1. **PURPOSE:** This procedure describes a standardized protocol for preparing libraries for sequencing on an Illumina sequencing platform, from enteric genomic DNA using Illumina's Nextera XT library preparation kit.

2. **SCOPE**: This protocol applies to all PulseNet WGS certified laboratories preparing genomic DNA for whole genome sequencing on enteric isolates for submission of sequence data to PulseNet using the Nextera XT library preparation kit. Participating PulseNet laboratories may adopt this SOP to their workflow as necessary.

3. **DEFINITIONS:**
   - 3.1. **ATM:** Amplicon Tagment Mix
   - 3.2. **BSC:** Biosafety cabinet
   - 3.3. **CAA:** Clean Amplified Plate
   - 3.4. **CAN:** Clean Amplified NTA
   - 3.5. **CDC:** Centers for Disease Control and Prevention
   - 3.6. **DNA:** Deoxyribonucleic acid
   - 3.7. **DNase:** Deoxyribonuclease
   - 3.8. **dsDNA:** Double-stranded DNA
   - 3.9. **EBT:** Elution Buffer with Tween
   - 3.10. **GHS:** Globally Harmonized System
   - 3.11. **HS:** High Sensitivity
   - 3.12. **HT1:** Hybridization buffer
   - 3.13. **NPM**: Nextera PCR Master Mix
   - 3.14. **NT:** Neutralize Tagment Buffer
   - 3.15. **NTA:** Nextera XT Tagment Amplicon Plate
   - 3.16. **PCR:** Polymerase Chain Reaction
   - 3.17. **PHL:** Public Health Laboratory
   - 3.18. **PN:** PulseNet
   - 3.19. **PPE:** Personal Protective Equipment
   - 3.20. **QC:** Quality Control
   - 3.21. **RSB:** Resuspension Buffer
   - 3.22. **SDS:** Safety Data Sheet
   - 3.23. **SOP:** Standard Operating Procedure
   - 3.24. **TCY:** Skirted thermocycler plate
   - 3.25. **TD:** Tagment DNA Buffer
   - 3.26. **Tris-HCL:** Tris hydrochloride
   - 3.27. **WGS**: Whole Genome Sequencing

4. **RESPONSIBILITIES:**
   - 4.1. **PulseNet Public Health Laboratories:**
     - 4.1.1. Perform quality assessment of genomic DNA prior to library preparation
     - 4.1.2. Prepare DNA libraries, and QC as necessary, for subsequent WGS
     - 4.1.3. 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:**

5.2.1. Nextera XT DNA Library Prep reagent TD Buffer has GHS classification Category 1B for reproductive toxicity. Personal injury can occur through inhalation, ingestion, skin and eye contact. See the Illumina kit SDS for additional information.

5.2.2. Agencourt AMPure XP beads contain <0.1% sodium azide (GHS Category 2 for Oral toxicity, Category 1 for aquatic toxicity) and must be disposed of in accordance with governmental safety standards.

5.2.3. Ethanol is flammable (GHS Flammability Category 2); take precautions when handling, storing and disposing of ethanol in the laboratory.

6. REAGENTS:

6.1.1. Nextera XT Library Prep Kit PulseNet (96 samples plus EBT, Illumina Cat# FC-131-1001) **OR** Nextera XT DNA Sample Preparation Kit (24 samples, Illumina Cat# FC-131-1024 or 96 samples, Illumina Cat# FC-131-1096)

NOTE: *Any additional reagents supplied with the original Nextera XT kit are not necessary for this protocol and may be discarded appropriately. Some contain formamide and must be disposed of as chemical waste in accordance with your laboratory's chemical waste stream.*

- Box 1 of 2. Store at -15°C to -25°C
    - ATM
    - TD
    - NPM
    - RSB
- Box 2 of 2. Store at 2-8°C or at 20-25°C
    - NT

6.1.2. Nextera XT Index Kit (1 of the following, stored at -15°C to -25°C)

6.1.2.1. Nextera XT Index Kit for 24 Indexes, 96 Samples (FC-131-1001)
- Index 2 Primers (S502-S504 & S517)
- Index 1 Primers (N701-N706)

