

1. **PURPOSE:** To describe the standardized laboratory protocol for whole genome sequencing of bacterial organisms on the MiSeq benchtop sequencer.
2. **SCOPE:** To provide the PulseNet participants with a single protocol for performing Nextera XT library prep and MiSeq run setup, thus ensuring inter-laboratory comparability of the generated results.

3. **DEFINITIONS:**

- 3.1. **WGS:** Whole Genome Sequencing
- 3.2. **DNA:** Deoxyribonucleic acid
- 3.3. **DNase:** Deoxyribonuclenase
- 3.4. **PCR:** Polymerase chain reaction
- 3.5. **CDC:** Centers for Disease Control and Prevention
- 3.6. **SOP:** Standard Operating Procedure
- 3.7. **ATM:** Amplicon Tagment Mix
- 3.8. **TD:** Tagment DNA Buffer
- 3.9. **NPM:** Nextera PCR Master Mix
- 3.10. **RSB:** Resuspension Buffer
- 3.11. **NT:** Neutralize Tagment Buffer
- 3.12. **TCY:** Skirted Thermocycler Plate
- 3.13. **NTA:** Nextera XT Tagment Amplicon
- 3.14. **CAA:** Clean Amplified Plate
- 3.15. **EDTA:** Ethylendiaminetetraacetic acid
- 3.16. **NaOH:** Sodium Hydroxide
- 3.17. **EBT:** Elution Buffer with Twain
- 3.18. **HT1:** Hybridization buffer
- 3.19. **PR2:** Incorporation buffer
- 3.20. **QC:** Quality Control
- 3.21. **SAV:** Sequencing Analysis Viewer
- 3.22. **MSR:** MiSeq Reporter

4. **RESPONSIBILITIES/PROCEDURE**

- 4.1. **Biosafety warning:** Foodborne pathogens are capable of causing serious disease. Always use a minimum of Biosafety level 2 practices and extreme caution when transferring and handling strains of this type. Work in a biological safety cabinet when handling organisms with a low infectious dose. Disinfect or dispose of all plastic ware and glassware that come in contact with the cultures in a safe manner.

**NOTE:** The MiSeq Reagent Cartridges contain formamide, an aliphatic amide that is a potential reproductive toxin. Ensure spent containers and unused contents are disposed of in accordance with governmental safety standards.

- 4.2. **Reagents, supplies and equipment needed for DNA extraction and quality control**
  - 4.2.1. Trypticase soy agar with 5% sheep blood (TSA-SB) or comparable media
  - 4.2.2. 1 µl inoculation loops

<b>VERSION:</b>	<b>REPLACED BY:</b>	<b>AUTHORIZED BY:</b>	<b>Page 1 of 46</b>
-----------------	---------------------	-----------------------	---------------------

<b>LABORATORY STANDARD OPERATING PROCEDURE FOR PULSENET NEXTERA XT LIBRARY PREP AND RUN SETUP FOR THE ILLUMINA MISEQ</b>	<b>CODE: PNL32</b>		
	Effective Date:		
	1	27	16

- 4.2.3. 1.5 ml microcentrifuge tubes
- 4.2.4. 2 - 5 ml tube
- 4.2.5. Incubator
- 4.2.6. Vortex
- 4.2.7. Water bath(s) or thermal block(s) accommodating 1.5 ml tubes and capable of 56°C ± 1
- 4.2.8. Tabletop centrifuge for high rpm spinning (up to 13,000-14,000 rpm)
- 4.2.9. 10 µl, 20 µl, 100 µl, 200 µl, 1000 µl single channel pipettes
- 4.2.10. Filtered Sterile Pipette tips
- 4.2.11. Recommended kit for DNA extraction: Qiagen DNeasy Blood & Tissue Kit for 50 (Cat. No. 69504) or 250 (Cat. No. 69506) extractions (store at 20 – 25°C)
  - 4.2.11.1. AL Buffer
  - 4.2.11.2. ATL Buffer
  - 4.2.11.3. Proteinase K
  - 4.2.11.4. AW1 Buffer
  - 4.2.11.5. AW2 Buffer
  - 4.2.11.6. AE Buffer
  - 4.2.11.7. Collection tubes
  - 4.2.11.8. Spin columns
- 4.2.12. Molecular-grade Ethanol (95 – 100%) (Fisher Scientific, Cat. No. BP2818-500)
- 4.2.13. RNase A (100 mg/ml) (Qiagen, Cat. No. 19101)
- 4.2.14. Enzymatic Lysis buffer (prepared in-house, see instructions in step 4.5.1.1.4.)
  - 4.2.14.1. 1 M Tris-HCl, pH 8.0
  - 4.2.14.2. 0.5 M EDTA
  - 4.2.14.3. Triton X-100
  - 4.2.14.4. DNase-free, molecular -grade water
- 4.2.15. Lysozyme (Suggested: Sigma-Aldrich, Cat. No. L4919)
- 4.2.16. 1 M Tris-HCl, pH 8.0 for diluting to 10 mM (DNA elution/rehydration) (Suggested: Sigma-Aldrich, Cat. No. T3038-1L or ThermoFisher Scientific, Cat. No. 15568-025)

**NOTE:** DNA extractions to be used for next generation library prep and sequencing (i.e. MiSeq), must not contain EDTA. This chemical has been found to inhibit certain processes during library prep (i.e. tagmentation), and is found in the elution / rehydration buffers of many commercial kits.
- 4.2.17. Alternative kit for DNA extraction: Promega Wizard Genomic DNA Purification Kit (Cat. No. A1120, A1125, or A1620) (store at 20 – 25°C)
  - 4.2.17.1. Additional reagents required for this kit:
    - 4.2.17.1.1. 100% Isopropanol (Fisher Scientific, Cat. No. BP26324)
    - 4.2.17.1.2. 70% Ethanol
- 4.2.18. Qubit® 3.0 Fluorometer (Invitrogen, Cat. No. Q33216) or 2.0 Fluorometer for quantification of double-stranded DNA
  - 4.2.18.1. Qubit dsDNA BR Assay kit for 100 (Invitrogen, Cat. No. Q32850) or 500 (Invitrogen, Cat. No. Q32853) assays
  - 4.2.18.2. Qubit Assay Tubes (500 ct.) for use with the Qubit Fluorometer (Invitrogen, Cat. No. Q32856)

<b>VERSION:</b>	<b>REPLACED BY:</b>	<b>AUTHORIZED BY:</b>	<b>Page 2 of 46</b>
-----------------	---------------------	-----------------------	---------------------

<b>LABORATORY STANDARD OPERATING PROCEDURE FOR PULSENET  NEXTERA XT LIBRARY PREP AND RUN SETUP FOR THE ILLUMINA  MISEQ</b>	<b>CODE: PNL32</b>				
	Effective Date: <table border="1" style="display: inline-table; vertical-align: middle;"> <tr> <td style="width: 20px; text-align: center;">1</td> <td style="width: 20px; text-align: center;">27</td> <td style="width: 20px; text-align: center;">16</td> </tr> </table>			1	27
1	27	16			

4.2.19. NanoDrop 2000 UV-Vis or equivalent spectrophotometer (Fisher, Cat. No. ND-2000-US-CAN) for determination of 260/280 values

**NOTE1:** The software needed to run this system requires a Windows Vista, XP, 7 or higher operating system.

**NOTE2:** The CF-1 Calibration Check Fluid (Fisher, Cat. No. CHEM-CF-1; 0.5ml ampule) will be needed to calibrate the instrument every 6 months.

**OPTIONAL:** DNA Clean & Concentrator-5 for 50 (Zymo Research, Cat. No. D4013) or 200 preps (Zymo Research, Cat. No. D4014)

**4.3. Reagents, supplies and equipment needed for library prep, run setup, and post-run washes**

**NOTE:** Library prep (pre-PCR) should be done in a designated clean space or biological safety cabinet to reduce or eliminate the possibility of contamination.

