1. **PURPOSE:** This procedure describes a standardized laboratory protocol for DNA library preparation of enteric bacterial organisms using the Illumina® DNA Prep kit (formerly known as Nextera DNA Flex), for subsequent sequencing on an Illumina platform, thus ensuring inter-laboratory comparability of sequencing results and optimizing sequencing data output and quality.

2. **SCOPE:** For use by PulseNet WGS certified laboratorians when preparing libraries from DNA from enteric organisms using the Illumina® DNA Prep kit for sequencing on Illumina platforms for submission of sequencing data to PulseNet. Laboratories may amend this procedure as necessary for use within their laboratories after validation per their laboratory's guidelines.
   NOTE1: *Instructions for combining Norovirus and Cyclospora amplicons in the same run with the PulseNet organisms are outlined in the appendices PNL35-1 and PNL35-2, respectively.*
   NOTE2: *This SOP deviates from the protocol provided by Illumina because the original Illumina protocol was optimized for 2 x 150 (300c) sequencing. PulseNet's protocol has been optimized to allow for 2 x 250 (500c) sequencing. If your laboratory follows the original Illumina SOP, the workbooks provided with the PulseNet SOP will not generate accurate calculations.*

3. **DEFINITIONS:**
   3.1. **BLT:** Bead-Linked Transposome
   3.2. **CD Index:** Combinatorial Dual Index
   3.3. **DNA:** Deoxyribonucleic Acid
   3.4. **dsDNA:** Double-Stranded DNA
   3.5. **EPM:** Enhanced PCR Mix
   3.6. **GHS:** Globally Harmonized System
   3.7. **HS:** High Sensitivity
   3.8. **IEM:** Illumina Experiment Manager
   3.9. **IPB:** Illumina Purification Beads
   3.10. **LRM:** Local Run Manager
   3.11. **Mb:** Megabase
   3.12. **Ng:** Nanogram
   3.13. **nM:** Nanomolar
   3.14. **PCR:** Polymerase Chain Reaction
   3.15. **PHL:** Public Health Laboratory
   3.16. **PN:** PulseNet
   3.17. **PPE:** Personal Protective Equipment
   3.18. **PulseNet Central:** PulseNet team at CDC comprising of the Outbreak Detection and Surveillance Unit and the Next Generation Subtyping Methods Unit
   3.19. **PulseNet/OutbreakNet SharePoint**: A closed, web-based collaboration application used for communication among PulseNet participants.
   3.20. **QC:** Quality Control
   3.21. **RSB:** Resuspension Buffer
   3.22. **SDS:** Safety Data Sheet
   3.23. **SOP:** Standard Operating Procedure
   3.24. **SPB:** Sample Purification Beads
   3.25. **TB1:** Tagmentation Buffer 1
   3.26. **TSB:** Tagment Stop Buffer
   3.27. **TWB:** Tagment Wash Buffer
   3.28. **UD Index:** Unique Dual Index
   3.29. **WGS:** Whole Genome Sequencing

4. RESPONSIBILITIES:

4.1. **PulseNet Public Health Laboratory:**

4.1.1. Prepare DNA libraries, and QC as necessary, for subsequent WGS.

4.1.2. Re-sequence any isolates that do not meet quality thresholds.

4.1.3. Inform PulseNet Central, as necessary, about any complications with laboratory protocols or suspected reagent issues.

4.2. **PulseNet Central:**

4.2.1. Perform additional sequence quality analysis in order to provide feedback and troubleshooting support for PHLs as necessary.

4.2.2. Notify PN PHL if any sequences submitted do not meet quality thresholds.

4.2.3. Communicate any suspected reagent issues to PHLs as necessary.

4.2.4. Maintain and review SOPs on a regular basis and post on SharePoint.

5. SAFETY:

5.1. **Biosafety Warning:** This document describes handling of DNA and associated products and does not describe best practices for handling of biological infectious material.

5.2. **Chemical Safety Warning:** Take proper precautions and wear appropriate PPE when handling potentially hazardous chemicals. Ensure that chemicals, spent containers, and unused contents are disposed of in accordance with local and governmental safety standards. **See all relevant SDSs for additional information.**

5.2.1. Illumina® DNA Prep Kit:

5.2.1.1. TSB: GHS Category 1 for eye damage/irritant and is harmful to aquatic life.

5.2.1.2. TB1: GHS Category 4 for acute toxicity (dust/mist), Category 2A for eye irritant and Category 1B for reproductive toxicity. Contains N, N=Dimethylformamide.

5.2.1.3. EPM: GHS Category 4 for acute oral toxicity and Category 1 for specific organ toxicity. Contains tetramethylammonium chloride.

5.2.2. Ethanol is flammable (GHS Flammability Category 2).

6. REAGENTS:

6.1. Illumina® DNA Prep (M) Tagmentation Kit (any of the following):

6.1.1. 96 samples, with SPB, Illumina Cat# 20018705
NOTE: *This kit will be obsolesced: the last order date December 31, 2022.*

6.1.2. 24 samples, with SPB, Illumina Cat# 20018704
NOTE: *This kit will be obsolesced: the last order date December 31, 2022.*

6.1.3. 96 samples, with IPB, Illumina Cat# 20060059

6.1.4. 24 samples, with IPB, Illumina Cat# 20060060

Kit components:

- Beads + Buffers. Store at room temperature – **NOTE:** *One component in the box may need to be refrigerated.*
  - SPB **(store upright at 2-8°C, do not freeze)** or IPB **(store upright)**
  - TSB
  - TWB
- PCR + Buffers. Store at -25 to -15°C.
  - RSB
  - TB1
  - EPM

- Tagmentation (M) Beads. Store at 2-8°C.
  BLT (store upright)

6.2. Indexes: choose either 6.2.1. or 6.2.2.
NOTE1: *The index kit(s) needed depends on the sample throughput of the laboratory and the level of multiplexing desired. It is recommended not to use the same index pair in two consequent runs on the same instrument to prevent carry-over from run to run.*
NOTE2: *The CD and UD indexes **cannot** be mixed on the same run.*