6.1.2.2. Nextera XT Index Kit v2 Set A for 96 Indexes, 384 Samples (FC-131-2001)
- Index 2 Primers (S502-S508 & S517)
- Index 1 Primers (N701-707, N710-N712, N714 & N715)

6.1.2.3. Nextera XT Index Kit v2 Set B for 96 Indexes, 384 Samples (FC-131-2002)
- Index 2 Primers (S502, S503, S505-S508 & S511)
- Index 1 Primers (N716, N718-N724, N726-N729)

6.1.2.4. Nextera XT Index Kit v2 Set C for 96 Indexes, 384 Samples (FC-131-2003)
- Index 2 Primers (S513, S515-S518 and S520-S522)
- Index 1 Primers (N701-N707,N710-N712,N714 & N715)

6.1.2.5. Nextera XT Index Kit v2 Set D for 96 Indexes, 384 Samples (FC-131-2004)
- Index 2 Primers (S513, S515-S518, S520-S522)
- Index 1 Primers (N716, N718-N724, N726-N729)

6.1.3. Agencourt AMPure XP Beads (60ml, Beckman Coulter Cat# A63881 or 5ml, Beckman Coulter Cat# A63880). Store at 2-8°C, away from light.
6.1.4. EBT (Elution Buffer with Tween 20), prepared in house:
- 100 µl of Tween 20
- 99.9 ml of 10mM Tris-HCl, pH 8.0

6.1.5. Ethanol, molecular grade, 95-100% (Fisher Cat# BP2818-500 or equivalent)
6.1.6. Ethanol, lab-grade, 70% or equivalent for disinfection purposes (Fisher Cat# 04-355-309 or equivalent)
6.1.7. Molecular-grade water (Fisher Cat# BP24701 or equivalent)
6.1.8. Qubit® dsDNA High Sensitivity (HS) Assay kit (100 samples, Invitrogen Cat# Q32851 or 500 samples, Invitrogen Cat# Q32854)
- dsDNA HS Reagent (Component A). Protect from light.
- dsDNA HS Buffer (Component B)
- dsDNA HS Standard #1 (Component C). Store at 4°C
- dsDNA HS Standard #2 (Component D). Store at 4°C

6.1.9. Tris-HCl, 1M, pH 8.0 (Sigma-Aldrich Cat# T3038-1L or equivalent)
6.1.10. Tween 20 (Sigma-Aldrich, Cat# P9416-50ML or equivalent) Diluted to 0.5%
***NOTE**: Over time the detergent will separate out of the solution and this should be avoided by preparing only 1 L of 0.5% Tween at a time.*

7. **SUPPLIES:**
7.1.1. Qubit® Assay Tubes (Invitrogen Cat# Q32856 or equivalent (clear, thin-wall 0.5-mL PCR tubes)).
7.1.2. Pipette tips, sterile, filtered: 20 µl, 200 µl and 1000 µl volumes (Rainin Cat# 17001865, 17001863 & 17001864 or equivalent)
7.1.3. Conical tubes, 5ml and/or 15ml (Fisher Cat# 14-959A and/or Fischer Cat# 14-959-53A or equivalent)
7.1.4. Solution basins, sterile (Fisher Cat# 13-681-504 or equivalent)
7.1.5. Deepwell storage "MIDI" plates, 96 well (Fisher Cat# AB-0859 or equivalent)
7.1.6. Hard shell low profile, thin-wall, skirted PCR "TCY" plates, 96 well (BioRad Cat# HSP-9601)
7.1.7. 96-Well PCR Plate, 0.2mL (Fisher 05-408-210 or equivalent)
7.1.8. Microseal A film (BioRad Cat# MSA-5001)
7.1.9. Microseal B film (BioRad Cat# MSB-1001)
7.1.10. Microcentrifuge tubes, 1.5 mL (ThermoFisher Cat# AM12400 or equivalent)
7.1.11. Serological pipets, 1ml to 10ml volumes (various catalog numbers)
7.1.12. Gloves (sizes and materials will be lab specific)
7.1.13. Ice

