR program. The PCR step adds Index 1 (i7) adapters, Index 2 (i5) adapters, and sequences required for sequencing cluster generation.

Preparation

1. Prepare the following consumables:
    - EPM-Invert to mix, then centrifuge briefly.
    - Index adapter plate
    - EPM should be kept on ice during use.
2. Centrifuge the Index adapter plate at 1000 × g for 1 minute to settle liquid away from the seal. Pipette slowly to minimize foaming.
3. Save the following BLT PCR program on a thermal cycler using the appropriate number of PCR cycles indicated in the table:

TOTAL DNA INPUT	NUMBER OF PCR CYCLES
1-9	12
10-24	8
25-49	6
50-99	5
100-500	5
Blood/Saliva	5

BLT PCR

Temp (C)	Time	Cycles
68	3 minutes	1
98	3 minutes	1

98	45 seconds	10
62	30 seconds	
68	2 minutes	
68	1 minutes	
10	∞	

Procedure

1. For each sample, combine the following volumes to prepare the PCR Master Mix. Multiply each volume by the number of samples being processed.
   - EPM (22 µl)
   - Nuclease-free water (22 µl)

Component	**Volume (µl)**	**No. samples**	**Total**
EPM	22		
Nuclease free water	22		

   Reagent overage is included in the volume to ensure accurate pipetting.
2. Vortex, and then centrifuge the PCR Master Mix at 280 x g for 10 seconds.
3. With the plate on the magnetic stand, use a 200 µl multichannel pipette to remove and discard supernatant.
   Foam that remains on the well walls does not adversely affect the library.
4. Remove from the magnetic stand.
5. Immediately add 40 µl PCR Master Mix directly onto the beads in each sample well.
6. Immediately pipette to mix until the beads are fully resuspended. Alternatively, seal the plate and use a plate shaker at 1600 rpm for 1 minute.

7. Seal the sample plate and centrifuge at 280x g for 3 seconds.
8. Add 10µl of the appropriate index adapters to each sample.
9. Using a pipette set to 40 µl, pipette 10 times to mix. Alternatively, seal the plate and use a plate shaker at 1600 rpm for 1 minute.
10. Seal the plate with Microseal 'B' and then centrifuge at 280x g for 30 seconds.
11. Place on the preprogrammed thermal cycler and run the BLT PCR program.

SAFE STOPPING POINT: If you are stopping, store at 2°C to 8°C for up to 30 days.

Clean Up Libraries

This step uses double-sided bead purification procedure to purify the amplified and indexed libraries.

Preparation

1. Vortex RSB to mix.
2. Allow IPB to reach room temperature and vortex before each use. Dispense it slowly due to its viscosity.
3. Prepare fresh 80% EtOH from absolute ethanol.

Procedure

1. Resuspend IPB as follows.
    - To mix, invert the bottle manually for 2 minutes, at a rate of 1 inversion per second. While inverting, rotate the bottle 90 degrees every 30 sec
    - If beads are still adhered to the walls of the container, invert the bottle manually for an additional 1 minute.
2. Add 40 µl nuclease-free water to each well of a MIDI plate labeled plate 1.
3. Add 45 µl IPB to each well to the same MIDI plate
4. Centrifuge the 96-well plate containing your amplified libraries at 280 g for 1 minute to collect contents at the bottom of the well.
5. Place the plate with the libraries on the magnetic stand and wait until the liquid is clear (5 minutes).
6. Transfer 45 µl supernatant from each well of the PCR plate to the corresponding well of a the MIDI plate with the IPB and nuclease-free water.
7. Pipette each well 10 times to mix.
8. Seal the plate and incubate at room temperature for 5 minutes.
9. Place on the magnetic stand and wait until the liquid is clear (-5 minutes).
10. During incubation, thoroughly vortex the IPB (undiluted stock tube), and then add 15 µl to each well of a new MIDI plate.
11. Transfer 125 ul supernatant from each well of the first plate into the corresponding well of the new MIDI plate containing 15 ul undiluted IPB.
12. Pipette each well in the MIDI plate 10 times to mix.