6.2.1. Nextera DNA CD Indexes. Store at -25°C to -15°C.

6.2.1.1. 24 Dual Index, tube format: H503, H505, H506, H517, H710, H705, H706, H707, H711, H714 (24 samples, Illumina Cat# 20018707)
NOTE: *This kit will be obsolesced: the last order date February 24. 2023; the last shipment date May 26, 2023.*

6.2.1.2. 96 Dual Index, plate format (96 samples, Illumina Cat# 20018708)

6.2.2. IDT for Illumina UD Dual Indexes. Store at -25°C to -15°C.
NOTE1: *These indexes have TEN base pair adapters; therefore, the number of index read cycles on the instrument must be adjusted to 10 (from 8 for CD indexes).*
NOTE2: *The Nextera DNA UD indexes set C or D cannot be combined on the same run with DNA/RNA UD Indexes Set A, B, C or D.*

6.2.2.1. DNA/RNA UD Indexes, set A, Tagmentation (96 indexes, 96 samples, Illumina Cat# 20027213)

6.2.2.2. DNA/RNA UD Indexes, set B, Tagmentation (96 indexes, 96 samples, Illumina Cat# 20027214)

6.2.2.3. DNA/RNA UD Indexes, set C, Tagmentation (96 indexes, 96 samples, Illumina Cat # 20042666)
NOTE: *This kit is replacing "IDT for Illumina – Nextera DNA Unique Dual Indexes Set C, Cat# 20027215 – last order date February 24, 2023*

6.2.2.4. DNA/RNA DNA UD Indexes, set D, Tagmentation (96 indexes, 96 samples, Illumina Cat # 20042667)
NOTE: *This kit is replacing "IDT for Illumina – Nextera DNA Unique Dual Indexes Set D, Cat# 20027216 – last order date February 24, 2023*

6.2.2.5. Nextera DNA UD Indexes, sets A-D (384 indexes, 384 samples, Illumina Cat # 20027217)
NOTE: *This kit will be obsolesced: the last order date February 24. 2023; the last shipment date May 26, 2023.*

6.3. Ethanol, molecular grade, 95-100% (Fisher Scientific Cat# BP2818-500 or equivalent)

6.4. Ethanol, lab-grade, 70% or equivalent for disinfection purposes (Fisher Scientific Cat# 04-355-305 or equivalent)

6.5. Water, Molecular grade (Fisher Scientific Cat# BP24701 or equivalent)

6.6. Invitrogen™ Qubit™ dsDNA HS Assay Kit: choose either 6.6.1. or 6.6.2.

6.6.1. Concentrated assay reagent kit (100 assays, Fisher Scientific Cat# Q32851 **OR** 500 assays, Fisher Scientific Cat# 32854) with the following components:
- dsDNA HS Reagent, Component A (room temperature, protect from light)
- dsDNA HS Buffer, Component B (room temperature)
- dsDNA HS Standard #1, Component C (≤ 4°C)
- dsDNA HS Standard #2, Component D (≤ 4°C)

6.6.2. 1x ready-to-use assay reagent kit (100 assays, Fisher Cat# Q33230 **OR** 500 assays Fisher Scientific Cat# Q33231) with the following components (2-8°C):
- dsDNA HS Working Solution, Component A (protect from light)
- dsDNA HS Standard #1, Component B

- dsDNA HS Standard #2, Component C

7. SUPPLIES:

7.1. 96 well PCR plates, skirted, hard shell low profile, thin-wall (BioRad Cat# HSP-9601 or equivalent)
7.2. Deepwell storage "MIDI" plates, 96 well (Fisher Scientific Cat# AB-0859 or equivalent) **– Optional**
7.3. Ice
7.4. Microcentrifuge tubes, 1.5 ml, sterile (Fisher Scientific Cat# 05-408-129 or equivalent)
7.5. Microseal A film (BioRad Cat# MSA-5001 or equivalent)
7.6. Microseal B adhesive seal (BioRad Cat# MSB-1001 or equivalent)
7.7. Microseal F adhesive foil seal (BioRad Cat# MSF-1001 or equivalent) **– Optional**
7.8. Pipette tips, sterile, filtered: 20 µl, 200 µl and 1000 µl volumes (Rainin Cat# 30389225, 30389239 & 30389212 or equivalent)
7.9. Qubit™ Assay Tubes (Fisher Scientific Cat# Q32856 or equivalent (clear, thin-wall 0.5-ml PCR tubes)).
7.10. Solution basins, sterile (Fisher Scientific Cat# 13-681-504 or equivalent)

8. EQUIPMENT:

8.1. Ice buckets/containers
8.2. Invitrogen™ DynaMag™-96 Side Skirted Magnet (Fisher Scientific Cat# 12-027 or equivalent)
8.3. Microcentrifuge for quick spins
8.4. Micropipettes, capable of volumes from 1 µl to 1000 µl. Single and multichannel (20 µl and 100µl volumes).
NOTE: *Two sets of pipettes are suggested, one for working with pre-amplified product and reagents and another set for working with post-PCR amplified product and reagents.*
8.5. Microplate centrifuge
8.6. Qubit™ 4.0 Fluorometer (older versions 2.0 or 3.0), or equivalent for quantification of dsDNA
8.7. Thermal cycler with heated lid, compatible with 96-well 0.2 ml PCR plate
8.8. Vortex

9. PROCEDURE: The Illumina DNA Prep checklist (PNL35.W3) may be used during library preparation in the laboratory as a record of what steps have been completed. This checklist will also calculate master mix volumes. A quick guide (PNL35.JA1) outlining the library preparation method is also available as a laboratory reference for the library preparation procedure. The DNA Prep workbook (PNL35.W1) may be used for planning sequencing runs (on the iSeq, MiniSeq or MiSeq), including index assignment and may also be printed and used in the laboratory to record reagent lots and other run-specific information. The Index tracking workbook (PNL35.W4) may be used for assignment of indexes and for inventory purposes.