- 4.3.1. MiSeq Desktop Sequencer
- 4.3.2. Qubit 2.0 or 3.0 Fluorometer and tubes (see 4.2.18 for details)
- 4.3.3. Qubit dsDNA High Sensitivity (HS) Assay kit for 100 (Invitrogen, Cat. No. Q32851) or 500 (Invitrogen, Cat. No. Q32854) assays
- 4.3.4. 96-well thermal cycler with heated lid
- 4.3.5. Microplate centrifuge
- 4.3.6. Vortex
- 4.3.7. DNA template (Prepared as described in section 4.5)
- 4.3.8. Nextera XT Library Prep Kit PulseNet, 96 samples plus EBT (Illumina, Cat. No. FC-133-1001) or Nextera XT DNA Sample Preparation Kit, 24 samples (Illumina, Cat. No. FC-131-1024) and 96 samples (Illumina, Cat. No. FC-131-1096)
  - 4.3.8.1. Box 1 of 2 (stored at -15° to -25°C):
    - 4.3.8.1.1. ATM (Amplicon Tagment Mix)
    - 4.3.8.1.2. TD (Tagment DNA Buffer)
    - 4.3.8.1.3. NPM (Nextera PCR Master Mix)
    - 4.3.8.1.4. RSB (Resuspension Buffer)
  - 4.3.8.2. Box 2 of 2 (stored at 2° – 8°C or room temperature):
    - 4.3.8.2.1. NT (Neutralize Tagment Buffer)
- 4.3.9. NexteraXT Index Kit for 96 indices, 384 samples (Illumina, Cat. No. FC-131-1002) (stored at -15° to -25°C) and replacement caps (included in index kit order, otherwise contact Illumina customer support to receive replacement caps)
- 4.3.10. MiSeq Reagent Kit: v2 300 or 500 Cycles (Illumina, Cat. No. MS-102-2002 or MS-102-2003), and v3 600 cycles (Illumina, Cat. No. MS-102-3003)

**NOTE:** The v2 300 cycle kits can be used for *Salmonella* (12 samples to be run per cartridge), *Listeria* (16 samples can be run per cartridge), and *Campylobacter* (22 samples can be run per cartridge), as these organisms can be combined in a single run as long as the total genome size for all organisms

<b>VERSION:</b>	<b>REPLACED BY:</b>	<b>AUTHORIZED BY:</b>	<b>Page 3 of 46</b>
-----------------	---------------------	-----------------------	---------------------

is 60 Mb or less. The v2 500 cycle kits (2x250bp) are the minimum number of cycles for *E. coli* (16 samples run per cartridge). The v3 600 cycle kits (run at 2x250bp or 2x300bp) can also be used for greater capacity. Other organisms can be run on these cartridges for a DNA load equivalent of 80 Mb (v2 500 cycles) or 200 Mb (v3 500 cycles) if a more simplified workflow is desired and longer run times are acceptable.

- 4.3.10.1. Box 1 of 2 (stored at -15° to -20°C):
  - 4.3.10.1.1. Reagent cartridge
  - 4.3.10.1.2. Hybridization Buffer (HT1)
- 4.3.10.2. Box 2 of 2 (stored at 2° – 8°C):
  - 4.3.10.2.1. Flow cell
  - 4.3.10.2.2. Incorporation Buffer (PR2)
- 4.3.11. **OPTIONAL:** 10 nM PhiX library (Illumina, Cat. No. FC-110-3001)
  - 4.3.11.1. **NOTE:** PhiX can be run as a control to troubleshoot run issues. Can be used to troubleshoot potential library preparation or MiSeq instrument issues and run either as a spike-in of the library run or as a library *per se* in the case of instrumentation issues. See Appendix PNL32-7 for details on how to prepare this library.
- 4.3.12. Agencourt AMPure XP Beads, 60 ml (Beckman Coulter, Cat. No. A63881) or 5 ml bottles (Beckman Coulter, Cat. No. A63880) (store at 2° – 8°C)
  - NOTE:** This reagent can be stored at 2° – 8°C for up to 1 year. The 5 ml bottle is enough for 200 samples.
- 4.3.13. Ambion Magnetic Stand-96 (Life Technologies; Cat. No. AM10027)
- 4.3.14. 10 µl, 20 µl, 100 µl, 200 µl, 1000 µl single channel pipettes (two sets for working with pre- and post-PCR amplified product)
  - NOTE:** the second set (post-tagmentation/PCR amplification) should include at least one Rainin Pipet-Lite XLS P1000 (Rainin, Cat. No. L-1000XLS) with corresponding filtered sterile extra-long tips (Rainin, Cat. No. RT-L1000XF) or equivalent for piercing the film over the sample loading well and loading the sample in the cartridge.
- 4.3.15. 10 µl and 200 µl multichannel pipettes
- 4.3.16. Filtered Sterile Pipette tips
- 4.3.17. Heat block accommodating 0.5 ml or 1.5 ml tubes capable of 96°C ± 1.
- 4.3.18. 5 ml and 15 ml conical tubes
- 4.3.19. Sterile solution basins
- 4.3.20. Lint-free wipes
- 4.3.21. Lens cleaning tissue
- 4.3.22. Molecular-grade Ethanol (95-100%) (Fisher Scientific, Cat. No. BP2818-500)
- 4.3.23. 70% lab-grade Ethanol or equivalent disinfectant, for sterilizing surfaces
- 4.3.24. 1 M NaOH solution (Sigma-Aldrich, Cat. No. 72082-100ML or Fisher Scientific, Cat. No. SS266-1) (Dispense in 100µl aliquots in 1.5ml centrifuge tubes and store at -20°C. NaOH rapidly expires upon opening so a new fresh bottle is needed for MiSeq sequencing. The bottle is only good for 6 months upon opening, but frozen aliquots are good for up to 2 years)

<b>VERSION:</b>	<b>REPLACED BY:</b>	<b>AUTHORIZED BY:</b>	<b>Page 4 of 46</b>
-----------------	---------------------	-----------------------	---------------------

<b>LABORATORY STANDARD OPERATING PROCEDURE FOR PULSENET NEXTERA XT LIBRARY PREP AND RUN SETUP FOR THE ILLUMINA MISEQ</b>	<b>CODE: PNL32</b>				
	Effective Date: <table border="1"> <tr> <td>1</td> <td>27</td> <td>16</td> </tr> </table>			1	27
1	27	16			

- 4.3.25. EBT (Elution Buffer with 0.1% Tween 20)
  - 4.3.25.1. For in-house preparation: Add 100 µl of Tween 20 to 99.9 ml of 10 mM Tris-HCl, pH 8.0
- 4.3.26. Tween 20 (Sigma-Aldrich, Cat. No. P9416-50ML)

**NOTE: Prepare only 1 L of 0.5% Tween 20 solution at a time so that the buffer can be used in a timely manner. This will also prevent separation of the detergent over time due to preparation in larger quantities.**
- 4.3.27. Sodium Hypochlorite (bleach) (Suggested: Fisher Scientific, Cat. No. SS290-1)
- 4.3.28. Reservoir tube provided by Illumina for the bleach wash. Contact Technical Support to request them
- 4.3.29. 1 M Tris-HCl, pH 8.0
- 4.3.30. 96-well storage (MIDI) plates (Fisher Scientific, Cat. No. AB-0859)
- 4.3.31. Hard-Shell Thin-Wall 96-Well Skirted PCR Plates (“TCY” plates) (Suggested: Bio-Rad, Cat. No. HSP-9601; 50-pack)
- 4.3.32. Microseals ‘A’ film (Bio-Rad, Cat. No. MSA-5001)
- 4.3.33. Microseal ‘B’ adhesive seals (Bio-Rad, Cat. No. MSB-1001)
- 4.3.34. DNase-free, molecular -grade water
- 4.3.35. 1.5 ml microcentrifuge tubes
- 4.3.36. Ice buckets / containers
- 4.3.37. Pipet-Aid
- 4.3.38. 1 ml serological pipets (for formamide waste removal from the cartridge post-run)
- 4.3.39. Microfuge for low (up to 6,000 rpm) rpm spinning
- 4.3.40. Wash bottles containing:
  - 4.3.40.1. 0.5% Tween 20
  - 4.3.40.2. 100% Ethanol
  - 4.3.40.3. DNase-free, molecular -grade water
- 4.3.41. Wash Tray (supplied by Illumina)
- 4.3.42. **OPTIONAL:** UPS back-up for the MiSeq (Recommended: Cyberpower AVR Series Line Interactive 1.5 kVA UPS, Cat. No. IM1M14017)
- 4.3.43. **OPTIONAL:** External encrypted hard drive for data transfer if BaseSpace is not available (Suggested: Ironkey H200 External Biometric Hard Drive by Imation, 320 GB – 1 TB)

**4.4. Day 0:**

**4.4.1. Isolate preparation**

- 4.4.1.1. Streak an isolated colony from test cultures to TSA-SB plate (or comparable media). Incubate cultures at 37°C ± 1 for 16 – 24 hours.

**NOTE:** From the original slant streak out a plate for isolation, select a colony from this plate then streak it out for DNA extraction. Minimize the number of passages from slant to plate used for DNA isolation.

<b>VERSION:</b>	<b>REPLACED BY:</b>	<b>AUTHORIZED BY:</b>	<b>Page 5 of 46</b>
-----------------	---------------------	-----------------------	---------------------

**4.5. Day 1:**

**4.5.1. DNA extraction / template preparation using the Qiagen DNeasy Blood & Tissue Kit**

**NOTE:** If using the Promega Wizard DNA extraction procedure, see Appendix PNL32-1. A minimum of 4 - 6 hours must be allocated to complete the process using this kit.

**4.5.1.1. Before beginning this procedure, first:**

4.5.1.1.1. Pre-heat water bath or heat block to 56°C ± 1.

4.5.1.1.2. Check DNeasy kit reagents and dilute where necessary (i.e. ethanol needs to be added to both wash buffers prior to the very first use).

4.5.1.1.3. Prepare the elution buffer, 10 mM Tris HCl pH 8.0, as follows:

4.5.1.1.3.1. Add 10 ml of 1 M Tris-HCl, pH 8.0 to 990 ml molecular grade water. Invert several times to mix (store at 2 – 8°C).

