1. **PURPOSE:** This procedure describes a standardized laboratory protocol for DNA library preparation of enteric bacterial organisms using the Illumina® DNA Prep kit that is heavily modified from the original SOP PNL35 in order to gain time and cost savings. This procedure has been validated for subsequent sequencing on the Illumina MiSeq 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 the Illumina MiSeq 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.

3. **DEFINITIONS:**
   - 3.1. **BLT:** Bead-Linked Transposome
   - 3.2. **DNA:** Deoxyribonucleic Acid
   - 3.3. **dsDNA:** Double-Stranded DNA
   - 3.4. **EPM:** Enhanced PCR Mix
   - 3.5. **GHS:** Globally Harmonized System
   - 3.6. **HS:** High Sensitivity
   - 3.7. **IEM:** Illumina Experiment Manager
   - 3.8. **IPB:** Illumina Purification Beads
   - 3.9. **LRM:** Local Run Manager
   - 3.10. **Mb:** Megabase
   - 3.11. **Ng:** Nanogram
   - 3.12. **nM:** Nanomolar
   - 3.13. **PCR:** Polymerase Chain Reaction
   - 3.14. **PHL:** Public Health Laboratory
   - 3.15. **PN:** PulseNet
   - 3.16. **PPE:** Personal Protective Equipment
   - 3.17. **PulseNet Central:** PulseNet team at CDC comprising of the PulseNet Response and Outbreak Management Team (database management) and the WGS core laboratory activity
   - 3.18. **PulseNet/OutbreakNet SharePoint**: A closed, web-based collaboration application used for communication among PulseNet participants
   - 3.19. **QC:** Quality Control
   - 3.20. **RSB:** Resuspension Buffer
   - 3.21. **SDS:** Safety Data Sheet
   - 3.22. **SOP:** Standard Operating Procedure
   - 3.23. **TB1:** Tagmentation Buffer 1
   - 3.24. **TSB:** Tagmentation Stop Buffer
   - 3.25. TWB: Tagmentation Wash Buffer
   - 3.26. **UD Index:** Unique Dual Index
   - 3.27. **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. TB1: GHS Category 4 for acute toxicity (dust/mist), Category 2A for eye irritant and Category 1B for reproductive toxicity. Contains N, N=Dimethylformamide.
  - 5.2.1.2. 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:
- 6.1.1. 96 samples, with IPB, Illumina Cat# 20060059
- 6.1.2. 24 samples, with IPB, Illumina Cat# 20060060

Kit components:

- Beads + Buffers. Store at room temperature.
  - IPB (store upright)
  - TSB
  - TWB
  - **NOTE:** *TSB and TWB not used in this procedure*
- PCR + Buffers. Store at -25 to -15°C.
  - RSB
  - TB1
  - EPM
- Tagmentation (M) Beads. Store at 2-8°C.
  - BLT (store upright)

6.2. IDT for Illumina UD Dual Indexes. Store at -25°C to -15°C.

NOTE: *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.*

- 6.2.1. DNA/RNA UD Indexes, set A, Tagmentation (96 indexes, 96 samples, Illumina Cat# 20027213)
- 6.2.2. DNA/RNA UD Indexes, set B, Tagmentation (96 indexes, 96 samples, Illumina Cat# 20027214)
- 6.2.3. DNA/RNA UD Indexes, set C, Tagmentation (96 indexes, 96 samples, Illumina Cat # 20042666)
- 6.2.4. DNA/RNA UD Indexes, set D, Tagmentation (96 indexes, 96 samples, Illumina Cat # 20042667)