NOTE1: *The only safe stopping points in the procedure are after the amplification step (9.4.), after the clean-up of the amplified libraries (9.5.) and after dilution to 4 nM (9.6.).*
NOTE2: *All multichannel mixing steps in this protocol may be replaced by the use of a plate shaker (1600 rpm for 1 minute) if desired.*
NOTE3: *Make sure the vortex is turned down once cells have been lysed during DNA extraction and for all steps during library prep.*
NOTE4: *It has been documented and observed that cluster density can be negatively affected by the use of ammonium-based cleaning products near sequencing equipment, including lab benches and pipets used for library preparation.* ***Do not use quaternary ammonium compounds or wipes near or on sequencing equipment!***

9.1. (Optional) Prepare the "Library Prep" Tab of the Illumina DNA Prep Workbook

NOTE1: *Use PNL35.W1 for indexes in a plate format or PNL35.W2 for 24 CD indexes in a tube format.*

NOTE2: *Do **NOT** delete rows or columns of the workbooks! There are many formulas and look-up tables that could be disturbed by doing so. There are numerous rows available for sample information; any rows which are not going to be used may be hidden by using the "Hide" feature in Excel. The workbook is designed with the following color scheme:*

- *White fields should be filled in*
- *Dark gray fields are optional*
- *Blue fields contain formulas, which will auto populate, and should not be altered*

9.1.1. Enter the Run ID using the format PulseNet lab ID-Instrument ID-run start date: labID-MXXXX-YYMMDD. (e.g., CDC-M3235-221004).

9.1.2. Enter the Sequencing Date, Sequencing Technician, select the Sequencing Kit Type/Chemistry from the drop-down box, Library Prep Date, and Library Prep Technician.

9.1.3. Enter the State Keys (the ID entered in the BioNumerics "Key" field).

NOTE: Important! *Fastq file names will be assigned on the Sample Sheet tab according to this State Key and the Run ID. These fields will be concatenated to create a unique prefix for the resulting fastq files (e.g., Sample1-CDC-M3235-221004)*

9.1.4. Enter the Genome Size Estimate (based on Table 1 below) for each organism in Column E of the workbook.

Organism	Estimated Genome Size (Mb)
***E. coli & Shigella* spp.**	**5**
***Salmonella* spp.**	**5**
***Vibrio* spp.**	**5**
Listeria monocytogenes	**3**
***Campylobacter* spp.**	**1.6**

Table 1. Estimated genome size (in Mb) by organism

9.1.5. Confirm that the number of isolates on the run is appropriate for the capacity of the instrument and reagent kit to be used. The recommended DNA load for each of the Illumina sequencing platforms and cartridges validated for use by PulseNet can be found in Table 2 below. Individual laboratories with experience and consistent library concentrations may be able to exceed the recommended DNA load while still reaching the required minimum coverage levels for all isolates on the run. The sum of the genome sizes (in Mb) for the samples on the run will be displayed on the workbook and is equal to the estimated DNA load of the run.

NOTE1: *The lower recommended DNA load for runs containing E. coli, Shigella* spp*., or Vibrio* spp*. is due to the fact that these organisms require a higher coverage (40x) for downstream analyses compared to the other organisms (20x or 30x). Therefore, more sequence read/data generated in the sequencing run is required for these organisms to meet coverage requirements*.

NOTE2: ***PulseNet never accepts, under any circumstances, sequences generated with cycling parameters below 300 cycles (2 x 150).***

Sequencing Kit (cycles)	DNA Load (Mb) Runs without *Vibrio*, *Escherichia* or *Shigella*	DNA Load (Mb) Runs containing *Vibrio*, *Escherichia* or *Shigella*
MiSeq		
v2 300	**90**	**90**
v2 500	**100**	**100**
v3 600	**200**	**175**
Micro (300)	**35**	**30**
Nano (500)	**13**	**13**
Nano (300)	**13**	**10**
MiniSeq		
Mid Output	**60**	**60**
High Output	**100**	**100**
iSeq		
iSeq v2	**35**	**25**

Table 2. Estimated DNA load capacity (in Mb) for Illumina reagent kits.

9.1.6. Determine which indexes will be used and enter the index plate well position (from the index plate). The index plate well may be entered manually, selected from the drop-down option or copied over from the index tracking worksheet.
NOTE1: *It is recommended not to use the same index pairs within two consecutive runs on the same sequencer to reduce the amount of carryover. The index tracking worksheets, PNL35.W4 may be used to reserve/keep track of used and available indexes for plated Nextera CD Indexes/IDT for Illumina UD Indexes.*
NOTE2*: CD and UD indexes cannot be combined on the same run. It is also not recommended to combine the old Nextera DNA UD indexes from sets C or D with the DNA/RNA UD Indexes Sets A, B, C or D.*

9.1.7. Enter the volume of input DNA (recommended 10 µl) to be added to each well at the beginning of the library preparation process.
NOTE1: *PulseNet has standardized the starting <u>volume</u> of DNA to 10 µl; however, the recommended <u>quantity</u> of input DNA per Illumina is 100-500 ng. Individual laboratories may adjust input DNA volumes to ensure DNA falls within this range if desired.*
NOTE2: *The minimum volume of input DNA is 2 µl. If DNA is too concentrated, perform a dilution to bring input DNA volume above 2 µl and proceed.*

9.1.8. The DNA Prep tab of the workbook may now be printed for use in the laboratory.

9.2. <u>Dilute and Tagment Input DNA</u>: DNA is fragmented and tagged with the adapter sequences and bound to the BLT during these steps. It is important that beads are well suspended at ALL steps in the procedure.
NOTE: *Ensure that DNA going into library preparation has been assessed for quality. The 260/280 value should be between 1.75 and 2.05. See PNL33 for more information.*

9.2.1. Allow BLT (from the refrigerator) and TB1 (from freezer) to come to room temperature.
NOTE: *Ensure that BLT is never frozen and is stored upright at all times so that the beads always remain submerged in the buffer.*

9.2.2. Label a 96-well PCR plate, or equivalent, with Run ID.

9.2.3. Add molecular grade water to each sample well (Total assay input volume is 30 µl; water volume is

20 µl if 10 µl of DNA will be added; volume is automatically calculated and displayed on the Workbook).

9.2.4. Add DNA to the molecular grade water (per volume entered in the Workbook, typically 10 µl) and mix well by gently pipetting approximately 5-10 times.

9.2.5. Vortex BLT for a **minimum** of 10 seconds and verify proper suspension of beads; repeat if necessary. Do not centrifuge.