4.5.1.1.4. For Gram positive DNA extractions: Prepare the enzymatic lysis buffer (ELB): 20 mM Tris, 2 mM EDTA, 1.2 % Triton:

5 ml 1 M Tris-HCl, pH 8.0

1 ml 0.5 M EDTA

3 ml Triton X-100

- Bring the volume up to 250 ml with molecular-grade water and mix gently to incorporate Triton (this may be done with a stir bar and stir plate).

**NOTE:** The 250 ml stock of enzymatic lysis buffer will last for 1 year at room temperature (15 – 30°C).

**4.5.1.2. Cell lysis of Gram positive organisms:**

4.5.1.2.1. Label one spin column and two 1.5 ml microcentrifuge tubes for each isolate, and prepare two additional collection tubes per sample.

**NOTE:** Only one 1.5 ml microcentrifuge tube for each isolate is required at this point.

4.5.1.2.2. Add 20 mg/ml of lysozyme to appropriate amount of the lysis buffer within 24 hours of use. For example, for 18 isolates: 3420 µl ELB + 68.4 mg lysozyme. See the following table for additional calculations.

<b>VERSION:</b>	<b>REPLACED BY:</b>	<b>AUTHORIZED BY:</b>	<b>Page 6 of 46</b>
-----------------	---------------------	-----------------------	---------------------

Num. of samples + 1 extra	Total ELB volume ( $\mu$ l)	Lysozyme (mg)
(5+1) = 6	1080	21.6
(10+1) = 11	1980	39.6
(18+1) = 19	3420	68.4
(20+1) = 21	3780	75.6

4.5.1.2.3. Dispense 180  $\mu$ l of prepared ELB-lysozyme mixture to each labeled 1.5 ml microcentrifuge tube.

**NOTE:** Thoroughly mix to ensure the lysozyme is fully incorporated before dispensing.

4.5.1.2.4. Fill a 1  $\mu$ l inoculating loop with overnight growth (see image below) and add to the 180  $\mu$ l ELB-lysozyme mixture in the 1.5 ml microcentrifuge tube. Vortex at high speed for approximately 5 – 10 seconds.



4.5.1.2.5. Incubate at  $56 \pm 1^\circ\text{C}$  for 30 min.

**NOTE:** Vortexing every 10 minutes throughout the incubation process will facilitate and improve cell lysis.

4.5.1.2.6. Add 4  $\mu$ l of RNase A solution and vortex at medium speed (e.g. speed '7' on a Daigger Vortex Genie) for 5 – 10 seconds.

4.5.1.2.7. Incubate at room temperature for 3 – 5 minutes.

4.5.1.2.8. Add 25  $\mu$ l Proteinase K and 200  $\mu$ l Buffer AL, and vortex for 5 – 10 seconds at medium speed.

4.5.1.2.9. Incubate at  $56 \pm 1^\circ\text{C}$  for 30 min.

4.5.1.2.10. Add 200  $\mu$ l of 95% – 100% ethanol and vortex for 5 – 10 seconds at medium speed.

**NOTE:** A clear or white precipitate may form on addition of ethanol. It is essential to apply all of the precipitate to the DNeasy spin column.

**4.5.1.3. Cell lysis of Gram negative organisms:**

4.5.1.3.1. Label one spin column and two 1.5 ml microcentrifuge tubes for each isolate, and prepare two additional collection tubes per sample.

**NOTE:** Only one 1.5 ml microcentrifuge tube for each isolate is required at this point.

<b>VERSION:</b>	<b>REPLACED BY:</b>	<b>AUTHORIZED BY:</b>	<b>Page 7 of 46</b>
-----------------	---------------------	-----------------------	---------------------

- 4.5.1.3.2. Dispense 180 µl of Buffer ATL to labeled 1.5 ml microcentrifuge tubes.
- 4.5.1.3.3. Fill a 1 µl inoculating loop with (see above image) overnight growth and add to 180 µl ATL Buffer in a 1.5 ml microcentrifuge tube, and vortex at high speed for approximately 5 – 10 seconds.
- 4.5.1.3.4. Add 20 µl Proteinase K to each sample tube and vortex at high speed for 5 – 10 seconds.
- 4.5.1.3.5. Incubate at 56°C ± 1 for 1 – 3 hours, vortexing every 20 minutes throughout the incubation process to facilitate and improve cell lysis.
- 4.5.1.3.6. Add 4 µl of RNase A solution and vortex at medium speed (e.g. ‘7’ on a Daigger Vortex Genie) for 5 – 10 seconds.
- 4.5.1.3.7. Incubate at room temperature for 3 – 5 min.
- 4.5.1.3.8. Add 200 µl Buffer AL and vortex for 5 – 10 seconds.
- 4.5.1.3.9. Add 200 µl of 95% – 100% ethanol and vortex 5 – 10 seconds.

**NOTE:** A clear or white precipitate may form on addition of Buffer AL. It is essential to apply all of the precipitate to the DNeasy spin column.

**4.5.1.4. DNA clean-up and elution:**

- 4.5.1.4.1. Pour the mixture into a DNeasy spin column set in a 2 ml collection tube.
- 4.5.1.4.2. Centrifuge at 10,000 rpm for 1 min and discard the flow-through and collection tube.
- 4.5.1.4.3. Place the DNeasy spin column in a new 2 ml collection tube and add 500 µl Buffer AW1 (invert buffer several times to mix before use).
- 4.5.1.4.4. Centrifuge at 10,000 rpm for 1 min and discard the flow-through and collection tube.
- 4.5.1.4.5. Place the DNeasy spin column in a new 2 ml collection tube and add 500 µl Buffer AW2 (invert buffer several times to mix before use).
- 4.5.1.4.6. Centrifuge at 13,000 – 14,000 rpm for 3 min to collect the flow-through in the collection tube and dry the membrane. Discard the flow-through and collection tube.

**OPTIONAL:** During this step, prepare a tube with enough 10 mM Tris-HCl pH 8.0 for 100 µl per sample + 1 extra, and incubate at 56°C ± 1 while samples are centrifuging.

- 4.5.1.4.7. Place the DNeasy spin column in a clean, labeled 1.5 ml microcentrifuge tube and add 100 µl 10 mM Tris-HCl pH 8.0 directly onto the DNeasy membrane. Be sure not to touch the membrane with the pipet tip.
- 4.5.1.4.8. Incubate at room temperature for 3 – 5 min.
- 4.5.1.4.9. Centrifuge at 10,000 – 11,000 rpm for 1 min to collect the eluate in the new tube.
- 4.5.1.4.10. The DNA is now ready for quality control.



**4.5.2. DNA Quality Control: Quantification Using the Qubit 2.0 or 3.0 Fluorometer and Quality check using the Nanodrop 2000 Spectrophotometer**

**4.5.2.1. Quantification Using the Qubit 2.0 or 3.0 Fluorometer**

4.5.2.1.1. **NOTE:** The Broad Range (BR) kit is designed to be accurate for initial sample concentrations from 100 pg/μl to 1,000 ng/μl. Use for quantitation of product containing higher concentrations of DNA (i.e. DNA extractions). The High Sensitivity Kit (HS) is designed to be accurate for initial sample concentrations from 10 pg/μl to 100 ng/μl. Use for quantitation of product containing a lower concentration of DNA (i.e. PCR product).

**4.5.2.1.2. Sample Preparation**

4.5.2.1.2.1. Set up and label one assay tube for each sample and the two standards.

4.5.2.1.2.2. Prepare the Qubit working solution using a 1:200 ratio of Qubit Reagent to Qubit Buffer for each sample and the two standards. See table for an example of calculations.

**NOTE:** Store the HS / BR Reagent away from light exposure, as it contains fluorescent components.

Working Solution reagents	1x	8 samples + 2 standards
<b>Qubit HS or BR Reagent</b>	1 μl	= 10 μl
<b>Qubit HS or BR Buffer</b>	199 μl	= 1990 μl
<b>Total</b>	200 μl	= 2000 μl

**NOTE:** If desired, increase the number of samples used in the calculation by at least one to ensure sufficient volume.

4.5.2.1.2.3. For each standard, dispense 190 μl of working solution and add 10 μl of the Qubit standards into the appropriately labeled assay tubes.

4.5.2.1.2.4. Dispense 198 μl Working Solution and 2 μl of input DNA to each sample assay tube. The final volume for each sample and standard should be 200 μl.

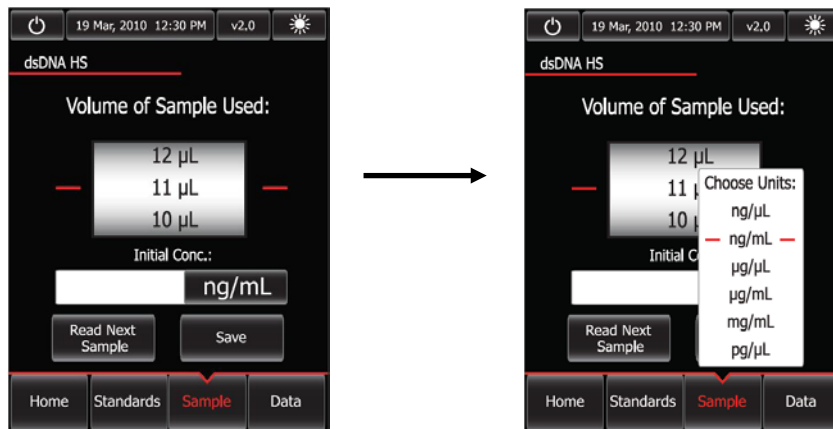
4.5.2.1.2.5. Vortex all tubes for 2 – 3 seconds.

