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.

2. **SCOPE:** For use by PulseNet WGS certified laboratorians when preparing libraries from DNA from enteric organisms using the Illumina® DNA Prep kit (formerly known as Nextera DNA Flex) 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.
   NOTE: *Instructions for combining Norovirus amplicons in the same run with the PulseNet organisms are outlined in the appendix PNL35-1.*

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. **LRM:** Local Run Manager
   - 3.10. **Mb:** Megabase
   - 3.11. **nM:** Nanomolar
   - 3.12. **PCR:** Polymerase Chain Reaction
   - 3.13. **PHL:** Public Health Laboratory
   - 3.14. **PPE:** Personal Protective Equipment
   - 3.15. **PR2:** Incorporation Buffer
   - 3.16. **PulseNet Central:** PulseNet team at CDC comprising of the Outbreak Detection and Surveillance Unit and the Next Generation Subtyping Methods Unit
   - 3.17. **QC:** Quality control
   - 3.18. **RSB:** Resuspension Buffer
   - 3.19. **SDS:** Safety Data Sheet
   - 3.20. **SOP:** Standard Operating Procedure
   - 3.21. **SPB:** Sample Purification Beads
   - 3.22. **TB1:** Tagmentation Buffer 1
   - 3.23. **TSB:** Tagment Stop Buffer
   - 3.24. **TWB:** Tagment Wash Buffer
   - 3.25. **UD Index:** Unique Dual Index
   - 3.26. **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. Communicate with PulseNet Central as necessary about any complications with laboratory protocols, suspected reagent issues, or suspected instrument 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. Communicate any suspected reagent issues to PHLs as necessary.
4.2.3. 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.
5.2.1. Illumina® DNA Prep Kit: See Illumina SDSs for additional information.
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); take precautions when handling, storing and disposing of ethanol in the laboratory.

6. REAGENTS:

6.1. Illumina® DNA Prep (M) Tagmentation Kit (96 samples, Illumina Cat# 20018705 or 24 samples, Illumina Cat# 20018704) with the following components:

- Beads + Buffers. Store at room temperature – **NOTE:** *One component in the box needs to be refrigerated.*
  SPB **(store upright at 2-8°C, do not freeze)**
  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)
6.2.1.2. 96 Dual Index, plate format (96 samples, Illumina Cat# 20018708)
6.2.2. IDT for Illumina UD Indexes. Store at -25°C to -15°C.
NOTE: *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).*
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. Nextera DNA UD Indexes, set C (96 indexes, 96 samples, Illumina Cat # 20027215)
6.2.2.4. Nextera DNA UD Indexes, set D (96 indexes, 96 samples, Illumina Cat # 20027216)
6.2.2.5. Nextera DNA UD Indexes, sets A-D (384 indexes, 384 samples, Illumina Cat # 20027217)

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. Serological pipets, 1 ml to 10 ml volumes (various catalog numbers)
7.11. Solution basins, sterile (Fisher Scientific Cat# 13-681-504 or equivalent)

8. EQUIPMENT:

8.1. Ice buckets/containers
8.2. Magnetic Stand-96 (Fisher Scientific Cat# AM10027 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. Pipet-Aid
8.7. Qubit™ 2.0 or 3.0 Fluorometer, or equivalent for quantification of dsDNA
8.8. Thermal cycler with heated lid, compatible with 96-well 0.2 ml PCR plate

8.9. Vortex

9. PROCEDURE: The Illumina DNA Prep checklist (PNL35.W3) may be used during the procedure if desired. The workbook has separate tabs for the MiSeq and iSeq. A quick guide (PNL35.JA1) is also provided for users who are familiar with the process as a reference.

NOTE: *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 4nM (9.6.).*

9.1. 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. The workbooks contain separate sample sheet tabs for MiSeq IEM and LRM and iSeq LRM.*

NOTE2: *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.

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: *Fastq files will be named according to this State Key and the Run ID.*

9.1.4. Enter the Genome Size Estimate (based on Table 1 below).

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. 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. This should not exceed the recommended DNA load for the sequencing kit to be used.