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. Ice
7.3. Microcentrifuge tubes, 1.5 ml, sterile (Fisher Scientific Cat# 05-408-129 or equivalent)
7.4. Microseal B adhesive seal (BioRad Cat# MSB-1001 or equivalent)
7.5. Microseal F adhesive foil seal (BioRad Cat# MSF-1001 or equivalent) **– Optional**
7.6. Pipette tips, sterile, filtered: 20 µl, 200 µl and 1000 µl volumes (Rainin Cat# 30389225, 30389239 & 30389212 or equivalent)
7.7. Qubit™ Assay Tubes (Fisher Scientific Cat# Q32856 or equivalent (clear, thin-wall 0.5-ml PCR tubes))
7.8. 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 (for the PCR plate, Fisher Scientific Cat# 12-027 or equivalent)
8.3. Promega MagneSphere™ Technology Magnetic Separation Stand (2-position for the 1.5 ml microcentrifuge tube, Fisher Scientific Cat# PR-Z5332 or equivalent)
8.4. Microcentrifuge for quick spins
8.5. 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.*
8.6. Microplate centrifuge
8.7. Pipet-Aid
8.8. Qubit™ 4.0 Fluorometer (older versions 2.0 or 3.0), or equivalent for quantification of dsDNA
8.9. Thermal cycler with heated lid, compatible with 96-well 0.2 ml PCR plate
8.10. Vortex

9. PROCEDURE: The PulseNet Rapid Library Prep checklist (PNL44.W2) 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. The PulseNet Rapid Library Prep workbook (PNL44.W1) may be used for planning sequencing runs, including index assignment using index plates of 96 index pairs 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 (9.3.) or after the*

RSB has been added to the ethanol-washed pool (9.4.).
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 PulseNet Rapid Library Prep Workbook.
NOTE1: *The workbook PNL44.W1 contains separate tabs for different software versions on the MiSeq: IEM, MiSeq LRM3/4 and MiSeq LRM versions earlier than 3; ensure that the proper tab is used for the software available on the MiSeq. Do* ***NOT*** *delete rows or columns of the workbook! 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.*
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. (e.g., CDC-M3235-231004)
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-M3235-231004)*
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**
***Cronobacter* spp.**	**5**
***Yersinia* spp.**	**5**

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 sequencing kit to be used (Table 2). 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. 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.
NOTE: ***PulseNet never accepts, under any circumstances, sequences generated with cycling parameters below 300 cycles (2 x 150).***

MiSeq Sequencing Kit (cycles)	DNA Load (Mb)
v2 300	80
v2 500	100

Table 2. Estimated DNA load capacity (in Mb) for MiSeq 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.
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. The index tracking worksheets, PNL35.W4 may be used to reserve/keep track of used and available indexes for plated IDT for Illumina UD Indexes.*

9.1.7. Enter the volume of input DNA (recommended 7.5 µl) to be added to each well at the beginning of the library preparation process. Total input DNA quantity should be 100-500 ng.

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

9.1.9. If using the Rapid Protocol Checklist (PNL44.W2), it is very important to enter the total number of samples with different genome sizes (*Campylobacter, Listeria*, non-*Campylobacter/Listeria*) into cells 1D, 1F and 1H. This information will be used to calculate master mixes and is VERY important in calculating appropriate IPB master mix volumes.

9.2. <u>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.
NOTE1: *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.*
NOTE2: *It is recommended to use freshly extracted DNA for this procedure instead of DNA that has undergone freeze-thaw cycles.*

9.2.1. Allow BLT (from 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

9.2.4. Add 7.5 µl DNA to the appropriate sample wells in the plate

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	2.5 µl
BLT	2.5 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 PulseNet Rapid Library Prep checklist, PNL44.W2 and master mix volumes will be auto calculated with overage included.*

9.2.8. Vortex the tagmentation master mix well

9.2.9. Add 5 µl 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 (12.5 µl volume), 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.

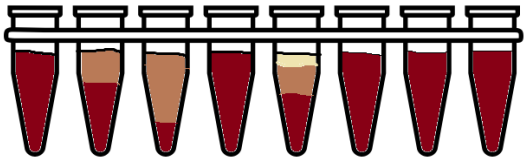

Figure 1. Example of homogeneous and non-homogeneous sample wells. Wells 1, 4, 6, 7 and 8 are well mixed. There should be no color striations ("sunsets") from the bottom to the top of the wells. Wells 2, 3 and 5 display this settling and should be re-mixed prior to incubation.

9.2.11. Seal the plate with Microseal B (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 12.5 µl)
NOTE: *It is recommended to pre-program a thermal cycler for this purpose.*

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9.3. <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.