9.2.6. Vortex TB1 to mix and perform a quick spin.

9.2.7. Prepare tagmentation master mix:

Reagent	Volume per Sample
TB1	10 µl
BLT	10 µl

Table 3: Reagent volumes per sample for tagmentation master mix

NOTE1: *It is recommended to increase the number of samples by 2-3 to ensure sufficient master mix volume (3 is recommended if preparing a higher number of samples).*

NOTE2: *The number of samples being prepared may be entered into the Illumina DNA Prep checklist, PNL35.W3 ("checklist") and master mix volumes will be auto calculated.*

9.2.8. Vortex the tagmentation master mix well.

9.2.9. Add 20µl of tagmentation master mix to each sample well.

NOTE: *If many samples (i.e., greater than 8) are being prepped at once, it may be necessary to re-vortex the tagmentation master mix occasionally to ensure that it does not settle during this step.*

9.2.10. Pipet gently to mix well, resuspending the beads (using a multichannel pipette for mixing is recommended). Inspect the wells to ensure that samples and master mix are homogeneous before incubation. See Figure 1 below for additional information.

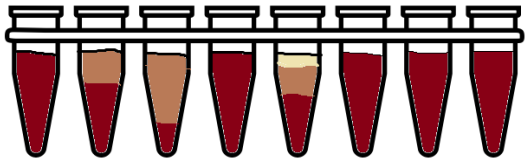


Figure 1. Example of homogeneous and non-homogeneous sample wells. Wells 1, 4, 6, 7 and 8 are well mixed. There should be no color striations ("sunsets") from the bottom to the top of the wells. Wells 2, 3 and 5 display this settling and should be re-mixed prior to incubation

9.2.11. Seal the plate with Microseal A (or equivalent) and incubate the plate at 55°C for 15 minutes, followed by a 10°C hold on a thermal cycler with the lid heated at 100°C (volume is 50 µl).

NOTE: *It is recommended to pre-program a thermal cycler for this purpose.*

9.3. <u>Clean Up Tagmented DNA</u>: DNA (now tagged with adapters and bound to the BLT) will be washed prior to amplification.

NOTE: *A deep-well MIDI plate may be used for the washing steps below if desired. This may improve formation of the pellet and decrease cross-contamination risks. The samples will need to be transferred to a 96-well skirted PCR plate for thermal cycler steps.*

9.3.1. Ensure that TSB is at room temperature and check that no precipitate is present (if it is, warm at 37°C for up to 10 minutes or until dissolved and vortex).

9.3.2. Add 10 µl of TSB to each sample.

9.3.3. Pipet gently to mix. Inspect the wells to ensure that samples and master mix are homogeneous prior to incubation. Refer to Figure 1.

9.3.4. Seal the plate with Microseal A or equivalent.

9.3.5. Incubate at 37°C for 15 minutes and hold at 10°C on a thermal cycler with a lid pre-heated to 100°C.
NOTE: *It is recommended to pre-program a thermal cycler for this purpose.*
9.3.6. While samples are incubating, thaw EPM on ice and thaw indexes at room temperature.
9.3.7. After samples reach 10ºC, remove the plate from the thermal cycler, and perform a quick spin.
9.3.8. Place on a magnet for 3 minutes (or until beads form a tight pellet).
NOTE: *If a tight pellet is not formed after 3 minutes, gently pipet the samples that haven't formed a pellet and allow to sit on the magnet for up to 3 minutes.*
9.3.9. Remove and discard supernatant.
9.3.10. Remove the plate from the magnet and add 100 µl TWB directly to the pellet.
NOTE*: A solution basin and multichannel pipet may be used for TWB washes if desired.*
9.3.11. Pipet gently (to avoid foaming) to mix until beads are fully resuspended.
NOTE: *Pipet with care. Some foaming may occur, but excessive foaming of TWB should be avoided, as foaming can lead to sample loss.*
9.3.12. Place back on the magnet for 3 minutes (or until beads form a tight pellet).
9.3.13. Remove and discard the supernatant.
9.3.14. Remove from the magnet and gently add 100 µl of TWB directly to the pellet.
9.3.15. Pipet gently to mix (avoiding foaming) until beads are fully resuspended.
9.3.16. Place back on the magnet for 3 minutes (or until beads form a tight pellet).
9.3.17. Remove and discard supernatant.
9.3.18. Remove from the magnet, add 100µl of TWB directly to the beads and gently pipet to resuspend.
9.3.19. Place back on the magnet for 3 minutes, allowing TWB to remain in the wells (to prevent drying of beads) while proceeding to amplification steps.

9.4. <u>Amplify Tagmented DNA:</u> The indexes are added to the tagmented DNA, and amplification occurs. DNA will be released from the BLT beads during PCR.

9.4.1. Vortex and quick spin the thawed EPM briefly prior to use.
9.4.2. Prepare PCR master mix:

Reagent	Volume per Sample
EPM	20 µl
Molecular grade water	20 µl

Table 4: Reagent volumes per sample for PCR master mix.

NOTE1: *It is recommended to increase the number of samples for master mix calculation by 2-3 to ensure sufficient master mix volume (3 is recommended if preparing a higher number of samples).*
NOTE2: *The number of samples being prepared may be entered into the Illumina DNA Prep checklist and master mix volumes will be auto calculated.*
9.4.3. Vortex and quick spin the PCR master mix.
9.4.4. Remove TWB from wells.
NOTE: *Removal of TWB is crucial, as it can impede PCR. Using a small volume pipette to remove all residual TWB is recommended. Any foam remaining on the wells will not negatively impact the library.*
9.4.5. Remove from the magnet and immediately add 40 µl of PCR master mix to each sample.
NOTE: *It is recommended to ensure that each pellet is submerged or wetted by the master mix prior to moving on to the next well and to add master mix to all wells prior to mixing in order to avoid beads drying out.*
9.4.6. Gently pipet to mix well, ensuring resuspension of the pellet.

9.4.7. Add indexes to samples:

9.4.7.1. If using an index plate, each well contains a unique index pair in a single-use volume. Add 10 µl of appropriate index pair from the index plate well to each sample well as indicated by the workbook (PNL35.W1). After wells have been used/pierced they **must be sealed** using lab tape or Microseal F or equivalent to prevent index cross-contamination.
NOTE: *It is recommended to pierce the foil of the desired well on the index plate with a new pipette tip, then to use a fresh pipette tip to remove the indexes from the wells.*

9.4.7.2. If using the Nextera DNA CD Indexes 24 Index Kit (index tubes), pair i7 and i5 indexes to create unique index pairs for each sample within a run. Add 5 µl of the i7 index and 5 µl of the i5 index to the appropriate sample well as indicated by the workbook (PNL35.W2).