NOTE: *The lower recommended DNA load for runs containing E. coli, Shigella, or Vibrionaceae is due to the fact that these organisms require a higher coverage (40x) for downstream analyses compared to the other organisms (20x or 30x). More sequence reads are required for these organisms to meet coverage requirements*.

Sequencing Kit (cycles)/Organism	DNA Load (Mb)
MiSeq	
v2 300	**90**
v2 500	**100**
v3 600, no *E. coli/Shigella/Vibrio	**200**

v3 600, *E. coli/Shigella/Vibrio	**175**
Micro (300), no *E. coli/Shigella/Vibrio*	**35**
Micro (300), *E. coli/Shigella/Vibrio*	**30**
Nano (500)	**13**
Nano (300), no *E. coli/Shigella/Vibrio*	**13**
Nano (300), *E. coli/Shigella/Vibrio*	**10**
MiniSeq	
***Mid Output**	**60**
***High Output**	**100**
iSeq	
E. coli/Shigella/Vibrio	**25**
no *E. coli/Shigella/Vibrio*	**35**

Table 2. Estimated DNA load capacity (in Mb) for Illumina reagent kits.
* Still being evaluated for these kits.

9.1.6. Determine which indexes will be used and enter (or select from the drop-down) the well position (from the index plate) into the Library Prep tab of the workbook.
NOTE: *It is recommended not to use the same index pairs within two consecutive runs on the same sequencer to reduce the amount of carryover. See index tracking worksheets, PNL35.W4 for plated Nextera CD Indexes/IDT for Illumina UD Indexes and PNL35.W2 for 24 sample index kit, to help select and track index usage.*

9.1.7. Enter the volume of input DNA 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.*

9.2. <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.
<u>NOTE</u>: *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. Bring BLT (from refrigerator) and TB1 (from freezer) 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.*

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

9.2.2. Add molecular grade water to each sample well (volume is automatically calculated and displayed on the Workbook).

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

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

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

9.2.6. 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.*

9.2.7. Vortex the tagmentation master mix well.
9.2.8. 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.*
9.2.9. 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.

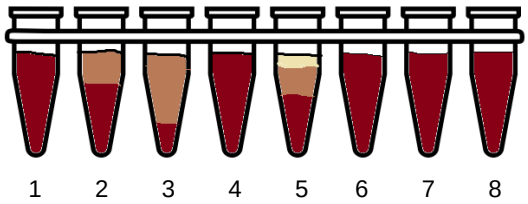

Figure 1. Example of homogeneous and non-homogeneous sample wells. Wells 1, 4, 6, 7 and 8 are well mixed. There should be no color striations ("sunsets") from the bottom to the top of the wells. Wells 2, 3 and 5 display this settling and should be re-mixed prior to incubation.

9.2.10. 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.*

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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 for precipitate (if present, warm at 37°C for up to 10 minutes and vortex).
9.3.1. Add 10 µl of TSB to each sample.
9.3.2. Pipet gently to mix and resuspend the beads. Inspect the wells to ensure that samples and master mix are homogeneous prior to incubation. Refer to Figure 1.
9.3.3. Seal the plate with Microseal A or equivalent.
9.3.4. 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.*
9.3.5. While samples are incubating, thaw EPM on ice and thaw indexes at room temperature.
9.3.6. After samples reach 10ºC, remove the plate from the thermal cycler, and perform a quick spin.
9.3.7. Pipet gently to resuspend the beads, and place on a magnet for 3 minutes (or until beads form

a tight pellet).

9.3.8. Remove and discard supernatant.

9.3.9. Remove the plate from the magnet and add 100 µl TWB directly to the pellet.
NOTE*: A solution basin may be used for TWB if desired.*

9.3.10. 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.*

9.3.11. Place back on the magnet for 3 minutes (or until beads form a tight pellet).

9.3.12. Remove and discard the supernatant.

9.3.13. Remove from the magnet and gently add 100 µl of TWB directly to the pellet.

9.3.14. Pipet gently to mix (avoiding foaming) until beads are fully resuspended.

9.3.15. Place back on the magnet for 3 minutes (or until beads form a tight pellet).

9.3.16. Remove and discard supernatant.

9.3.17. Add 100µl of TWB directly to the beads and gently pipet to resuspend.

9.3.18. 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 during this step.