13. Discard the first plate.
14. Pipette each well 10 times to mix.
15. Incubate the sealed MIDI plate at room temperature for 5 minutes.
16. Place on the magnetic stand and wait until the liquid is clear (-5 minutes).
17. Without disturbing the beads, remove and discard supernatant.
18. Wash beads; with the plate on the magnetic stand, add 200 µl fresh 80% EtOH without mixing and incubate for 30 seconds.
19. Without disturbing the beads, remove and discard supernatant,
20. Repeat step 18 to wash beads a second time with the 80% ethanol.
21. Use a 20 ul pipette to remove and discard residual EtOH
22. Air-dry on the magnetic stand for 5 minutes. Proceed with the next steps if you see cracks in the beads. These may not be seen easily in a MIDI plate but can be observed in lower plates.
23. Remove from the magnetic stand.
24. Add 32 µl RSB to the beads and pipette to resuspend.
25. Incubate at room temperature for 2 minutes.
26. Place the plate on the magnetic stand and wait until the liquid is clear (-2 minutes.
27. Transfer 30 µl supernatant to a new 96-well PCR plate.

SAFE STOPPING POINT

If you are stopping, seal the plate with Microseal 'B' adhesive seal or Microseal 'F' foil seal, and store at -25°C to -15°C for up to 30 days.

Dilute Libraries to the Starting Concentration

This step dilutes libraries to the starting concentration for your sequencing system and it involves the dilution of samples to the final loading concentration.

1. Calculate the molarity value of the library or pooled libraries using the following formula.
   • For libraries qualified on a Bioanalyzer, use the average size obtained for the library.
   • For all other qualification methods, use 600 bp as the average library size.
   (Concentration in ng/ul)/ 660 g/mol x average library size x10^6 = Molarity (nM)

2. Using the molarity value, calculate the volumes of nuclease-free water and library needed to dilute libraries to the starting concentration for your system.

Sequencing system	**Starting concentration (nM)**	**Final loading concentration pM)**
HiSeq 2500 and HiSeq 2000 (high output modes)	2	12
HiSeq 2500 (rapid run mode)	2	8.5
HiSeq X, HiSeq 4000, and HiSeq 3000	2-3	200-300
iSeq 100 (v1 reagents)	2	200
iSeq 100 (v2 reagents)	2	100
MiniSeq	2	1.2-1.3
MiniSeq (v2and v3 reagents)	4	12
MiSeq i100*	0.8	80
NextSeq 550 and NextSeq 500	2	1.2-1.3
NextSeq 2000	2	750
NovaSeq 6000	Refer to the NovaSeq 6000 documentation on the Illumina support site	Refer to the NovaSeq 6000 documentation on the Illumina support site
NovaSeq 6000	2	12

3. Dilute libraries using nuclease-free water
   - Libraries quantified as a multiplexed library pool- Dilute the pool to the starting concentration for your system
   - Libraries quantified individually- Dilute each library to the starting concentration for your system.
4. Add a maximum of 5ul of each diluted library to a tube to create a multiplexed library pool in a 1.7ml microcentrifuge tube.

Sequencer Loading and Operation

The following instructions detail how to prepare your libraries for sequencing, loading onto the sequencer, and performing a sequencing run on an Illumina MiSeq. For other sequencers please refer to the product documentation from Illumina.

Preparation

1. MiSeq reagents need to be thawed before sequencing. Remove the reagent kit from the -20°C freezer and thaw it in a water bath using room temperature water for approximately 30 minutes. If you do not have a water bath, an ice box or other water-tight container with room temperature water works just as well. Ensure that the water level does not exceed the "Fill Line" indicated on the cartridge. Once thawed, place the reagents cartridge on ice or in a refrigerator until use. Use the cartridge within 24 hours of thawing.
2. Remove the hybridization buffer (HT1 buffer) from the reagent box before discarding and thaw on the bench. Place the buffer on ice after thawing.
3. Prepare a fresh 0.2M (0.2N) NaOH solution that will be used to denature the DNA library pool.
4. Power cycle the MiSeq. From the Home screen, select Manage Instrument then Shut Down. Toggle the power switch to the OFF positions and wait at least 60 seconds. Toggle the power switch back to the ON position, and wait for the machine to boot up.
5. Confirm that a sample sheet has been created for the pool.
6. Optionally, wash the MiSeq prior to use. Open the MiSeq Control Software and select the Wash Instrument icon. Then select Post-Run Wash. The machine will prompt you to fill the MiSeq wash tray with the MiSeq Wash Solution (0.5% Tween Solution).