4.5.2.1.2.6. Incubate all tubes for a minimum of 2 minutes at room temperature and read within 1 hr. The short time frame is required to minimize the exposure of the reagent to light.

**NOTE:** Ensure that the assay is performed entirely at room temperature. Do not hold the assay tubes in your hand before a measurement, because holding the tubes warms the solution and results in a low reading.

**4.5.2.1.3. Qubit 2.0 Fluorometer Operation**

- 4.5.2.1.3.1. Touch the screen to activate the device.
- 4.5.2.1.3.2. When prompted to choose the sample type, select “DNA”.
- 4.5.2.1.3.3. When prompted to choose the assay type select “dsDNA Broad Range” or “dsDNA High Sensitivity” depending on the assay used.
- 4.5.2.1.3.4. When asked whether new standards are available, select “yes”.
- 4.5.2.1.3.5. Insert Standard 1 and push “read”.
- 4.5.2.1.3.6. Insert Standard 2 and push “read”.  
**NOTE:** If the instrument is unable to read the standards, start the process over by preparing new standards and samples using the instructions outlined in step 4.5.2.1.2.1.
- 4.5.2.1.3.7. Insert the first user sample and push “read.”
- 4.5.2.1.3.8. Once the initial read is complete, push “calculate stock concentration” and select 2 µl on the volume roller wheel, in the Dilution Calculator Screen (the units should be ng/µl when proceeding with Nextera XT library prep).



- 4.5.2.1.3.9. Push “Save”. A progress bar will appear indicating that the displayed data is being saved to the instrument.  
**NOTE:** The “Save” button can be pushed multiple times without creating duplicate entries.

<b>VERSION:</b>	<b>REPLACED BY:</b>	<b>AUTHORIZED BY:</b>	<b>Page 10 of 46</b>
-----------------	---------------------	-----------------------	----------------------

4.5.2.1.3.10. Proceed to the next user sample and repeat steps 4.4.3.1.3.7. – 4.4.3.1.3.9. until all samples have been read.

4.5.2.1.3.11. **Data Transfer**

4.5.2.1.3.11.1. When all samples have been read, push the “data” button at the bottom right of the screen.

4.5.2.1.3.11.2. Insert the flash drive in the designated slot. Highlight the reads for the samples of interest and push the flash drive icon on the lower left of the screen to transfer the data. A progress bar will be displayed to show the status of the process.

**NOTE:** A green dot on the USB icon indicates that the instrument recognizes the flash drive. If the flash drive is not present, not inserted properly, or cannot be recognized, a red dot will be displayed instead.

4.5.2.1.3.11.3. Remove the flash drive. The saved .CSV file can now be opened in Microsoft Excel on any computer workstation.

4.5.2.1.3.11.4. Samples are listed, from top to bottom, **in the order of the most recently read to the first read.**

4.5.2.1.3.11.5. Rename the samples accordingly. The default sample names under the “Name” column when the file is opened in Excel will be the time at which each sample was read.

**OPTIONAL:** DNA concentration values can also be written down in lieu of using the flash drive, primarily for situations where only a few samples have been quantitated.

**NOTE: You will need at least 10 ng/μl to proceed with library prep.**

4.5.2.1.4. **Qubit 3.0 Fluorometer Operation**

4.5.2.1.4.1. Touch the screen to activate the device.

4.5.2.1.4.2. When prompted to choose the sample type select “dsDNA.”

4.5.2.1.4.3. When prompted to choose the assay type select “dsDNA Broad Range” or “dsDNA High Sensitivity” depending on the assay used.

4.5.2.1.4.4. Touch “read standards.”

4.5.2.1.4.5. At the prompt, insert standard 1 and select “read standard 1.”

4.5.2.1.4.6. At the prompt, insert standard 2 and select “read standard 2.”

**NOTE: If the instrument is unable to read the standards, start the process over by preparing new standards and samples using the instructions outlined in step 4.5.2.1.2.1.**

4.5.2.1.4.7. Select “run samples.”

- 4.5.2.1.4.8. In the sample volume screen, select the sample volume (1-20  $\mu$ L) by touching the + or – buttons on the wheel.
- 4.5.2.1.4.9. Select the units for the output sample concentration from the pulldown menu.



- 4.5.2.1.4.10. Insert a sample tube into the sample chamber, close the lid, and select “read tube.”
- 4.5.2.1.4.11. Proceed to the next user sample and repeat step 4.5.2.1.4.10. until all samples have been read.

**NOTE:** If the results are within the assay’s range, the concentration values are displayed. The top value (in large font) is the concentration of the original sample. The bottom value is the dilution concentration.

**4.5.2.1.4.12. Data Transfer**

- 4.5.2.1.4.12.1. Select “data.”

**NOTE:** The blue chevron above the sample number reverses the order of the data entries.

- 4.5.2.1.4.12.2. Insert the flash drive in the designated slot and select “export.”
- 4.5.2.1.4.12.3. Select the assay to be exported to the flash drive.

**NOTE:** The screen will display all assays that have been saved onto the instrument in chronological order, the most recent will be at the top.

- 4.5.2.1.4.12.4. Select “export.”

**NOTE:** The export data screen displays the number of successful samples saved onto the flash drive.

- 4.5.2.1.4.12.5. Select “done.”

- 4.5.2.1.4.12.6. Touch the home icon.

- 4.5.2.1.4.12.7. Remove the flash drive. The saved .CSV file can now be opened in Microsoft Excel on any computer workstation

- 4.5.2.1.4.12.8. Samples are listed, from top to bottom, **in the order of the most recently read to the first read.**

<b>VERSION:</b>	<b>REPLACED BY:</b>	<b>AUTHORIZED BY:</b>	<b>Page 12 of 46</b>
-----------------	---------------------	-----------------------	----------------------

4.5.2.1.4.12.9. Rename the samples accordingly. The default sample names under the “Name” column when the file is opened in Excel will be the time at which each sample was read.

**OPTIONAL:** DNA concentration values can also be written down in lieu of using the flash drive, primarily for situations where only a few samples have been quantitated.

**NOTE: You will need at least 10 ng/μl to proceed with library prep.**

**4.5.2.2. Quality Check Using the Nanodrop 2000 Spectrophotometer**

4.5.2.2.1. Select the Nanodrop 2000 icon on the desktop of the Nanodrop workstation.

4.5.2.2.2. Select the appropriate application from the screen, in this case “nucleic acid”, and follow prompts to begin initialization. When asked if you would like to open up your last workbook, choose “yes” if you would like to append results to previously saved data, or choose “no” if you would like to begin a new workbook.

**NOTE:** The arm of the Nanodrop must be down during initialization.

4.5.2.2.3. Lift the arm of the Nanodrop and use a lint-free laboratory wipe dampened with distilled H<sub>2</sub>O to wipe down the top and bottom pedestals.

4.5.2.2.4. Pipette 2 μl of blanking solution (10 mM Tris-HCl pH 8.0) onto the bottom pedestal and lower the arm after loading. Ensure that “Add to report” is selected in the upper left quadrant of the screen so that measurements are saved, and click the “Blank” button on the screen (upper left).

**NOTE:** Ensure that there are no bubbles in the sample on the stand so that an accurate read is obtained.

4.5.2.2.5. After the instrument has read the blank, lift the arm and wipe both pedestal and arm with a dry laboratory wipe to ready the Nanodrop for measuring samples.

4.5.2.2.6. Pipette 2 μl of undiluted DNA sample onto the bottom pedestal and lower the arm.

4.5.2.2.7. Enter the sample name in the “Sample ID” field (upper right) and click “Measure” (upper left). When the measurement has been taken, the results will populate the screen.

**NOTE:** After the first sample is measured, follow the prompt to save your workbook.

4.5.2.2.8. You must save your workbook before continuing.

4.5.2.2.9. Wipe both pedestals clean with a lint-free wipe dampened with dH<sub>2</sub>O and repeat steps 4.5.2.2.6. – 4.5.2.2.9. for all samples.

- 4.5.2.2.10. After all samples have been measured, click on the “Reports” icon in the lower left quadrant of the screen to print and/or export data if desired.
- 4.5.2.2.11. The value for the 260/280 field (i.e. protein content) should be between **1.75 and 2.05 (this includes a 0.05 or 3% error rate)**.  
**NOTE:** If 260/280 values are consistently within range over the course of several runs, then it is not necessary to routinely read the values on the Nanodrop for subsequent extractions.
- 4.5.2.2.12. Store DNA extractions to be used within two weeks at 2 – 8°C. For long term storage, keep extractions at -15 to -80°C.