8. **EQUIPMENT:**
8.1.1. Qubit 2.0, or 3.0 Fluorometer. Or equivalent for quantifying double-stranded DNA
8.1.2. Thermal cycler, 96-well with heated lid, capacity for 0.2 ml PCR plates
8.1.3. Microplate centrifuge
8.1.4. Vortex
8.1.5. Ambion Magnetic Stand-96 (Life Technologies Cat# AM10027) or equivalent
8.1.6. 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.*

8.1.7. Ice buckets/containers
8.1.8. Pipet-Aid
8.1.9. Microcentrifuge for quick spins

9. **PROCEDURE*:***

The steps described below for Sample Plate and Sample Sheet setup on the MiSeq in the Experiment Manager program on the Illumina instrument must be performed prior to the start of the run. Refer to the appropriate instrument SOP for detailed instructions on how to complete these steps on the instrument.

9.1. **Prepare the "Initial Dilution" tab/worksheet of the Nextera XT Library Prep Workbook (PNL34.W1) as described below**:

9.1.1. Enter the State Key (Column B, This will be the PulseNet state ID entered in the BioNumerics "Key" field).
NOTE: ***Effective after PFGE is dropped. Up until then the sample ID will be the PNUSA_id assigned by the PulseNet Database Team.***

9.1.2. Enter the 260/280 Nanodrop purity values into Column G, the Genome Size Estimate (based on Table 1 below) into Column H, and the DNA concentrations (determined from the Qubit) into Column I.

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

Table 1. Estimated Genome Size (in Mb) Per Organism

9.1.3. Confirm that the number of isolates on the run is appropriate for the sequencing kit and the instrument to be used. The sum of the genome sizes (in Mb) for the samples on the run (from column H) is the estimated DNA load of the run. This cannot exceed the DNA load allowance of the sequencing kit to be used. See Table 2 for kit capacities.

Illumina Sequencing Kit	DNA Load (Mb)
MiSeq v2, 300c	**60**
MiSeq v2, 500c	**80**
MiSeq v3, 600c* (mixed runs)	**200**
MiSeq v3, 600c* (*E. coli/Shigella* runs)	**175**
MiSeq Micro, 300c	**35**
MiSeq Nano, 500c	**13**
MiniSeq Mid Output	**60**
MiniSeq High Output	**100**

Table 2. Recommended DNA Load (in Mb) Capacity for Illumina Sequencing Kits
*DNA loads for v3 600 cycle cartridges are based on sequencing runs of 500 cycles using this chemistry

9.1.4. Determine which set of indices to use for each sample, and enter them into Column E (Index 1) and Column F (Index 2) of the "Initial dilution" tab.

NOTE1: *Do not use the same indices in back-to-back runs to avoid read carryover.*

NOTE2: *When runs contain a low number of samples (8 or less), index selection should be made to optimize color balancing. See Illumina's guide "Index Adapters: Pooling Guide" for more information to avoid using indices which overlap with each other.*

9.1.5. Print the "Initial dilution" tab of the Nextera XT Library Prep Workbook to be used as a guide for normalization of the DNA.

9.2. **Normalize the DNA:** *Accurate DNA quantitation is essential for a successful library prep and subsequent cluster generation, therefore it is imperative to mix thoroughly during normalization steps. Tagmentation and PCR setup should be performed in a clean space separate from post-PCR processes in order to reduce the possibility of amplicon contamination.*

NOTE1*: Genomic DNA elutions used for WGS should not contain EDTA, which is known to interfere with library preparation. See PulseNet's recommended extraction method using the Qiagen DNeasy Blood & Tissue Kit (PNL33) for more information.*

NOTE2*: DNA used for library preparation should have a 260/280 value of 1.75 – 2.05. See PNL33 for more information.*

9.2.1. Label the dilution plate (96-well PCR plate), designate wells for 10 ng/µl, 1 ng/µl, 0.2 ng/µl dilutions, as well as for "NTA" wells. The labeling scheme below is recommended for the dilution plate. Figure 1 (below) is an example of the labeling scheme for a run with 16 samples. This plate may be referred to as the "NTA plate".