Procedure

1. Dilute the library pool to 4 nM with Elution Buffer using the (C1)*(V1)=(C2)*(V2) formula.
2. Aliquot 5 µL of the 4 nM dilution into a new 1.5 mL tube.
3. Add 5 µL of 0.2 N NaOH to the 5 µL of diluted library pool. Vortex, spin, and let stand at room temperature for 5 minutes to denature the DNA in the sample. Denatured library pool concentration is now at 2 nM.
4. Add 990 µL of Hybridization Buffer to the 2 nM denatured library pool. Mix by inversion and spin. DNA in the pooled library is now single-stranded and at concentration of 20 pM.
5. Dilute to the final loading concentration of 10 pM (adjust as needed) as follows:

Component	**Volume**
Hybridization buffer	300 uL
Library at 20 pM	300 uL
Total Volume	600 uL

6. Invert the MiSeq reagent cartridge and visually verify that all reagent wells have completely thawed.
7. Tap the cartridge on the counter to settle all reagents to the bottom of the kit.
8. Using a 1 mL pipette tip, pierce the foil for position 17 (surrounded by an orange circle). Discard the pipette tip after use.
9. Add 600 µL of the 10 pM library pool to the hole created in the previous step. Avoid touching the pierced foil with the tip of the pipette, and avoid dispensing onto the walls of the well.
10. Remove the flow cell from the 4°C refrigerator and carefully take it out of the container.
11. Over a sink or paper towels, rinse the flow cell thoroughly with laboratory-grade water
12. Blot the flow cell dry with lint-free tissue wipes verifying that the flow cell is completely dry and free of smudges or blemishes. Avoiding touching the two black port holes at the top of the flow cell.
13. Wipe flow cell glass with alcohol wipe. Then blot dry with lint-free tissue wipes verifying that the flow cell is completely dry and free of smudges or blemishes.
14. Immediately proceed with loading the MiSeq. Do not let the kit sit for long periods with the sample inside.
15. Transport the loaded MiSeq cartridge, flow cell, and PR2 buffer to the MiSeq.
16. On the home screen, select *Sequence*.
17. The MiSeq will prompt for the flow cell to be loaded.
    a. Open the flow cell holder door and press the white button to release the used flow cell that is being held in place.
    b. Place the new, cleaned flow cell into the flow cell holder. The black gaskets will be facing upwards and the notch on the flow cell should be on the right-hand side.
    c. Gently push the flow cell to the back of its slot while you close the flow cell holder and the flow cell holder door.
    d. Select *Next* once the flow cell RFID is identified.
18. The MiSeq will now prompt that the reagents be put into place and a sample sheet be selected.
    a. Open the large chiller door and the small reagent chiller door.
    b. Lift the PR2 sipper and remove the bottle of wash buffer.
    c. Empty the MiSeq waste container. **Note**: MiSeq waste contains trace amounts of formamide and should be disposed of properly using your institution's hazardous waste procedure though usually it's safe to dispose of in the sink.
    d. Uncap the new bottle of PR2 buffer and place it into the MiSeq. **Note**: Place the cap from the new bottle of buffer onto the wash bottle to keep it clean between washes.
    e. Lower the PR2 sipper.
    f. Remove the wash cartridge from the small reagent chiller and replace it with the loaded MiSeq reagent cartridge. **Note**: Dump the remaining wash solution in the sink and let the wash cartridge dry before reusing.

    g. Close the small reagent chiller and the larger chiller door.
19. Select the Sample Sheet from the location that it was saved in.
20. Select *Load Sample Sheet* and confirm that the run and cycle parameters match what is desired.
21. Select *Start Flow Check* to perform the pre-run fluidics check. This will take approximately five minutes.
22. Select *Start* when the flow check has completed.
    a. If the flow check fails, remove the flow cell and clean it again.
    b. If the flow check fails a second time, perform a volume test.
23. A MiSeq 2 x 150 V2 run will take approximately 24 hours to complete. Important run statistics will be available after approximately 1 hour. Clusters passing filter should be less than 1000 k/mm 2 for V2 chemistry. If higher, consider stopping the run and starting another run with the loading library concentration decreased by 2 pM.
24. After completion, perform a post-wash run.