**4.6. Day 1 or 2:**

**NOTE:** The steps for Sample Plate and Sample Sheet setup in the Experiment Manager program on the Illumina MiSeq can be completed at any point in this protocol, but must be performed prior to the start of the run. When sequencing less than 7 samples or for newly trained laboratory personnel, we recommend creating the Sample Plate and Sample Sheet at the beginning of the library prep. **Refer to Appendix PNL32-3** for detailed instructions on how to complete these steps. **Additionally, be sure to strictly adhere to the Sample Name (WGSID-labID-MXXXX-YYMMDD; e.g. PNUSAE123456-CDC-M1234-160101), Sample ID (WGS ID only; e.g. PNUSAE123456 for PulseNet, or GenomeTrakr equivalent), and Run ID (labID-MXXXX-YYMMDD) naming formats to ensure that the data is appropriately filtered in downstream analysis workflows.**

**4.6.1. Library Prep**

- 4.6.1.1. Enter the 260/280 Nanodrop values, the Genome Size Estimate and the DNA concentrations determined from the Qubit BR dsDNA Assay Kit (Section 4.5.2.1) into the appropriate columns on the Initial Dilution tab of the Nextera XT Library Prep Workbook (Appendix PNL32-4).

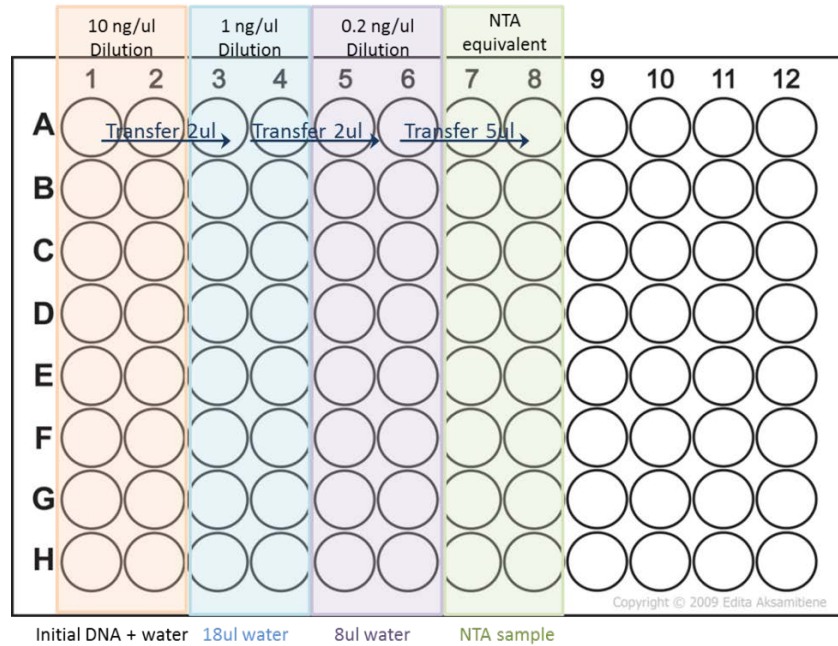
**NOTE1: When preparing to set up a run, consider the total DNA load allowance for the reagent kit to be used prior to selecting the number of isolates. The DNA load (sum of Mb for all isolates) for a v2 500 cycle cartridge is ~80 Mb, and ~60 Mb for a v2 300 cycle cartridge. For example, for a v2 500 cycle cartridge 16 isolates with a 5 Mb genome could be prepped for a total DNA load of 80 Mb, or 200 Mb for a v3 500 cycle run. Alternatively, 8 isolates with a 5 Mb genome, 11 isolates with a 3 Mb genome, and 4 isolates with a 1.6 Mb genome could also be run with the v2 500 cycle kit.**

**NOTE2: The genome size estimates for *E. coli*, *Salmonella*, *Campylobacter*, *Listeria*, *Vibrio cholerae*, and other *Vibrio spp.* are as follows: *E. coli* and *Salmonella* – 5 million bases (or megabases, Mb); *Campylobacter* – 1.6 million bases; *Listeria* – 3 million bases;**

<b>VERSION:</b>	<b>REPLACED BY:</b>	<b>AUTHORIZED BY:</b>	Page 14 of 46
-----------------	---------------------	-----------------------	---------------

***Vibrio cholerae* – 5 million bases; other *Vibrio spp.* – 4.5 million bases.**

- 4.6.1.2. Print the Initial Dilution tab to use as a guide for serial dilutions.
- 4.6.1.3. Label the dilution plate (as shown below) before beginning DNA dilutions.



\*Diagram illustrates initial dilution plate setup for 16 samples.

- 4.6.1.4. Follow the dilution directions on the Nextera XT Library Prep worksheet to normalize DNA concentration to 0.2 ng/μl, adding water first and then the input DNA.

**NOTE: Tagmentation and PCR setup should be performed in a clean space separate from all other post-PCR processes in order to reduce the possibility of cross-contamination.**

- 4.6.1.5. Pipette up and down approximately 5 – 10 times to thoroughly mix each dilution before proceeding to the next one. **This is VERY IMPORTANT.** Accurate DNA quantitation is essential for a successful library prep and subsequent cluster generation on the MiSeq.

**4.6.1.6. Tagmentation of Input DNA**

- 4.6.1.6.1. Turn on a thermal cycler before performing the following procedure as some steps below are time sensitive.
- 4.6.1.6.2. Remove ATM and TD from -15 to -25°C freezer and thaw at room temperature. Store on ice until ready to use.
- 4.6.1.6.3. Invert (do not vortex) the ATM and TD 3 – 5 times.
- 4.6.1.6.4. Remove the new vial of NT from the 2 – 8°C refrigerator.

<b>VERSION:</b>	<b>REPLACED BY:</b>	<b>AUTHORIZED BY:</b>	<b>Page 15 of 46</b>
-----------------	---------------------	-----------------------	----------------------

**NOTE:** As a convenience, NT may be stored at room temperature near the thermal cycler.

- 4.6.1.6.5. Visually inspect NT to insure there is no precipitate. If there is a precipitate, vortex until all particulates are resuspended.
- 4.6.1.6.6. Selecting two new lanes (“NTA equivalent” in the above plate diagram) from the same plate used for the dilutions, add 10 µl of TD buffer to each sample well.
- 4.6.1.6.7. Add 5 µl of input DNA at 0.2 ng/µl (1 ng total) to each sample well containing TD buffer. Change tips between samples. A multichannel pipette can be used.
- 4.6.1.6.8. Add 5 µl of ATM to each sample well containing input DNA and TD buffer, changing tips between samples.  
**NOTE: Tagmentation is time sensitive. It is important to perform steps 4.6.1.6.2. – 4.6.1.6.14. quickly.**
- 4.6.1.6.9. Using a multichannel pipette, gently pipette up and down 5 times to mix. Change tips between samples. The total volume in each well is 20 µl.
- 4.6.1.6.10. Cover the plate (now the NTA plate) with a fresh microseal A. Make sure the seal is tightly sealed over the sample wells.
- 4.6.1.6.11. Centrifuge the NTA at 800 – 1200 rpm (or 280 x g) for approximately 1 minute.
- 4.6.1.6.12. Place the NTA in the 96-well block of thermal cycler.
- 4.6.1.6.13. Upon initial set up, create and save a “TAGMENT” program on the thermocycler with the following conditions:

55°C for 5 minutes  
 Hold at 10°C

**NOTE:** Because this step is time and temperature sensitive, **do not leave** the thermal cycler. TAGMENT run time is less than 7 minutes.

- 4.6.1.6.14. Run the “TAGMENT” Program.
- 4.6.1.6.15. Aliquot 7 µl of NT per sample well into strip tubes during TAGMENT run time.  
**NOTE:** Aliquots of NT may be prepped ahead of time.
- 4.6.1.6.16. As soon as the sample temperature reaches 10°C, carefully remove the microseal and add 5 µl of NT to each well.  
**NOTE: Because this step is time and temperature sensitive, use a multichannel pipette to add the NT.**
- 4.6.1.6.17. Gently pipette up and down 3-5 times to mix.
- 4.6.1.6.18. Cover the plate with a fresh microseal.
- 4.6.1.6.19. Centrifuge the plate at 800 - 1200 rpm (or 280 x g) for approximately 1 minute.
- 4.6.1.6.20. Incubate the plate at room temperature for at least 5 minutes.

<b>VERSION:</b>	<b>REPLACED BY:</b>	<b>AUTHORIZED BY:</b>	<b>Page 16 of 46</b>
-----------------	---------------------	-----------------------	----------------------



**4.6.1.7. Amplification of Tagmented DNA**

4.6.1.7.1. Remove NPM and the appropriate Index Primers from -15 to -25°C storage and thaw at room temperature (see Illumina’s Dual Indexing Principles and Pooling Guidelines, pg. 44). Store on ice until ready to use.

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

4.6.1.7.2. It is recommended to maintain color balance for each base of the index read being sequenced and to calibrate the cameras. Otherwise, index read sequencing could fail due to registration failure. Attempt to use at least two unique and compatible barcodes for each index (index 1 and index 2). Indices are color balanced in pairs 701/702, 703/704, and 705/706 for index 1, as well as 503/504 and 505/506 for index 2. When choosing indices for a run, ensure there is a set of at least one color balanced index pair per sequencing run (e.g. 701/503, 701/504, 702/503, 702/504). See Illumina’s technical note for examples of “good” and “bad” color balanced pairs:

[http://www.illumina.com/documents/products/technotes/technote\\_nextera\\_low\\_plex\\_pooling\\_guidelines.pdf](http://www.illumina.com/documents/products/technotes/technote_nextera_low_plex_pooling_guidelines.pdf).