9.4.8. Pipet a minimum of 10 times to mix.

9.4.9. Inspect the wells to ensure that all samples are homogeneous. This step is crucial for effective indexing and subsequent product recovery. Refer to Figure 1.

9.4.10. Seal the plate with Microseal A or equivalent, place onto thermal cycler, and run the following pre-programmed settings with a heated lid (100°C):

Step 1: 68°C for 3 minutes
Step 2: 98°C for 3 minutes
Step 3: 5 cycles
98°C for 45 seconds
62°C for 30 seconds
68°C for 2 minutes
Step 4: 68°C for 1-minute
Step 5: Hold at 10°C
Total volume: 50 µl

9.4.11. Centrifuge plate at 280 x g for 1 minute.
NOTE: *This is a safe stopping point. The plate may be sealed with Microseal B or equivalent and stored at 2-8°C or left on the thermal cycler at 10°C for up to 3 days.*

9.5. Clean up and Size Select Amplified Libraries: This dual bead clean-up procedure is for purification and size selection of libraries. Target fragment sizes are 800-1000 bp.
NOTE1: *The steps listed below are critical for efficient size selection, product recovery, and thus cluster generation and sequencing. Always check pipette tips for correct volumes and ensure that no beads have accidentally been aspirated on steps where supernatant is being removed. If beads have accidentally been aspirated or the bead pellet has been disturbed, allow the pellet to re-form and repeat the step. It is also important to ensure that beads are well suspended at all "pipet to mix steps" as described below.*
NOTE2: *The target fragment size of 800-1000 bp has been optimized for the PulseNet protocol (instead of the 500 bp fragment size targeted by the original Illumina DNA Prep procedure) in order to better facilitate the use of 500 cycle sequencing chemistry.*

9.5.1. Prepare reagents:

9.5.1.1. If using SPB, bring it to room temperature (at least 30 minutes) from refrigerator.

9.5.1.2. Bring RSB to room temperature (from frozen) and vortex to mix.
NOTE: *RSB is in a 50 ml conical. In order to facilitate faster thawing, it is recommended to aliquot and freeze back smaller volumes after opening for the first time.*

9.5.1.3. Dilute enough fresh 80% ethanol for all samples:

Reagent	Volume per sample	Example: 20 samples
100% ethanol	0.4 ml	8 ml
Molecular grade water	0.1 ml	2 ml

Table 5: Reagent volumes per sample for 80% ethanol.

9.5.1.4. Vortex and invert SPB/IPB several times to fully resuspend the beads.

9.5.1.5. Prepare **SPB/IPB master mix**:

Reagent	Volume per Sample
SPB or IPB	40.8 µl
Molecular grade water	44.2 µl

Table 6: Reagent volumes per sample for SPB/IPB master mix

NOTE1: *It is recommended to increase the number of samples by 3-4 to ensure sufficient volume of master mix.*

NOTE2: *The number of samples being prepared may be entered into the DNA Prep checklist and master mix volumes will be auto calculated.*

9.5.2. If plate was retrieved from cold storage, centrifuge plate at 280 x g for 1 minute.

9.5.3. Place plate on the magnet for 5 minutes (or until beads have formed a tight pellet).
NOTE: *If any wells do not have tight pellets after 5 minutes, gently resuspend them and allow time for tight pellet to form.*

9.5.4. Transfer 45 µl of supernatant (now containing the DNA) to new wells.
NOTE1: *A MIDI plate may be used for steps 9.5.5. through 9.5.24. if desired. This may improve pelleting of the beads and reduce the tendency of the beads to be pulled off of the magnet during removal of the final product during final elution step.*
NOTE2: *If sample loss has occurred during tagmentation and PCR, it is important to maintain the appropriate ratio of SPB/IPB master mix to DNA (1.88x) for recovery of appropriate library lengths. Measure and transfer as much of the resulting supernatant as possible and add the corresponding volume of SPB/IPB master mix from the Table 7 below. Do not adjust subsequent volume of stock SPB/IPB later in the protocol, as this will negatively affect product recovery yield.*

Volume of DNA recovered after tagmentation (µl)	Volume of SPB/IPB Master mix to be added (µl)
40	75.2
35	65.8
30	56.4
25	47
20	37.6
15	28.2

Table 7. Adjusted SPB/IPB master mix volumes in the case of sample loss.

9.5.5. Remove sample plate from the magnet.

9.5.6. Vortex SPB/IPB master mix thoroughly and add 85µl to each sample (or appropriate volume from Table 7 above, if applicable).

9.5.7. Pipet to mix a minimum of 10 times; using a multichannel pipet is recommended.
NOTE*: Use caution when mixing as the volume will be > 100 µl.*