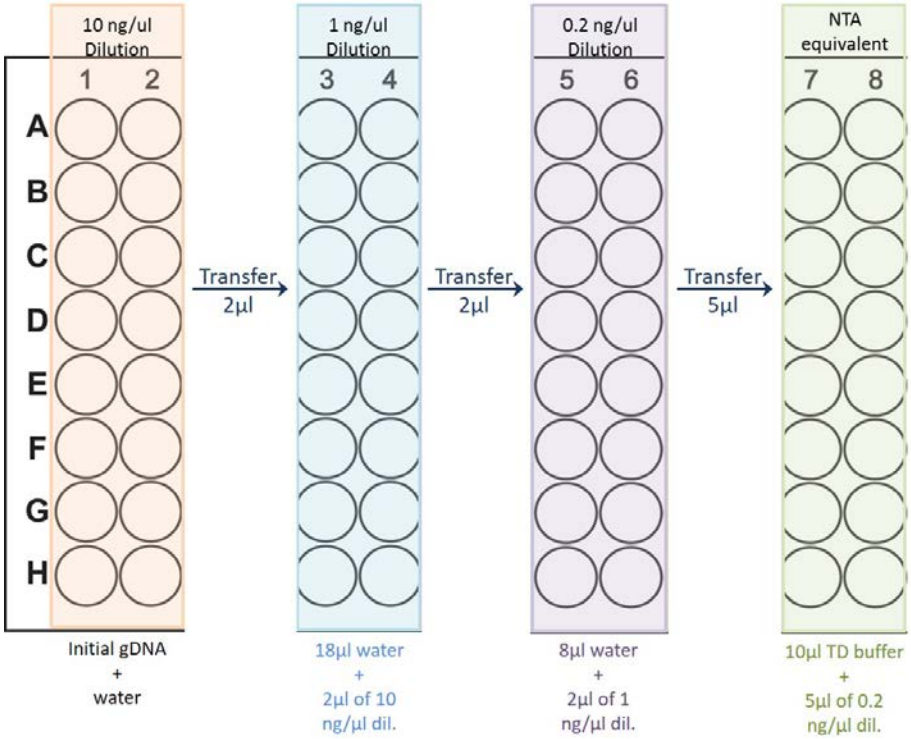

Figure 1: Suggested Dilution Plate Set Up and Labeling Scheme for Normalization of 16 Samples.

9.2.2. Prepare the wells for dilution by adding molecular grade water as described below:
- Add 18 µl to the"1 ng/µl" dilution wells (columns 3 & 4 in the example above)
- Add 8 µl to "0.2 ng/µl" dilution wells (columns 5 & 6 in the example above)
- Add the volume designated in Column K of the worksheet into the "10 ng/µl" dilution wells (columns 1 & 2 in the example above)

9.2.3. Normalize the DNA to 10 ng/µl in the first set of wells by adding the appropriate volume of DNA (Column J on the worksheet).
9.2.4. Mix well by pipetting up and down approximately 5-10 times.
9.2.5. Transfer 2 µl from the 10 ng/µl wells into the wells containing 18 µl of molecular grade water in order to normalize the DNA down to 1ng/µl, and mix well by pipetting up and down 5-10 times.
9.2.6. Transfer 2 µl of the 1 ng/µl DNA into the wells containing 8 µl of molecular grade water in order to complete normalization to 0.2 ng/µl and mix thoroughly by pipetting up and down 5-10 times.