4.6.1.7.3. **It is also important to rotate through the indices from run to run to prevent read carryover. The same index pairs should not be used within 6 consecutive runs on the same sequencer so as to reduce the amount of carryover generated during the run, which can interfere with downstream analyses. The following table illustrates a suggested index rotation scheme. This index rotation can also be utilized in the Nextera XT Library Prep Workbook by selecting the preferred run option from the dropdown menu above the Index 1/2 columns.**

RUN1:	Index 1	Index 2	RUN2:	Index 1	Index 2	RUN3:	Index 1	Index 2	RUN4:	Index 1	Index 2	RUN5:	Index 1	Index 2	RUN6:	Index 1	Index 2
N701	S517		N701	S503		N701	S505		N701	S507		N709	S517		N709	S505	
N702	S517		N702	S503		N702	S505		N702	S507		N710	S517		N710	S505	
N703	S517		N703	S503		N703	S505		N703	S507		N711	S517		N711	S505	
N704	S517		N704	S503		N704	S505		N704	S507		N712	S517		N712	S505	
N705	S517		N705	S503		N705	S505		N705	S507		N709	S502		N709	S506	
N706	S517		N706	S503		N706	S505		N706	S507		N710	S502		N710	S506	
N707	S517		N707	S503		N707	S505		N707	S507		N711	S502		N711	S506	
N708	S517		N708	S503		N708	S505		N708	S507		N712	S502		N712	S506	
N701	S502		N701	S504		N701	S506		N701	S508		N709	S503		N709	S507	
N702	S502		N702	S504		N702	S506		N702	S508		N710	S503		N710	S507	
N703	S502		N703	S504		N703	S506		N703	S508		N711	S503		N711	S507	
N704	S502		N704	S504		N704	S506		N704	S508		N712	S503		N712	S507	
N705	S502		N705	S504		N705	S506		N705	S508		N709	S504		N709	S508	
N706	S502		N706	S504		N706	S506		N706	S508		N710	S504		N710	S508	
N707	S502		N707	S504		N707	S506		N707	S508		N711	S504		N711	S508	
N708	S502		N708	S504		N708	S506		N708	S508		N712	S504		N712	S508	

<b>VERSION:</b>	<b>REPLACED BY:</b>	<b>AUTHORIZED BY:</b>	<b>Page 17 of 46</b>
-----------------	---------------------	-----------------------	----------------------

- 4.6.1.7.4. Gently invert tubes to mix and briefly centrifuge.  
**NOTE:** It is very important to centrifuge the index tubes. Liquid can become trapped inside the cap after inverting and can be inadvertently lost.
- 4.6.1.7.5. Add 15 µl of NPM to each well of the NTA containing tagmented DNA. Change tips between samples.
- 4.6.1.7.6. Add 5 µl of the appropriate Index 1 primer (orange caps) to the designated wells of the NTA, discarding caps and replacing with fresh orange caps as each primer is used.
- 4.6.1.7.7. Add 5 µl of the appropriate Index 2 primer (white caps) to the designated wells of the NTA. Discard the caps and replace with fresh white caps as each index primer is used.
- 4.6.1.7.8. Using a multichannel, gently pipette up and down to mix.
- 4.6.1.7.9. Securely cover the plate with a fresh microseal A.  
**NOTE:** This is a critical step. The plate needs to be tightly sealed with a microseal to avoid sample evaporation. Using a thermal cycler with a tight-fitting lid will ensure that a minimal amount of product is lost.
- 4.6.1.7.10. Centrifuge at 800-1200 rpm (or 280 x g) for approximately 1 minute.
- 4.6.1.7.11. Place the NTA in a thermal cycler.
- 4.6.1.7.12. Upon initial set up, create and save a “NAP\_XT” program on the thermocycler, 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
- 4.6.1.7.13. Run the “Nap\_XT” program.

**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 GENOME TRAKR AND ILLUMINA NEXTERA XT PROTOCOLS.**

<b>VERSION:</b>	<b>REPLACED BY:</b>	<b>AUTHORIZED BY:</b>	<b>Page 18 of 46</b>
-----------------	---------------------	-----------------------	----------------------

**4.6.1.8. Pre-Clean Up Qubit Quantification**

**NOTE:** 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.

- 4.6.1.8.1. Remove the NTA from thermal cycler or 4°C.
- 4.6.1.8.2. Centrifuge the NTA for approximately 1 minute to collect condensation.
- 4.6.1.8.3. Determine the concentration (ng/μl) of each sample in the NTA using the Qubit dsDNA HS Assay Kit. (See section 4.5.2.1. DNA Quantification using the Qubit 2.0 or 3.0 Fluorometer for instructions.)
- 4.6.1.8.4. Record concentrations on the Normalization and Pooling worksheet (Appendix PNL32-5, column D) of the NexteraXT Library Prep Worksheet.

**NOTE:** Samples for which DNA concentration is below the limit of detection should be discarded.

**4.6.1.9. PCR Product Clean-Up**

- 4.6.1.9.1. Bring the AMPure XP beads and RSB to room temperature.
- 4.6.1.9.2. Prepare a fresh 80% ethanol dilution.
  - 4.6.1.9.2.1. Approximately 0.5 ml is needed per sample. For 16 samples, mix 6.4 ml 100% ethanol with 1.6 ml dH<sub>2</sub>O.

**NOTE:** It is important to always prepare fresh 80% ethanol for wash steps. Ethanol can absorb water from air impacting sequencing results.

- 4.6.1.9.2.2. Centrifuge the NTA at 800-1200 rpm (or 280 x g) for approximately 30 seconds to collect condensation. (If the optional Pre-Clean Up Qubit Quantification was performed, this step may be skipped.)
- 4.6.1.9.2.3. Label a MIDI plate CAA (Clean Amplified Plate).
- 4.6.1.9.2.4. Transfer 40 μl of the PCR product for each sample from the NTA to the CAA.
- 4.6.1.9.2.5. Vortex the AMPure XP beads until well suspended (15 – 30 seconds).
- 4.6.1.9.2.6. Add 20 μl (0.5X volume) of AMPure XP beads to each well of the CAA.
  - NOTE:** Avoid bead carryover by confirming no liquid droplets are on your pipette tip. This will affect the ratio of beads to PCR reaction and affects the insert size.
- 4.6.1.9.2.7. Gently pipette up and down 5 – 10 times to mix.
- 4.6.1.9.2.8. Incubate at room temperature, without shaking, for at least 5 minutes (10 minute incubation may improve library quality).
- 4.6.1.9.2.9. Place the CAA on the magnetic stand for a minimum of 2 minutes or until the supernatant has cleared.

<b>VERSION:</b>	<b>REPLACED BY:</b>	<b>AUTHORIZED BY:</b>	<b>Page 19 of 46</b>
-----------------	---------------------	-----------------------	----------------------

- 4.6.1.9.2.10. With the CAA on the magnetic stand, carefully remove and discard the supernatant.
- 4.6.1.9.2.11. If any beads are inadvertently aspirated into the tips, dispense the beads back to the CAA and let the plate rest on the magnetic stand another 2 minutes or until the supernatant has cleared.
- 4.6.1.9.2.12. With the CAA 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
- 4.6.1.9.2.13. Incubate the CAA on the magnetic stand for 30 seconds, then carefully remove and discard the supernatant by pipetting.
- 4.6.1.9.2.14. With the CAA on the magnetic stand, perform a second 80% ethanol wash.
- 4.6.1.9.2.15. Incubate the CAA on the magnetic stand for 30 seconds, then carefully remove and discard the supernatant by pipetting.
- 4.6.1.9.2.16. With the CAA on the magnetic stand, allow the beads to air-dry for 10 – 11 minutes. Use a low volume tip to remove any remaining ethanol droplets.  
**NOTE:** Exceeding the maximum air-dry period (11 minutes) can make resuspension difficult and is not recommended. Over-drying is indicated by cracks in the bead pellets.
- 4.6.1.9.2.17. Remove the CAA from the magnetic stand.
- 4.6.1.9.2.18. Vortex the RSB tube.
- 4.6.1.9.2.19. Add 53 µl of RSB to each well of the CAA.
- 4.6.1.9.2.20. Gently pipette up and down at least 10 times to mix, changing tips after each well.
- 4.6.1.9.2.21. Incubate at room temperature for 2 minutes.
- 4.6.1.9.2.22. Place the CAA on the magnetic stand for 2 minutes or until the supernatant has cleared.
- 4.6.1.9.2.23. Label a new TCY plate with “CAN” (Clean Amplified NTA Plate), the run ID number, initials, and date.
- 4.6.1.9.2.24. Carefully transfer 50 µl of the supernatant from the CAA to the CAN while still on the magnetic plate. Ensure you are not transferring any beads as this will affect the subsequent Qubit reading.