9.4.1. Vortex 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, PNL35.W3 ("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; using a small volume pipette is recommended to ensure removal of residual TWB before proceeding.
NOTE: *Removal of TWB is crucial, as it can impede PCR. However, 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 in 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.*

9.4.6. Gently pipet to mix, resuspending the pellet.

9.4.7. Add indexes to samples:

9.4.7.1. If using the <u>Nextera CD/IDT for Illumina UD Index plate</u>, each well contains a unique index pair in a single-use volume. Add 10 µl of appropriate index pair from the index plate to each sample well as indicated by the workbook (PNL35.W1).
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, followed by resealing the open wells (Microseal F or equivalent) on the index plate after use.

9.4.7.2. If using the Nextera DNA CD Indexes 24 Index Kit, pair i7 and i5 indexes to create unique index pairs for each sample within a run. Add 5 ul of the i7 index and 5 ul 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 for up to 3 days.*

9.5. Clean up Amplified Libraries: This dual bead clean-up procedure is for purification and size selection of libraries. Target fragment sizes are 800-1000 bp.

NOTE: *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.*

9.5.1. Prepare reagents:

9.5.1.1. Bring SPB to room temperature (at least 30 minutes) from refrigerator.

9.5.1.2. Bring RSB to room temperature (from frozen) and vortex to mix.

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

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

9.5.1.5. Prepare SPB master mix:

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

Table 6: Reagent volumes per sample for SPB 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 Illumina DNA Prep checklist, PNL35.W3 ("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, and pipet gently to resuspend.

9.5.3. Place plate on the magnet for 5 minutes (or until beads have formed a tight pellet).

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.*

NOTE2: *If sample loss has occurred during tagmentation, it is important to maintain the appropriate ratio of SPB master mix to DNA (1.88x) for recovery of appropriate library lengths. Transfer as much of the resulting supernatant as possible and add the corresponding volume of SPB master mix from the Table 7 below to samples with less than 45 µl of product available. Do not adjust subsequent volumes (i.e. stock SPB solution) later in the protocol, as this will negatively affect product recovery.*

Volume of DNA recovered after tagmentation (µl)	Volume of SPB 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 master mix volumes in the case of sample loss.

9.5.5. Remove sample plate from the magnet.

9.5.6. Vortex SPB 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.

9.5.11. After incubation, with the plate still on the magnet, transfer 125 µl of supernatant (containing DNA) to new wells.

NOTE: *If volumes of SPB 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 to the supernatant.

NOTE: *Be sure to add from the stock tube of <u>undiluted</u> SPB, NOT the previously made SPB*

master mix. Accidentally using the SPB 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; longest 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. 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.
9.5.21. Pipet gently and thoroughly to mix.
9.5.22. Incubate at room temperature for 2-5 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 during this step. To prevent aspirating beads, decreasing the recovery volume to 25 µl is acceptable.*
NOTE2: *A quantification step (Qubit or equivalent) may be performed at this point on the individual libraries and results may be recorded in the Workbook if desired. Checking post-clean up library concentrations is recommended for new users of the Illumina DNA Prep kit and for troubleshooting purposes. See PNL33 for specific instructions on operating the Qubit.*
NOTE3: *This is a safe stopping point. The plate may be sealed with Microseal B or equivalent and stored at -20°C until use, or for long-term storage.*

9.6. Pool Libraries.

9.6.1. Pool 5 µl of each library into a new well, and pipet well to mix.
NOTE: *Use a pooling volume of 2.5 µl for Campylobacter spp. instead of 5 µl.*
9.6.2. Quantify the pool, using the Qubit or equivalent. See SOP PNL33 for instructions on operating the Qubit.
9.6.3. Enter the concentration 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). 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 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.*