9.3. **Tagmentation of Input DNA:** *Tagmentation is time sensitive. It is important to perform steps 9.3.5- 9-3.10. quickly and ensure that the pre-programmed thermal cycler is turned on prior to beginning the tagmentation steps.*

9.3.1. Remove ATM and TD from -15 to -25°C freezer and thaw at room temperature. Invert 3 – 5 times to mix (do not vortex) and quick spin. Store on ice until ready to use.
9.3.2. Retrieve the NT, either from 2 – 8°C or from room temperature, and visually inspect to make sure there is no precipitate. If precipitate is present, vortex until all particulates are resuspended.
9.3.3. Prepare the "NTA" wells by adding 10 µl of TD buffer to each.
9.3.4. Transfer 5 µl of 0.2 ng/µl normalized input DNA (1 ng total) to each well containing TD buffer.
9.3.5. Quickly add 5 µl of ATM to each "NTA" sample well.
9.3.6. Using a multichannel pipette, gently pipette up and down 5 times to mix.
NOTE: *The total volume in each well is now 20 µl.*
9.3.7. Cover the plate with microseal A, ensuring that the wells are tightly sealed.
9.3.8. Centrifuge the plate at 800 – 1200 rpm (or 280 x g) for approximately 1 minute.
9.3.9. Run the "TAGMENT" program on the thermocycler with the following conditions:
55°C for 5 minutes
Hold at 10°C
Heated lid recommended
NOTE: ***Run time is less than 7 minutes; because this step is time and temperature sensitive, do not leave the thermal cycler.***
9.3.10. While the program is running, 7 µl of NT per sample may be aliquoted into strip tubes.
NOTE*: Aliquots of NT may alternatively be prepped ahead of time.*
9.3.11. **As soon as** the sample temperature reaches 10°C, carefully remove the microseal and use a multichannel pipette to add 5 µl of aliquoted NT to each sample well.
NOTE: *Because this step is time and temperature sensitive, a multichannel pipet is recommended for adding the NT buffer.*
9.3.12. After quickly adding NT to each well, gently pipette up and down 3-5 times with a multichannel pipet to mix.
9.3.13. Cover the plate with fresh microseal A.
9.3.14. Centrifuge the plate at 800 - 1200 rpm (or 280 x g) for approximately 1 minute.
9.3.15. Incubate the plate at room temperature for at least 5 minutes, but not to exceed 20 minutes.

9.4. **Amplification of Tagmented DNA**

9.4.1. Remove NPM and the appropriate Index Primers from -15 to -25°C and thaw at room temperature. Store on ice until ready to use.

NOTE: *It is not recommended for the indices to exceed 5 freeze/thaw cycles.*

9.4.2. Gently invert tubes to mix and briefly centrifuge prior to use.
NOTE: *It is very important to centrifuge the index tubes. Liquid can become trapped inside the cap after inverting and can be inadvertently lost.*

9.4.3. Add 15 µl of NPM to each well containing tagmented DNA.

9.4.4. Add 5 µl of the appropriate Index 1 primer (orange caps) to the designated wells, according to the "Initial dilution" tab of the Workbook.
NOTE: *Discard the index caps and replace with fresh caps as each index primer is used.*

9.4.5. Add 5 µl of the appropriate Index 2 primer (white caps) to the designated wells.
NOTE: *Discard the caps and replace with fresh white caps as each index primer is used.*

9.4.6. Use a multichannel pipette to gently mix.

9.4.7. Securely cover the plate with a fresh microseal A.
NOTE: *The plate needs to be tightly sealed to avoid sample evaporation. Using a thermal cycler with a tight-fitting, heated lid will ensure that a minimal amount of product is lost.*

9.4.8. Centrifuge at 800-1200 rpm (or 280 x g) for approximately 1 minute.

9.4.9. Run the "NAP_XT" program with the following conditions:

72°C for 3 minutes
95°C for 30 seconds
12 cycles – 95°C for 10 seconds
55°C for 30 seconds
72°C for 30 seconds
72°C for 5 minutes
Hold at 4°C

- 50 µl per reaction
- Heated Lid

THIS IS A SAFE STOPPING POINT. THE PLATE MAY REMAIN IN THE THERMAL CYCLER, OR CAN BE STORED AT 2 – 8°C, FOR UP TO TWO DAYS. THIS IS ALSO A STOPPING POINT FOR GENOMETRAKR PROTOCOLS.