**THIS IS A SAFE STOPPING POINT. IF USING THE PULSENET SOP, SEAL THE CAN WITH MICROSEAL ‘B’ ADHESIVE AND STORE AT -15 TO -25°C FOR 1 – 3 MONTHS. IF USING THE GENOMETRAKR OR ILLUMINA NEXTERA XT SOP, THE PLATE CAN BE STORED AT 2 – 8°C FOR UP TO 2 DAYS.**

<b>VERSION:</b>	<b>REPLACED BY:</b>	<b>AUTHORIZED BY:</b>	<b>Page 20 of 46</b>
-----------------	---------------------	-----------------------	----------------------

**4.6.1.10. DNA Library QC check and Qubit Quantification**

4.6.1.10.1. Determine the concentration (ng/μl) of each sample in the CAN plate using the Qubit dsDNA HS Assay Kit. (See section 4.5.2.1., DNA Quantitation using the Qubit 2.0 or 3.0 Fluorometer for instruction.)

4.6.1.10.2. Enter the concentrations into the Qubit Output column on the Normalization and Pooling worksheet of the Nextera XT Library Prep Workbook (Appendix PNL32-5).

**NOTE1:** The DNA concentration after post-PCR clean-up should be between 1.34 ng/μl and 4 ng/μl Qubit quantification. If the measured concentration is above 4 ng/μl then assign the value for that sample as 4 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 4 ng/ul likely consist of a majority of fragments smaller than the optimal insert size (800 – 1500bp). In order to adjust for this occurrence, the samples are diluted to a higher concentration by assigning the 4 ng/ul 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 Qubit. These low concentration samples should not be diluted before pooling libraries. If 25% or more of the samples are below the ideal minimum concentration, consider delaying the run (depending on the 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.

**4.6.2. Setting up a run on the MiSeq**

**NOTE1: At least 100 GB disk space (20% of the hard drive) will be needed before a run can be started.** It is also important to **reboot the MiSeq at least once a week** (and recommended after deleting large amounts of data) as a part of routine maintenance of the machine. This will clear the virtual memory ensuring that the memory capacity for future runs is at a maximum.

**NOTE2:** The cartridge takes approximately 1 hour to thaw in a water bath at room temperature and once thawed can be stored on ice or at 2 – 8°C for up to 24 hours (see Appendix PNL32-6 for instructions). Alternatively, the cartridge can be thawed in its original packaging at 2 – 8°C (8-12 hours), and then stored at 2 – 8°C for up to 7 days. If thawing using this procedure, proceed directly to step 4.6.2.2.

**4.6.2.1. Preparing your Reagent Cartridge for the MiSeq**

4.6.2.1.1. Retrieve a pre-thawed reagent cartridge and HT1 from refrigerator or thaw according to methods in Appendix PNL32-6.

<b>VERSION:</b>	<b>REPLACED BY:</b>	<b>AUTHORIZED BY:</b>	<b>Page 21 of 46</b>
-----------------	---------------------	-----------------------	----------------------

- 4.6.2.1.2. Invert the reagent cartridge 10 times to mix the thawed reagents, and then visually inspect that all positions are thawed. Ensure the reagent in Position 1 is fully mixed and free of precipitates.
- 4.6.2.1.3. Gently tap the cartridge on the bench to reduce air bubbles in the reagents.
- 4.6.2.1.4. Place the reagent cartridge and HT1 on ice or set aside at 2 – 8°C until the sample is ready to load on the cartridge.

**4.6.2.2. Preparing Reagents and Normalizing the Libraries**

- 4.6.2.2.1. Remove a 100 µl aliquot of 1 N NaOH from freezer and place on ice to thaw.
- 4.6.2.2.2. If library in the CAN plate is frozen, thaw on ice, then centrifuge CAN plate at 800 – 1200 rpm (or 280 x g) for 1 minute in a centrifuge that accommodates microtiter plates.
- 4.6.2.2.3. Dilute Samples using the Normalization and Pooling worksheet of the Nextera XT Library Workbook (Appendix PNL32-5).
- 4.6.2.2.4. For each library, dispense EBT into a clean well of the CAN plate according to the volumes listed in the EBT column under Sample Dilution.
- 4.6.2.2.5. Next, dispense the listed volume of each library to the corresponding well containing the EBT according to the Sample column. Change tips between each sample.
- 4.6.2.2.6. Mix the CAN plate dilutions by pipetting samples up and down 5-7 times with a multichannel pipet.

**4.6.2.3. Pooling the Sample Libraries**

- 4.6.2.3.1. For each sample, transfer the volume listed in the Pooling Volume column into a single well on the CAN plate for a total pooled volume of 50 µl.  

**NOTE:** The pooling factor is based on the genome size. When genomes of different sizes are run together on the MiSeq, the amount of each single library is added proportionately to genome size to reduce the over-representation of small genomes in the pooled library.
- 4.6.2.3.2. Mix the pooled library by pipetting up and down 10 times. Place on ice.

**4.6.2.4. Denaturation of the Pooled library DNA**

- 4.6.2.4.1. Add 400 µl of molecular-grade water to the 100 µl aliquot of 1N NaOH to make a 0.2 N solution, and invert the tube several times to mix.
- 4.6.2.4.2. Combine 5 µl of the pooled sample library and 5 µl of the freshly-made 0.2 N NaOH in a new microcentrifuge tube.
- 4.6.2.4.3. Incubate for 5 minutes at room temperature to denature the DNA.

<b>VERSION:</b>	<b>REPLACED BY:</b>	<b>AUTHORIZED BY:</b>	<b>Page 22 of 46</b>
-----------------	---------------------	-----------------------	----------------------

4.6.2.4.4. **IMMEDIATELY** add 990 µl of pre-chilled HT1 to the tube containing 10 µl of denatured pooled library. If a dilution concentration of 2 nM was used, then 1 ml of a 10 pM denatured pooled library is obtained after the addition of HT1.

4.6.2.4.5. Place the denatured DNA on ice until ready to proceed to the next steps.

4.6.2.5. **Dilute to Desired Final Loading Concentration**

4.6.2.5.1. Refer back to the Normalization and Pooling worksheet (Appendix PNL32-5) for the dilution concentration (2 nM, 3 nM, or 4 nM). Then refer to the denatured pooled library table (from 2 nM, 3 nM, or 4 nM dilution) below to prepare the final dilution of the pooled library according to the final loading concentration that is appropriate for the instrument and application.

**NOTE:** The optimal final loading concentration is determined empirically for a particular instrument. Refer to previous run metrics for guidance on a target loading concentration. The 10 pM concentration is a good starting point, then increase or decrease the concentration until the cluster density falls within the 600 – 1200 K/mm<sup>2</sup> target.

4.6.2.5.2. Refer back to the worksheet for your dilution concentration. Using either the 2 nM, 3 nM, or 4 nM tables below, dilute the pooled library in enough pre-chilled HT1 to achieve the desired Final Loading Concentration.

	10 pM denatured pooled library (from 2 nM dilution)			15 pM denatured pooled library (from 3 nM dilution)			20 pM denatured pooled library (from 4 nM dilution)		
Final Loading Concentration	<b>8 pM</b>	<b>9 pM</b>	<b>10 pM</b>	<b>9 pM</b>	<b>10 pM</b>	<b>12 pM</b>	<b>10 pM</b>	<b>12 pM</b>	<b>15 pM</b>
Denatured Pooled Library	800 µl	900 µl	1000 µl	600 µl	667 µl	800 µl	500 µl	600 µl	750 µl
HT1	200 µl	100 µl	0 µl	400 µl	333 µl	200 µl	500 µl	400 µl	250 µl

4.6.2.5.3. Invert several times to mix the DNA solution.

4.6.2.5.4. Place the denatured and diluted DNA library on ice or at 2 – 8°C until you are ready to load your samples onto the MiSeq reagent cartridge.

**OPTIONAL:** The denatured PhiX control may be spiked in at this point. See Appendix PNL32-7 for instructions.

4.6.2.6. **Loading the MiSeq and Starting a Run**

4.6.2.6.1. Retrieve the Flow Cell and Incorporation buffer (PR2) that are part of the MiSeq Reagent Box v2 stored at 2 – 8°C.

4.6.2.6.2. Clean the flow cell as follows:

4.6.2.6.2.1. While wearing clean, powder-free gloves, carefully remove the flow cell from the container (leave the fluid in the container) without touching the glass of the flow cell.

4.6.2.6.2.2. Lightly rinse the flow cell assembly with molecular-grade water, making sure that both the glass and plastic casing are thoroughly rinsed of the excess salts.

4.6.2.6.2.3. Using care around the black port gasket, pat dry the flow cell and cartridge using a lint-free wipe. Take particular care in the area of the gasket and adjacent glass, making sure to remove any excess fluid.

4.6.2.6.2.4. Wet a clean piece of lens paper with 70% ethanol to clean the flow cell glass, making sure that the glass is free of streaks and fingerprints, and that the flow cell is completely free of lint or tissue fibers.