9.5.8. Incubate at room temperature for 5 minutes.

9.5.9. Place on the magnet for 3-5 minutes (or until beads form a tight pellet).

9.5.10. During incubation, vortex the stock SPB/IPB.
9.5.11. After incubation, with the plate still on the magnet, transfer 125 µl of supernatant (containing DNA) to new wells (longest DNA fragments are being left behind).
NOTE: *If volumes of SPB/IPB were changed due to product loss, the volume of supernatant transferred to new wells will be lower. Transfer as much of the supernatant as possible.*
9.5.12. Remove the plate from the magnet and add 15 µl of well-suspended **stock SPB/IPB** to the supernatant (undiluted beads to DNA ratio 0.12).
NOTE: *Be sure to add from the stock tube of undiluted SPB/IPB, NOT the previously made SPB/IPB master mix. Accidentally using the SPB/IPB master mix will dramatically decrease yield.*
9.5.13. Gently pipet a minimum of 10 times to mix.
9.5.14. Incubate at room temperature for 5 minutes.
9.5.15. Place on magnet for 3-5 minutes (or until beads form a tight pellet and supernatant clears).
9.5.16. Remove and discard supernatant (desired libraries are bound to the beads; shortest libraries in the supernatant are being discarded).
9.5.17. Perform the steps below (9.5.17.1. through 9.5.17.3.) twice, for a total of two washes:
9.5.17.1. While the plate is on the magnet, add 170 µl of fresh 80% ethanol.
NOTE1*: Do not add directly to the bead, do not mix, and do not remove from the magnet during the wash steps.*
NOTE2: *A solution basin may be used for the ethanol if desired.*
9.5.17.2. Incubate for 30 seconds.
9.5.17.3. Remove and discard supernatant.
9.5.18. After the second wash, use a small volume pipette to remove excess ethanol, if necessary.
9.5.19. Allow beads to air dry for 3-5 minutes.
NOTE: *Do not allow beads to over-dry or crack. It is advisable to err on the shorter side of drying time. If cracking is observed, immediately resuspend beads as described below regardless of drying time.*
9.5.20. Remove from magnet and add 32 µl of RSB. It is recommended to add RSB quickly to each sample pellet, then go back and mix with a multichannel pipet after all pellets have been wetted.
9.5.21. Pipet gently and thoroughly to mix.
9.5.22. Incubate at room temperature for 2-5 minutes.
NOTE: *Longer incubation (5-10 minutes) is preferred for optimal yield and recovery of longer libraries. If yield is low (less than 10 ng/µl), RSB incubation time may be increased to 8 minutes.*
9.5.23. Place on magnet for 3 minutes (or until supernatant is clear).
9.5.24. Transfer 30 µl of the supernatant into new wells (or into wells on a new plate) – this is the final product.
NOTE1: *If a skirted PCR plate is used for this step, the pellet may slide away from the magnet. To prevent aspirating beads, decreasing the recovery volume to 25 µl is acceptable.*
NOTE2: *A quantification step (Qubit dsHS kit or equivalent) may be performed at this point on the individual libraries and results may be recorded in the Workbook if desired. See PNL33 for specific instructions on operating the Qubit. Checking post-clean up library concentrations is recommended for new users of the Illumina DNA Prep kit and for troubleshooting purposes. Ideal library yield is >10 ng/µl. Sequencing libraries successfully with yields below 10 ng/µl is possible but indicates inefficient library preparation.*
NOTE3: *This is a safe stopping point. The plate may be sealed with Microseal B or equivalent and stored at -20°C for up to 30 days per Illumina recommendation. Longer term storage is possible but repeated freeze-thaw cycles of the libraries will result in loss of yield.*

9.6. Pool Libraries

9.6.1. Pool 5 µl of each library into a new well, with the exceptions noted below in Table 8, and pipet well to mix.

Organism	Instrument	Pooling volume (µl)
Campylobacter	MiSeq	2.5
Campylobacter	iSeq	2.0
Listeria	iSeq	2.0

Table 8. Exceptions to the 5 µl pooling volume for the Illumina DNA Prep libraries

NOTE: *Lower pooling volumes are required to achieve more a more balanced run. This will help prevent over-coverage due to small genome size and lower coverage requirements.*

9.6.2. Quantify the pool, using the Qubit dsHS kit or equivalent. See SOP PNL33 for instructions on operating the Qubit.

9.6.3. Enter the concentration (ng/µl) for the pool in the appropriate cell of the Workbook.

9.6.4. Calculate the molarity (nM) of the pool:
(Qubit reading ng/µl/ (660g/mol x 1000bp)) x 10^6
NOTE1: *The workbook will automatically calculate and display this value.*
NOTE2: *The formula in the workbook is based on an assumed average fragment size of 1000 base pairs (in blue above) which is optimal for 500 cycle sequencing (and deviates from the original Illumina SOP). If laboratories generate fragment sizes after library prep which vary greatly from 1000 bp in length, they may adjust this formula accordingly, as this will make the determination of the library concentration and dilution to loading concentration more accurate. For example, if fragment analysis reveals average library lengths at around 800 bp, the formula would be the following: (Qubit reading ng/µl/ (660g/mol x 800bp)) x 10^6 and therefore the molarity calculation cell would need to be changed to "=(PoolConcentration/(660*800))*10^6". Contact PulseNetNGSLab@cdc.gov for assistance if necessary.*

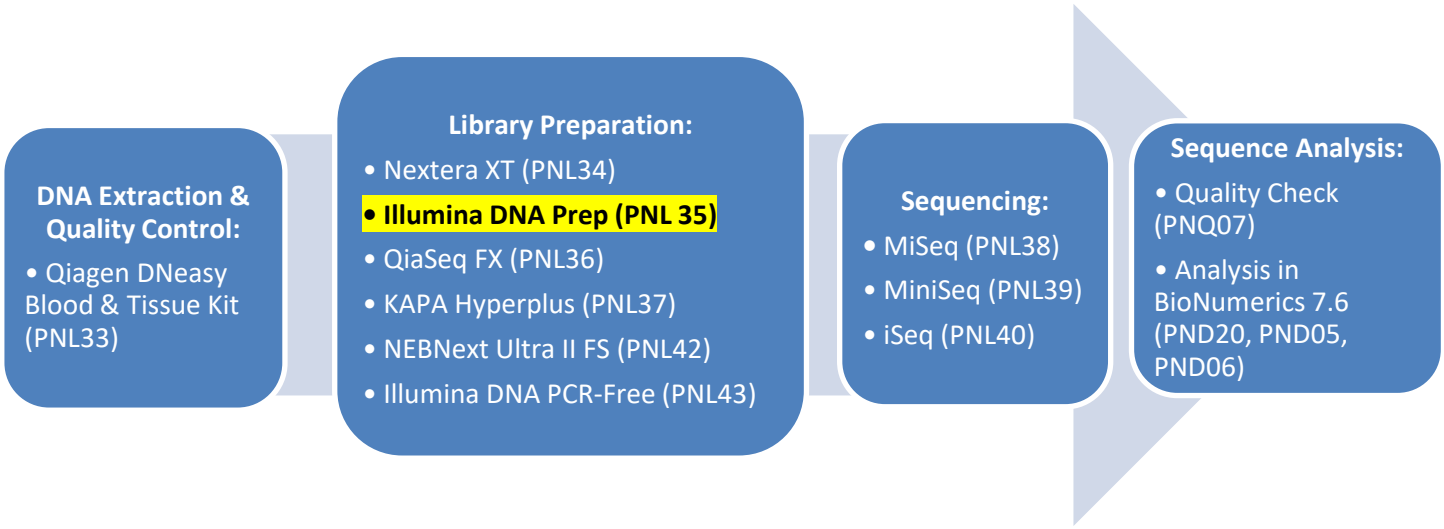
9.6.5. Calculate the volume (µl) of pool necessary to generate 50 µl of a 4 nM pool:
(200/Molarity of pool) = volume of pool for dilution
NOTE: *The workbook will automatically calculate and display this value.*