9.5. **Pre-Clean Up Qubit Quantification (Optional):** *This extra QC step may help troubleshoot if Post Clean-up PCR product recovery is problematic. It is also recommended for newly trained laboratory personnel.*

9.5.1. Remove the NTA plate from thermal cycler or 4°C.

9.5.2. Centrifuge the NTA plate for approximately 1 minute to collect condensation.

9.5.3. Determine the concentration (ng/µl) of each sample using the Qubit dsDNA HS Assay Kit. (See SOP PNL33 for detailed instructions.)

9.5.4. Record sample concentrations in Column E of the "Normalization and Pooling" tab of the Nextera XT Library Prep workbook.
NOTE: *Samples for which DNA concentration is below the limit of detection should not be sequenced. They may be discarded and the samples may need to either be re-tagmented, or re-extracted.*

9.6. **PCR Product Clean-Up:** *If, at any point during bead clean-up, some of the beads are inadvertently aspirated into the pipet tips, dispense everything back into the CAA plate and let the plate rest on the*

magnetic stand for another 2 minutes, or until the supernatant has cleared. It is recommended to use a multichannel pipette for parts of this process so that incubation times are strictly followed.

9.6.1. Bring the AMPure XP beads (from 2 – 8˚C) and RSB (-15 to -25˚C) to room temperature.
NOTE: *To prevent warming the stock solution of beads multiple times over their life-span and to minimize exposure to light, a single aliquot may be taken from the stock solution and allowed to come to room temperature. Vortex the stock solution **well** to ensure that they are fully resuspended (this is VERY important), remove the necessary volume into a sterile microcentrifuge tube and allow this tube to come to room temperature (preferably in an area protected from light).*

9.6.2. Prepare a fresh 80% ethanol dilution. Approximately 0.5 ml is needed per sample.
- Example: For 16 samples, mix 6.4 ml 100% ethanol with 1.6 ml molecular grade water.

NOTE: *It is important to always prepare fresh 80% ethanol for wash steps. Ethanol can absorb water from the air, changing its concentration and thus impacting sequencing results.*

9.6.3. Centrifuge the NTA plate at 800-1200 rpm (or 280 x g) for approximately 30 seconds to collect any condensation.
NOTE: *If the optional Pre-Clean Up Qubit Quantification was performed, this step may be skipped*.

9.6.4. Label a deep-well plate (the "MIDI" plate) with respective initials of the scientist and date of use. This plate will be referred to as the CAA plate ("Clean Amplified plate").

9.6.5. Transfer 40 µl of the PCR product to the CAA plate.

9.6.6. Vortex the AMPure XP beads until **well suspended** (15 – 30 seconds).

9.6.7. Add appropriate volume of AMPure XP beads to PCR product:

- For 0.5X ratio (recommended), use 20 µl of beads
- For 0.6X ratio, use 24 µl of beads
- For **dual bead** clean up, use 16 µl (0.4x) of beads for this step

NOTE1: ***Avoid bead carryover by confirming no liquid droplets are clinging to the pipette tips. This is important, as droplets will affect the ratio of beads to PCR reaction which affects the insert size.***
NOTE2: *If a wider range of recovered fragment sizes and/or increased post clean-up yield is desired, a 0.6x bead ratio may be used. Note that this enables retention of shorter fragment sizes, which could result in short read lengths when sequencing libraries prepped using this ratio. Therefore, 0.6x ratio is not recommended for users who experience short read lengths and consultation with PulseNet is suggested for assistance.*
NOTE3: *Dual bead clean up may be preferred for users who are experiencing variation in fragment recovery lengths. Note that this bead clean up method may results in a decrease in post-bean clean up yield.*

9.6.8. Gently pipette up and down 5 – 10 times to thoroughly mix PCR product with beads.

9.6.9. Incubate at room temperature, without shaking, for at least 5 minutes.
NOTE: *Increasing the incubation time to 10 minutes may improve library quality and yield.*