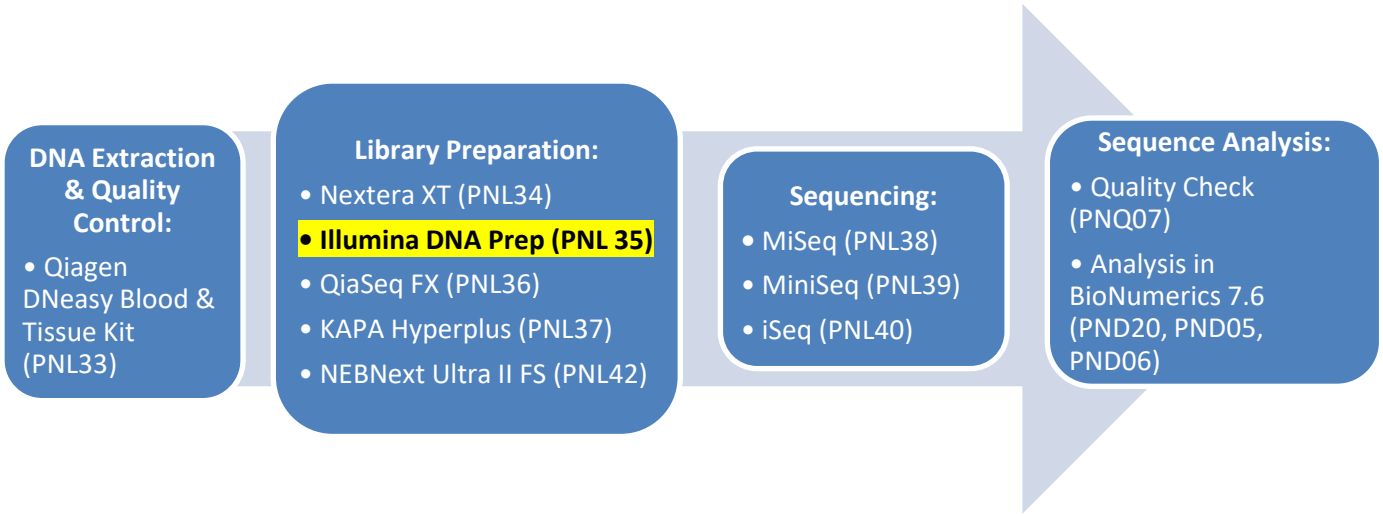
9.6.5. Calculate the volume (µl) of pool necessary to generate 50µl of a 4nM pool:
(200/Molarity of pool) = volume of pool for dilution
NOTE: *The workbook will automatically calculate and display this value.*

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 libraries are now ready for sequencing or the plate may be sealed with Microseal B or equivalent and stored at -20°C until use. 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 viral amplicons (i.e. Norovirus) to the PulseNet sequencing run, refer to Appendix PNL35-1 for instructions on how to spike in viral amplicon 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
PNL35.W2	Illumina DNA Prep Workbook, 24 CD Indexes
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

12. REFERENCES:

12.1. Illumina, Inc. Index Adapters Pooling Guide. (Doc.# 1000000041074 v10). July 2020. https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/experiment-design/index-adapters-pooling-guide-1000000041074-10.pdf

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

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.

15. APPROVAL SIGNATURES:

Approved By: Patricia C. Lafon -S (Affiliate) Digitally signed by Patricia C. Lafon -S (Affiliate) Date: 2021.04.13 10:19:53 -04'00' Date: 04/13/2021
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: 2021.04.12 13:16:19 -04'00' Date: 04/12/2021
PulseNet Next Generation Subtyping Methods Unit Chief

Approved By: Efrain M. Ribot -S Digitally signed by Efrain M. Ribot -S Date: 2021.04.12 12:51:23 -04'00' Date: 04/12/2021
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.
   NOTE: *For Norovirus samples, a prefix "noro_" needs to be added in front of the state keys to easily differentiate Norovirus samples from PulseNet samples.*
3. Pool equal volumes of libraries and dilute to 4 nM. Follow the calculations in Table 8 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 400bp) x 10^6:	40.15	=(PoolConcentration/(660*400))*10^6
Volume of pool to dilute (µl) = (nM)(x)=(4nM)(50ul):	4.98	=4*50/Molarity
Volume of RSB to dilute (µl) = 50 - volume of pool:	45.02	=50-Volume of pool

Table 8. 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.

Workflow for Combined PulseNet/Norovirus Sequencing Runs