9.3.1. While samples are incubating, thaw EPM on ice and thaw indexes at room temperature

9.3.2. Vortex and quick spin the thawed EPM briefly and quick spin the index plate prior to use

9.3.3. Prepare PCR master mix:

Reagent	Volume per Sample
EPM	5 µl
Molecular grade water	5 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 PulseNet Rapid Library Prep checklist and master mix volumes will be auto calculated with overage.*

9.3.4. Vortex and quick spin the PCR master mix

9.3.5. Remove plate from thermal cycler and place on magnet for 3 minutes or until clear

9.3.6. Discard the supernatant using a single channel pipette, taking care not to disturb the bead pellet (recommended to set the pipet to 11 µl). If beads are inadvertently disturbed or pulled up in the pipet, expel supernatant back into the well and allow to sit on the magnet until clear and try again.

9.3.7. Remove from the magnet and immediately add 10 µl of PCR master mix directly to the bead pellet for 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.*

9.3.8. Gently pipet to mix well, ensuring resuspension of the pellet

9.3.9. Add 2.5 µl of appropriate index pair from the index plate well to each sample well as indicated by the workbook (PNL44.W1). After wells have been used/pierced they **must be sealed** using 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.*

9.3.10. Pipet a minimum of 10 times to mix

9.3.11. 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.

9.3.12. Seal the plate with Microseal B 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: 8 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: 12.5 µl

NOTE: *Ensure plate is sealed well to avoid evaporation during PCR as this can result in product loss.*

9.3.13. Centrifuge plate at 280 x g for 10 s.

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.*

9.4. <u>Clean up and Size Select Amplified Libraries</u>: This dual bead clean-up procedure is for purification and size selection of libraries. Target fragment sizes are 800-1100 bp.

NOTE: *The steps listed below are critical for efficient size selection, product recovery, and thus cluster generation and sequencing. <u>Always</u> 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.*

9.4.1. 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.*

9.4.2. If plate was retrieved from cold storage, centrifuge plate at 280 x g for 10 s

9.4.3. Place plate on the magnet for 3-5 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.*

9.4.4. Transfer 11µl of supernatant (now containing the DNA) to new wells

9.4.5. Remove sample plate from the magnet

9.4.6. Pool 1.5 µl of *Campylobacter, 2* µl *Listeria* and 4 µl of the 5 Mb genomes into a single 1.5 ml microcentrifuge tube

NOTE1: *the PulseNet Rapid Library Prep checklist will calculate the total pool volume.*

NOTE2: *Lower pooling volumes are required for genomes smaller than 5 Mb to achieve more a more balanced run. This will help prevent over-coverage due to small genome size and lower coverage requirements.*

NOTE3: *Very small volumes are being pooled. Verify that the correct volumes are being transferred by carefully inspecting the pipette tips during pooling.*

9.4.7. Vortex and invert IPB several times to fully resuspend the beads

9.4.8. Prepare **IPB master mix**:

Reagent	Volume per Sample
IPB	5 µl
Molecular grade water	5 µl

Table 5: Reagent volumes per sample for IPB master mix

NOTE: *The volumes will be auto calculated with overage by the PulseNet Rapid Library Prep Checklist. The volume of mastermix is enough for the two size selection steps.*

9.4.9. Vortex the IPB master mix thoroughly

9.4.10. Add 2x volume of IPB master mix to the DNA pool

NOTE: *the PulseNet Rapid Library Prep checklist will calculate the IPB master mix volume to be added.*

9.4.11. Pipet or quick vortex to mix. Thorough mixing is critical!

9.4.12. Incubate at room temperature for 5 minutes

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

9.4.14. While on the magnet, transfer supernatant into a new microcentrifuge tube. Discard the old tube (selecting out the larger libraries that will be bound to the beads).