9.6.10. Place the CAA plate on the magnetic stand for a minimum of 2 minutes, or until the supernatant has cleared.

9.6.11. For DUAL BEAD clean up **ONLY**:

9.6.11.1. While the plate is on the magnet, carefully transfer 50 µl of the supernatant to a new set of wells (do NOT discard).
9.6.11.2. Remove the plate from the magnet and add 5 µl of AMPure XP beads (0.1x) to the recovered supernatant.
9.6.11.3. Gently pipet 5-10 times to mix.
9.6.11.4. Incubate at room temperature, without shaking for 5 to 10 minutes (longer incubation may improve library recovery and yield).
9.6.11.5. Place the plate on the magnetic stand for a minimum of 2 minutes, or until supernatant has cleared and proceed to the step below.

9.6.12. With the CAA plate on the magnetic stand, carefully remove and discard the supernatant. Ensure that no beads are accidentally aspirated by visually inspecting pipet tips prior to discarding the supernatant.
9.6.13. With the CAA plate on the magnetic stand, wash the beads by adding 200 µl of freshly prepared 80% ethanol to each well.
NOTE: *Do not resuspend the beads at any point during the wash steps, or remove the plate from the magnetic stand.*
9.6.14. Incubate the beads in ethanol on the magnetic stand for approximately 30 seconds, then carefully remove and discard the supernatant by pipetting.
9.6.15. With the CAA plate on the magnetic stand, perform a second wash by adding 200 µl of 80% ethanol, incubating for 30 seconds and then carefully removing and discarding the supernatant.
9.6.16. While the plate is still on the stand, use a low volume tip to remove any remaining ethanol droplets.
9.6.17. While still on the stand, allow the beads to air-dry for up to five minutes.
NOTE: *Exceeding the maximum air-dry period can make resuspension of DNA difficult and is not recommended. Over-drying is indicated by cracks in the bead pellets. It is recommended to observe the beads during the drying time and immediately re-suspend if the beads appear to be over-drying.*
9.6.18. Near the end of the drying period, vortex the RSB vial.
9.6.19. Remove the CAA plate from the magnetic stand and quickly add RSB to each sample well, volumes determined below:
- 0.5X or 0.6X bead ratios: 53 µl of RSB
- Dual bead clean-up: 33 µl of RSB

9.6.20. Gently pipette up and down, at least 10 times, to mix well.
9.6.21. Incubate at room temperature for 5 minutes.
9.6.22. Place the plate back on the magnetic stand for 2 minutes, or until the supernatant has cleared.
9.6.23. Label a new TCY plate with the run ID number, initials, and date. This plate will be referred to as the CAN (Clean Amplified NTA) plate.
9.6.24. With the CAA plate still on the magnetic stand, carefully transfer the supernatant to the CAN plate.
- 0.5X or 0.6X bead ratios: transfer 50 µl of supernatant
- Dual bead clean-up: transfer 30 µl of supernatant

THIS IS A SAFE STOPPING POINT. THE CAN PLATE MAY BE SEALED WITH MICROSEAL 'B' ADHESIVE AND STORED AT -15 TO -25°C FOR 1 – 3 MONTHS. IF USING THE GENOMETRAKR PROTOCOL, THE PLATE CAN BE STORED AT 2 – 8°C FOR UP TO 2 DAYS.

9.7. **DNA Library Qubit Quantification**

9.7.1. Determine the concentration (ng/µl) of each sample in the CAN plate using the Qubit dsDNA HS Assay Kit. (See SOP PNL33 for detailed instructions.)

9.7.2. Enter the concentrations into the Column F ("Qubit Output") on the Normalization and Pooling tab of the Nextera XT Library Prep Workbook.