9.6.6. Calculate the volume (µl) of RSB diluent required:
50 – volume of pool = volume of RSB required
NOTE: *The workbook will automatically calculate and display this value.*

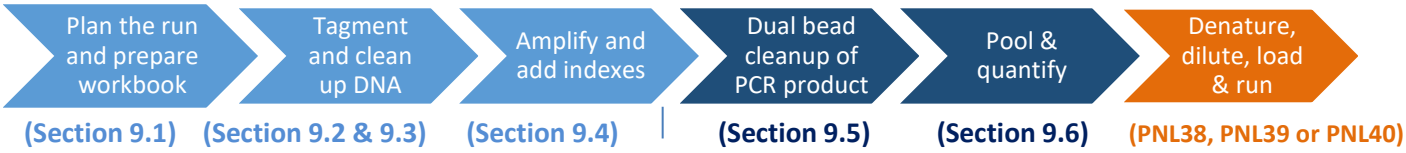
9.6.7. In a new well, dilute the pool to 4 nM by adding the calculated volume of library pool to the calculated volume of RSB.
NOTE1: *The pooled libraries are now ready for sequencing, or the plate may be sealed with Microseal B or equivalent and stored at -20°C for no more than 48 h. If continuing with sequencing, proceed to the appropriate instrument sequencing SOP for instructions to denature and/or dilute the pool to the proper loading concentration.*
NOTE2: *To include Norovirus or Cyclospora to the PulseNet sequencing run, refer to Appendices PNL35-1and PNL35-2, respectively, for instructions on how to spike in Norovirus and Cyclospora libraries to the 4 nM bacterial library pool.*

10. FLOW CHARTS:

10.1. PulseNet WGS Workflow:



10.2. Illumina DNA Prep Library Preparation Workflow:



11. RELATED DOCUMENTS:

Document Number	Title
PNL33	DNA Extraction and QC SOP
PNL38	Sequencing on the MiSeq SOP
PNL39	Sequencing on the MiniSeq SOP
PNL40	Sequencing on the iSeq SOP
PNQ07	Illumina Sequence Data QC SOP
PND20	BioNumerics RefID Database Workflow SOP
PND05	BioNumerics Organism-Specific Database Workflow SOP
PNL35.W1	Illumina DNA Prep Workbook, 96 CD/UD Indexes, separate sample sheets for MiSeq IEM, MiSeq LRM (vs 3 and earlier versions), MiniSeq LRM and iSeq LRM
PNL35.W2	Illumina DNA Prep Workbook, 24 CD Indexes, separate sample sheets for MiSeq IEM, MiSeq LRM (vs 3 and earlier versions) and iSeq LRM
PNL35.W3	Illumina DNA Prep Checklist Workbook
PNL35.W4	Illumina DNA Prep Index Tracking Template Worksheet
PNL35.JA1	Illumina DNA Prep Quick Guide Job Aid
VGB.NV.METHOD.002	RT-PCR Protocol for Amplification of Full-Length Norovirus GII Genomes
AMD.DR.C.001	State Cyclospora Outbreak Genotyping SOP

12. REFERENCES:

12.1. Illumina, Inc. Index Adapters Pooling Guide. (Doc.# 1000000041074 v11). https://support-docs.illumina.com/SHARE/IndexAdapterPooling/Content/SHARE/IndexAdapterPooling/Intro_dtP.htm

12.2. Illumina, Inc. Illumina DNA Prep Checklist (Doc.# 1000000033561 v05). June 2020. https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/illumina_prep/illumina-prep-checklist-1000000033561-05.pdf

12.3. Illumina, Inc. Illumina DNA Prep Reference Guide (Doc.# 1000000025416 v09). June 2020. https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/illumina_prep/illumina-dna-prep-reference-guide-1000000025416-09.pdf

12.4. Graphic 1 (basic design): Ko, Julie. PCR Tube. The Noun Project. https://thenounproject.com/search/?q=pcr&i=1702287

12.5. Illumina, Inc. Customer Notification. November 10, 2021. https://support.illumina.com/bulletins/2019/09/impact-of-ammonium-based-cleaning-products-on-sequencing-run-per.html

13. CONTACTS:

13.1. PulseNet NGS Lab troubleshooting account: PulseNetNGSLab@cdc.gov

14. AMENDMENTS:

14.1. **01/28/19:** New Document

14.2. **04/09/20:** Added notes throughout based on feedback and PHL experience, added Table 7, added workflows and iSeq/MiniSeq information. Made several appendices individual workbooks & added quick guide.

14.3. **03/30/2021:** Updated the SOP throughout with the new name for the Nextera DNA Flex kit: Illumina® DNA Prep. Updated catalog numbers and added the IDT for Illumina UD indexes. Updated the references section. Added appendix PNL35-1 for instructions to make combined runs with PulseNet organisms and viral amplicons and added a workflow chart for the procedure.

14.4. **02/01/2023:** Updated catalog numbers and added notes for library prep and index kits to be obsolesced by Illumina. Added language explaining all significant deviations from the Illumina SOP and language facilitating deviations from this SOP, such as using a higher DNA load capacity than the minimum recommended by PulseNet. Added library pooling instructions for iSeq. Added PNL35-2 for instructions to make combined runs with PulseNet organisms and Cyclospora amplicons and added a workflow chart for the procedure. Updated the workbooks to reflect the changes in the index kits (PNL35.W1, PNL35.W4), added the MiniSeq LRM sample sheet to PNL35.W1, added MiniSeq checklist to PNL35-W3, and added LRM3 sample sheet to PNL35.W2. Updated PNL35.JA1 to include use of IPB.