Genome Assembly

After sequencing, raw genomic data can be assembled into full *V. cholerae* genomes using several publicly available bioinformatics tools. The instructions below describe our recommended procedure for sequencing data generated on the Illumina platform, which leverages the *bacpage* bioinformatics pipeline for reference-based assembly.

The instructions below assume *bacpage* will be run on the command line, though *bacpage* is also available on Dockstore for use on the Terra cloud compute platform.

Preparation

1. Install the *bacpage* bioinformatics pipeline on the computer to be used for analysis. Detailed installation instructions can be found on the *bacpage* documentation site: https://cholgen.github.io/. Source code and resources for *bacpage* are available at: https://github.com/CholGen/bacpage. *bacpage* is also available on Dockstore for use on the Terra cloud compute platform.
2. Set up a project directory to ensure all sequencing data and analysis results are in the same place. To do so, create an empty project directory in your home folder (or another folder dedicated for sequencing analyses) using the `bacpage setup` command:

   ```
   bacpage setup [project-directory-name]
   ```

   Of note, we recommend that you replace `[project-directory-name]` with an informative name, such as `[date]_[sequencing-run-name]` (e.g., `20220609_cholera_run1`). Detailed instructions for setting up a project directory are also available on the *bacpage* documentation site here.

3. The project directory should have been created and will contain a single file, `config.yaml`, and a directory, `input/`.

Procedure

1. Locate FASTQ files containing raw reads from sequencer. Demultiplexed FASTQ files (two FASTQ files per input sample) will be automatically generated for all sequencing runs initiated by Local Run Manager directly on the machine or uploaded to BaseSpace Sequence Hub. On the machine, FASTQ files, labeled as `{SampleName}_S{0-99}_L001_R{1,2}_001.fastq.gz`, can be found in `Data\Intensities\BaseCalls` directory within the run folder.
   a. If you did not set up your run with as described above or need to manually demultiplex raw base calls into individual sample FASTQs, please follow the online instructions for using the `bcl2fastq` conversation software, available here.
2. Once FASTQ files have been located, transfer the files to a computer to perform the analysis. This can be done using a flash drive, or by downloading from BaseSpace Sequence Hub.

3. Move your FASTQ files into the `input/` directory created during the `bacpage setup` step above.
4. From the project directory, run `bacpage assemble` to perform reference-based assembly. Please refer to the documentation for *bacpage* for detailed usage instructions, which are modified periodically as updates are made to the *bacpage* code base (e.g., when adding new features): https://cholgen.github.io/.

Lineage Classification

V. cholerae genomic data can be rapidly classified into canonical lineages (AFR1-AFR17) using *vibecheck*. *Vibecheck* can be run on assembled genomes, after completing the procedure above, or on raw sequencing reads, after locating and transferring files to a computer for analysis. *Vibecheck* is also available on Dockstore for use on the Terra cloud compute platform.

Preparation

1. Install the *vibecheck* bioinformatics pipeline on the computer to be used for analysis. Detailed installation instructions can be found on the *vibecheck* documentation site: https://github.com/CholGen/vibecheck.

Procedure

1. Run *vibecheck* with the following command:

   ```
   vibecheck [query ...]
   ```

   Where `[query ...]` is the name of your input FASTA file or a pair of FASTQ files. A query FASTA file can contain as many sequences as you would like to be classified, while paired FASTQ files should only contain the data for one sample.
2. Inspect the output. *Vibecheck* will generate a CSV file (`lineage_report.csv`) containing the estimated lineage(s) for each sequence in the query file.

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