NOTE 1*: The DNA concentration after post-PCR clean-up should be between 1.34 ng/µl and 7 ng/µl. If the measured concentration is above 7 ng/µl then assign the value for that sample as 7 ng/µl in the Qubit output column. This is based on the assumption (determined by observed measurements over the course of several runs and users) that cleaned libraries with concentrations greater than 7 ng/µl most likely consist of a majority of fragments smaller than the optimal insert size (800 – 1000bp). In order to adjust for this occurrence, the samples are diluted to a greater concentration by assigning the 7 ng/µl value for normalization.*

NOTE2: *The ideal minimum concentration required for sequencing is 2 nM. However, a sample can still be run if it is below the ideal concentration as long as DNA is detected by the Qubit. These low concentration samples should not be diluted prior to pooling libraries.*

NOTE3: *If 25% or more of the samples are below the ideal minimum concentration, consider delaying the run (depending epidemiological importance of the isolate or whether it is associated with an outbreak investigation), and repeat the library prep procedure for samples with a low concentration.*

9.8. **Normalizing the Libraries**

9.8.1. If libraries are frozen, thaw on ice, then centrifuge at 800 – 1200 rpm (or 280 x g) for approximately 1 minute.

9.8.2. Select the desired dilution concentration in cell J4 of the Normalization and Pooling tab of the workbook.

NOTE: *The default starting dilution concentration is 2nM. If the desired final loading concentration is >10 pM, the starting concentration should be changed to 3 nM or 4 nM.*

9.8.3. Dispense EBT diluent into clean wells of the CAN plate, the volume for each sample is determined by values in Column I of the Normalization and Pooling tab of the workbook.

9.8.4. Mix the libraries well, then transfer the appropriate volume for each sample into its appropriate well containing EBT. The volume for each library is pre-populated in Column H of the workbook.

9.8.5. Pipet to mix 5-7 times. The libraries are now at a concentration of 2nM, unless otherwise selected in Cell J4 of the workbook.

9.9. **Pooling the Libraries**

9.9.1. Ensure each sample is well mixed, then transfer the volume listed in the Pooling Volume (Column K on the "Normalization and Pooling" tab) into a single well on the CAN plate or a 0.2 ml tube for a total volume of 50 µl.

NOTE: *The pooling factor is based on the genome size. When genomes of different sizes are run together, the amount of each single library is added proportionately to genome size to reduce the over-representation of small genomes in the pooled library.*

9.9.2. Mix the pooled library by pipetting up and down 10 times.

The pooled libraries are now ready for denaturation and sequencing. Please proceed to the appropriate instrument SOP for instructions to denature and to dilute the pool to the proper loading concentration. Libraries may be stored as described at the bottom of Section 7.6 of this SOP.

10. **FLOW CHART:** NA

11. **RELATED DOCUMENTS:**

Document Number	
PNL33	**DNA Extraction and QC SOP**
PNL38	**Sequencing on the MiSeq SOP**
PNQ07	**Illumina MiSeq Data QC**
PNL34.W1	**Nextera XT Library Prep Workbook**

12. **APPENDICES:** NA

13. **REFERENCES:**

13.1. Illumina, Inc. Nextera XT DNA Library Prep Reference Guide (#15031942). January 2016.
13.2. Illumina, Inc. Illumina Adapter Sequences. (#1000000002694). November 2018.
13.3. Illumina, Inc. Index Adapters Pooling Guide (#1000000041074). December 2018.

14. **CONTACTS:**

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15. **AMENDMENTS:**

15.1. **01/31/2019:**

- **Pulled procedures pertaining to setting up a run on the MiSeq out of PNL32 to create separate document (PNL34)**
- **Created PNL34.W1: Nextera XT Library Prep Workbook to be used for Sample Sheet Set-up and calculations during library preparation.**

16. **APPROVAL SIGNATURES**:

Approved By:_____ Date: _____
Author

Approved By: _____ Date: _____
PulseNet QA/QC Personnel

Approved By: _____N/A_____ Date: _____N/A_____
PulseNet Outbreak Detection and Surveillance Unit Chief

Approved By: _____N/A_____ Date: _____N/A_____
PulseNet PFGE Reference Unit Chief

Approved By: _____ Date: _____
PulseNet Next Generation Subtyping Methods Unit Chief

Approved By: _____ Date: _____
PulseNet Reference Outbreak Surveillance Team Lead