15. APPROVAL SIGNATURES:

Approved By: Patricia C. Lafon -S (Affiliate) Digitally signed by Patricia C. Lafon -S (Affiliate) Date: 2023.02.10 15:57:08 -05'00' Date: 02/10/2023
QA/QC Personnel

Approved By: N/A Date: NA
PulseNet Outbreak Detection and Surveillance Unit Chief

Approved By: Rebecca L. Lindsey -S Digitally signed by Rebecca L. Lindsey -S Date: 2023.02.09 12:47:25 -05'00' Date: 02/09/2023
PulseNet Next Generation Subtyping Methods Unit Chief

Approved By: Efrain M. Ribot -S Digitally signed by Efrain M. Ribot -S Date: 2023.02.07 09:04:34 -05'00' Date: 02/07/2023
PulseNet Reference Outbreak Surveillance Team Lead

16. APPENDICES:

Appendix PNL35-1. Combining PulseNet Organisms and Norovirus Amplicons on the Same Sequencing Run

NOTE: *There is no need to decrease the DNA load of the bacterial DNA. The viral amplicon libraries can be spiked into PulseNet sequencing runs that are considered "full runs" for the sequencing kit chosen.*

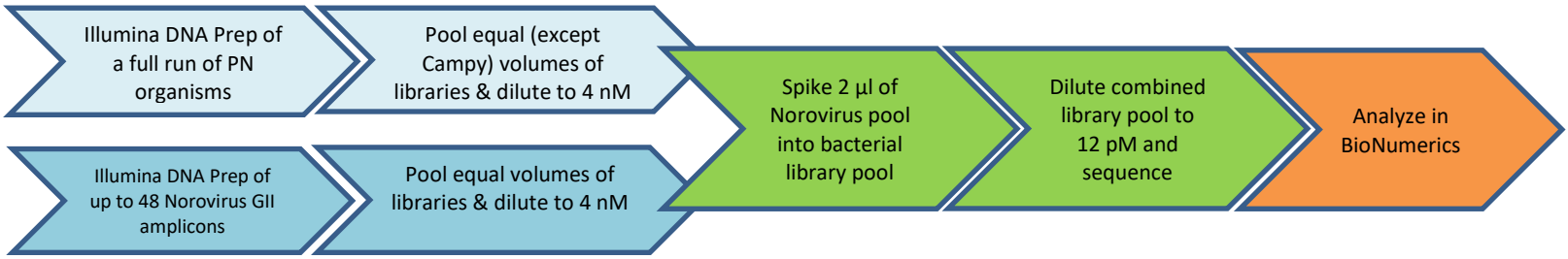
1. Prepare the amplicons (maximum of 48 samples) as described in VGB.NV.METHOD.002 RT-PCR Protocol for Amplification of Full-Length Norovirus GI and GII Genomes.
2. Prepare the sequencing libraries from the sample amplicons as described in PNL35.
3. Pool equal volumes of libraries and dilute to 4 nM. Follow the calculations in Table 9 for diluting the pool to 4 nM.
   NOTE: *The calculations are automated in workbooks PNL35.W1 and PNL35.W2.*

	Example Calculation	Equations
Pool concentration (Qubit value, ng/µl):	10.6	
Molarity (nM) = (conc/(660g/mol x 400 bp) x 10^6:	40.15	=(PoolConcentration/(660*400))*10^6
Volume of pool to dilute (µl) = (nM)(x)=(4 nM)(50 µl):	4.98	=4*50/Molarity
Volume of RSB to dilute (µl) = 50 - volume of pool:	45.02	=50-Volume of pool

Table 9. Calculations to dilute the Norovirus pool to 4 nM

4. Spike 2 µl of 4 nM amplicon library pool into the 4 nM bacterial library pool.
   NOTE: *The viral amplicon spike-in does not significantly affect the bacterial library pool molarity.*
5. Proceed to sequencing on MiSeq following the instructions in PNL38.
   5.1. For Norovirus samples, a prefix "noro_" needs to be added in front of the state keys to easily differentiate Norovirus samples from PulseNet samples.
   5.2. For index selection, make sure that all index pairs (bacterial and Norovirus) are unique to the run and have not been used in the previous run on this instrument.

Workflow for Combined PulseNet/Norovirus Sequencing Runs



Appendix PNL35-2. Combining PulseNet Organisms and Cyclospora Amplicons on the Same Sequencing Run

NOTE: *There is no need to decrease the DNA load of the bacterial DNA. The Cyclospora amplicon libraries can be spiked into PulseNet sequencing runs that are considered "full runs" for the sequencing kit chosen.*

1. Extract the Cyclospora DNA, prepare the amplicons (maximum of 24 samples) and DNA sequencing libraries as described in AMD.DR.C.001 State Cyclospora Outbreak Genotyping SOP.
2. Pool equal volumes of libraries, measure the concentration and size distribution and dilute to 4 nM. Follow the calculations in Table 10 for diluting the pool to 4 nM.
   NOTE1: *The calculations are automated in workbooks PNL35.W1 and PNL35.W2.*
   NOTE2: *The pool fragment size distribution typically varies between 300-350 bp. It is recommended determine the fragment size for every run.*

	Example Calculation	Equations
Pool concentration (Qubit value, ng/µl):	6.72	
Molarity (nM) = (conc/(660g/mol x avg library size in bp) x 10^6:	25.45	=(PoolConcentration/(660*400))*10^6
Volume of pool to dilute (µl) = (nM)(x)=(4 nM)(50 µl):	7.86	=4*50/Molarity
Volume of RSB to dilute (µl) = 50 - volume of pool:	42.14	=50-Volume of pool

Table 10. Calculations to dilute the Cyclospora pool to 4 nM

3. Spike 20 µl of 4 nM Cyclospora library pool into the 4 nM bacterial library pool.
4. Proceed to sequencing on MiSeq following the instructions in PNL38.
   4.1. For Cyclospora sample naming in the Library Prep tab of the workbook, follow the standardized format outlined in the SOP AMD.DR.C.001, step 4.9.1.
   4.2. For index selection, make sure that all index pairs (bacterial and Cyclospora) are unique to the run and have not been used in the previous run on this instrument.

Workflow for Combined PulseNet/Cyclospora Sequencing Runs