NOTE: *the PulseNet Rapid Library Prep checklist will calculate the volume to be transferred.*

9.4.15. Vortex IPB master mix thoroughly again and add 0.33x volume IPB master mix to the DNA pool

NOTE: *the PulseNet Rapid Library Prep checklist will calculate the IPB master mix volume to be added.*

9.4.16. Pipet or quick vortex to mix. Thorough mixing is critical!

9.4.17. Incubate at room temperature for 5 minutes

9.4.18. Dilute 1 ml of fresh 80% ethanol for all samples: 800 µl + 200 µl H_2O

9.4.19. Place the tube containing the pool on magnet for 3-5 minutes (or until beads form a tight pellet and supernatant clears)

9.4.20. Remove and discard supernatant (desired libraries are bound to the beads; shortest libraries in the supernatant are being discarded)

9.4.21. Perform the steps below (9.4.21.1. through 9.4.21.3.) twice, for a total of two washes:

9.4.21.1. While the plate is on the magnet, add 400 µl of fresh 80% ethanol

NOTE*: Do not add directly to the bead pellet, do not mix, and do not remove from the magnet during the wash steps.*

9.4.21.2. Incubate for 30 seconds

9.4.21.3. Remove and discard supernatant

9.4.22. After the second wash, use a small volume pipette to remove excess ethanol, if necessary

9.4.23. 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.*

9.4.24. Remove from magnet and add 32 µl of RSB directly to each bead pellet before pipetting to mix

9.4.25. Pipet gently and thoroughly to mix

9.4.26. Incubate at room temperature for 2-5 minutes

NOTE: *Longer incubation (5-10 minutes) is preferred for optimal yield and recovery of longer libraries.*

9.4.27. Place on magnet for 3-5 minutes (or until supernatant is clear)

9.4.28. Transfer 27-30 µl (make sure not to absorb any beads) of the supernatant into a new microfuge tube or a plate well – this is the final product and can be stored at -25C to -15C for up to 30 days.

9.5. Quantify and Dilute the Pool

9.5.1. Quantify the pool, using the Qubit dsHS kit or equivalent. See SOP PNL33 for instructions on operating the Qubit.
NOTE: Sequencing may proceed with pools that have a concentration of 7 ng/µl or higher. Pools that have concentrations lower than that may not be sufficient for sequencing, and library preparation may need to be repeated.

9.5.2. **OPTIONAL BUT HIGHLY RECOMMENDED:** determine the average fragment size of the pool.
NOTE: *This procedure typically generates fragments of 900-1,200 bp in size. Considerable variation has been observed in fragment size even among the preps from the same analyst. It is advisable to perform fragment analysis at least at the beginning to establish the level of the consistency in fragment size from a given analyst. If the fragment sizes are not consistently within 150 bp, routine fragment analysis of the pool is highly recommended. If fragment analysis is not possible, use 1000 bp as the assumed fragment size.*

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

9.5.4. 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. 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 1200 bp, the formula would be the following: (Qubit reading ng/µl/ (660g/mol x 1200bp)) x 10^6 and therefore the molarity calculation cell would need to be changed to "=(PoolConcentration/(660*1200))*10^6". This adjustment is done in cell D133 of the workbook. Contact PulseNetNGSLab@cdc.gov for assistance if necessary.*

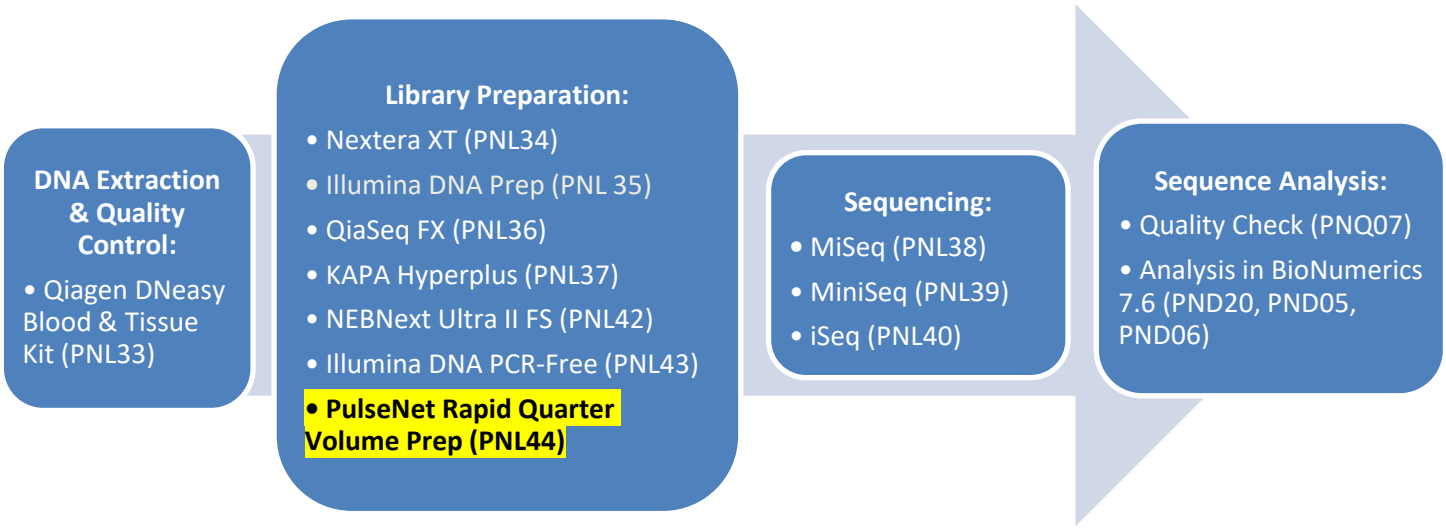
9.5.5. Calculate the volume (µl) of pool necessary to generate 25µl of a 4nM pool:
(200/Molarity of pool) = volume of pool for dilution
NOTE: *The workbook will automatically calculate and display this value.*

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

9.5.7. In a new well/tube, dilute the pool to 4 nM by adding the calculated volume of library pool to the calculated volume of RSB.
NOTE*: The pooled libraries are now ready for sequencing. Proceed to the appropriate instrument sequencing SOP for instructions to denature and/or dilute the pool to the proper loading concentration.*

10. FLOW CHARTS:

10.1. PulseNet WGS Workflow:



10.2. PulseNet Rapid Quarter Volume DNA Prep Library Preparation Workflow:

11. RELATED DOCUMENTS:

Document Number	Title
PNL33	DNA Extraction and QC SOP
PNL38	Sequencing on the MiSeq SOP
PNQ07	Illumina Sequence Data QC SOP
PND20	BioNumerics RefID Database Workflow SOP
PND05	BioNumerics Organism-Specific Database Workflow SOP
PNL44.W1	PulseNet Rapid Library Prep Workbook, 96 UD Indexes, separate sample sheets for MiSeq IEM, MiSeq LRM 3/4 and LRM versions earlier than 3
PNL44.W2	PulseNet Rapid Library Prep Checklist Workbook
PNL35.W4	Illumina DNA Prep Index Tracking Template Worksheet

12. REFERENCES:

12.1. Hickman R, Nguyen J, Lee TD et al. Rapid, High-Throughput, Cost Effective Whole Genome Sequencing of SARS-CoV-2 Using a Condensed One Hour Library Preparation of the Illumina DNA Prep Kit. MedRxiv preprint doi: https://doi.org/10.1101/2022.02.07.22269672; this version posted February 8, 2022.

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

12.3. Illumina, Inc. Illumina DNA Prep Reference Guide (Doc.# 1000000025416 v10). August 20221. https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/illumina_prep/illumina-dna-prep-reference-guide-1000000025416-10.pdf16).

12.4. 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

12. CONTACTS:

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

13. AMENDMENTS:

13.1. **01/16/2024:** New Document

14. APPROVAL SIGNATURES:

Approved By: Jennifer K. Adams Digitally signed by Jennifer K. Adams Date: 2024.01.18 08:46:40 -05'00' Date: 01/18/2024
QA/QC Personnel

Approved By: Patricia C. Lafon -S (Affiliate) Digitally signed by Patricia C. Lafon -S (Affiliate) Date: 2024.01.19 09:04:17 -05'00' Date: 01/19/2024
PulseNet WGS Technical Lead

Approved By: N/A Date: NA
PulseNet Response and Outbreak Management Team Lead

Approved By: Efrain M. Ribot -S Digitally signed by Efrain M. Ribot -S Date: 2024.01.18 09:32:23 -05'00' Date: 01/18/2024
PulseNet Reference Outbreak Surveillance Team Lead