**NOTE: Do not add ethanol directly to flow cell and avoid getting ethanol on the flow cell port gasket.**

4.6.2.6.2.5. Dry any excess alcohol with a lint-free lens cleaning tissue, and visually inspect to make sure that the flow cell ports are free of obstructions and that the gasket is well-seated around the flow cell ports.

4.6.2.6.3. **Loading the Flow Cell**

4.6.2.6.3.1. On the main Sequencing screen of the computer, select “Sequence” to initiate “Load Flow Cell” screen.

4.6.2.6.3.2. Raise the flow cell compartment door, and then press the release button to the right of the white flow cell latch.

4.6.2.6.3.3. Remove the old flow cell and discard. Visually inspect the flow cell stage to make sure it is free of lint. If lint or other debris is present, clean the flow cell stage using a lint-free tissue moistened with ethanol or isopropanol. Carefully wipe the surface of the flow cell stage until it is clean and dry.

4.6.2.6.3.4. Hold the flow cell by the plastic edges near the Illumina label. Make sure the label is facing upward and place the flow cell on the flow cell stage making sure that it sits flush to the stage, and that the two openings are directly over the two prongs set in the stage.

4.6.2.6.3.5. Gently press down on the flow cell latch to close it over the flow cell. You will hear a click when the flow cell latch is secure.



- 4.6.2.6.3.6. Check the lower-right corner of the screen to confirm that the flow cell RFID was successfully read.
- 4.6.2.6.3.7. Close the flow cell compartment door.
- 4.6.2.6.3.8. Select “Next” on the Load Flow Cell screen. The Load Reagents screen will open.

4.6.2.6.4.     **Loading the Reagents**

- 4.6.2.6.4.1. Gently invert the capped bottle to mix the Incorporation buffer (PR2), and then remove the lid.
- 4.6.2.6.4.2. Open the reagent cartridge door and raise the sipper handle in the right hand compartment until it locks into place.
- 4.6.2.6.4.3. Remove the MiSeq Wash Buffer bottle, re-cap, and set aside for future use.
- 4.6.2.6.4.4. Place the PR2 bottle in the indentation where the MiSeq Wash Buffer bottle was previously located.
- 4.6.2.6.4.5. Empty the contents of the waste bottle into the appropriate waste container, if necessary.
- 4.6.2.6.4.6. Slowly lower the sipper handle. Make sure that the sippers lower into the PR2 and waste bottles.
- 4.6.2.6.4.7. Check the lower-right corner of the screen to confirm that the RFID of the PR2 bottle was read successfully.

**NOTE: If the RFID is not read by the system for any step, the software prompts you through the steps to obtain a temporary bypass code and proceed with setting up the run. For more information, see Resolving RFID Read Failure in the MiSeq System User Guide.**

- 4.6.2.6.4.8. Select “Next” on the Load Reagents screen.

4.6.2.6.5.     **Loading the Sample Libraries onto Cartridge**

- 4.6.2.6.5.1. Thoroughly dry the reagent cartridge. Again, be sure there is no water on the top of the cartridge before proceeding.
- 4.6.2.6.5.2. Heat the denatured DNA library to 96 ± 1 °C for 2 minutes in a heat block, followed by immediate cooling in an ice-water bath (combining approximately 3 parts ice and 1 part water) for at least 5 minutes prior to loading. This will assure all DNA strands are denatured.
- 4.6.2.6.5.3. Use a separate, clean 1000 µl pipette tip to pierce the foil seal over the reservoir labeled “Load Samples.”
- 4.6.2.6.5.4. Using the Rainin RT-L1000XF pipette or equivalent and corresponding tips, pipette **600 µl** of the denatured DNA library into the “Load Samples” reservoir. Load into bottom of reservoir; do not allow droplets to form on the sides.

- 4.6.2.6.5.5.        **Gently tap the cartridge on a hard surface to ensure the contents of each reagent are collected at the bottom of the reservoirs.**  
                               **NOTE: Take care to avoid touching the foil seal as you dispense the sample.**
- 4.6.2.6.5.6.        Confirm the MiSeq screen states “load reagent cartridge” and the sippers in the chiller have been raised.
- 4.6.2.6.5.7.        Open the reagent chiller door, remove the wash tray, and dry bottom of chiller compartment if necessary using paper towels.  
                               **NOTE: Do not leave the reagent chiller door open for extended periods of time.**
- 4.6.2.6.5.8.        Hold the reagent cartridge on the end with the Illumina label, and slide the reagent cartridge into the reagent chiller until the cartridge stops.
- 4.6.2.6.5.9.        Close the reagent chiller door.
- 4.6.2.6.5.10.       Check the screen to confirm that the RFID of the reagent cartridge was read successfully.
- 4.6.2.6.5.11.       Close the reagent compartment door.
- 4.6.2.6.5.12.       Select “Change Sample Sheet” to select the sample sheet.
- 4.6.2.6.5.13.       Select “Next” on the Load Reagents screen. The Review screen opens.
- 4.6.2.6.5.14.       Start the MiSeq Run
  - 4.6.2.6.5.14.1. Review Run Parameters: review Experiment Name, Analysis Workflow, and Read Length. These parameters are specified in the sample sheet. Make sure to save any changes made.
- 4.6.2.6.5.15.       Select “Next”. The Pre-Run Check screen will open.
  - 4.6.2.6.5.15.1. Review Pre-Run Check: The system performs a check of all run components, disk space, and network connections before you start the run. If any items do not pass the pre-run check, a message appears on the screen with instructions to correct the error. For more information, see Resolving Run Setup Errors in the MiSeq System User Guide.
- 4.6.2.6.5.16.       When all items successfully pass the pre-run check, you are ready to start the run.
- 4.6.2.6.5.17.       Select “Start Run”.  
                               **NOTE: Once the run has started, do not open the flow cell compartment or the reagent compartment door, or touch the instrument monitor unless you plan to stop or pause the run.** The MiSeq is sensitive to vibration and performing tasks on the instrument during a run could cause the run to halt and could adversely impact sequencing results.

4.6.3. **Day 2 or 3**

4.6.3.1. **Exporting Data from the MiSeq**

- 4.6.3.1.1. Record run statistics (i.e. Q30, cluster density, clusters passing filter, estimated yield) in the table on the worksheet after the run has finished and before proceeding to the post-run wash.
- 4.6.3.1.2. Data is found in the D drive → Illumina → MiSeqOutput → Run folder (choose the most recent run folder) → Data → Intensities → BaseCalls. Within the BaseCalls folder the raw read data is in fastq.gz format, there will be 2 files per isolate for paired reads. This data can be copied onto an external hard drive or USB drive with sufficient space and moved to a computer workstation. Please note that it takes 20 minutes to 1 hour for the MiSeq to generate data post run. For Basespace users, the data will be located in the run folder on Basespace.

**NOTE: If fastq data is unavailable for a given isolate(s) due to incorrect index assignment or sample sheet error, the run can be requeued for analysis using either MiSeq Reporter (MSR) or corrective options in BaseSpace (see Appendix PNL32-9).**

4.6.3.2. **Post-Run Wash and Reagent Disposal**

- 4.6.3.2.1. Once the run is complete, select the Start Wash option at the bottom right-hand side of the screen.
- 4.6.3.2.2. Prepare a squirt bottle containing the wash buffer (0.5% Tween 20 solution in molecular grade water) and fill each reservoir of the wash tray with the wash buffer. A bleach wash (or template line wash) can also be performed at this point.
  - 4.6.3.2.2.1. **NOTE: Prepare only 1 L of 0.5% Tween 20 solution at a time so that the buffer can be used in a timely manner. This will also prevent separation of the detergent over time when prepared in larger quantities.**
  - 4.6.3.2.2.2. **NOTE2: A post-run wash with 0.01% bleach should be performed at least once a week followed by a post-run wash with Tween 20 wash solution only. See Appendix PNL32-8 for instructions.**
- 4.6.3.2.3. Open the reagent compartment door and reagent chiller door and remove the used cartridge from the chiller. Set cartridge aside.
- 4.6.3.2.4. Slide the wash tray into the reagent chiller until it stops, then close the reagent chiller door.
- 4.6.3.2.5. Raise the sipper handle, remove the PR2 bottle and replace with the MiSeq Wash Buffer bottle (also containing the 0.5% Tween 20 solution).
- 4.6.3.2.6. Remove the waste container and dispose of contents in a chemical waste receptacle.

**NOTE: The reagent in position 8 of the cartridge contains Formamide, which must be disposed of properly through a chemical waste system.**

- 4.6.3.2.7. Return the waste container to the reagent compartment, lower the sipper arm, and close the reagent compartment door.
- 4.6.3.2.8. Select “Next” to begin the post-run wash. This will take approximately 20 minutes.
- 4.6.3.2.9. Using a Pipet-Aid and a 1 ml serological pipette, remove the Formamide waste from the position 8 reservoir of the cartridge and add to the appropriate chemical waste container (formamide waste bottle). Once the reagent has been removed, the cartridge can be disposed of in the biohazardous waste receptacle.
- 4.6.3.2.10. The flow cell, wash bottle, wash tray, and waste bottle should always remain in place while the instrument is not in use.

**4.6.3.3. Performing a Standby Wash**

- 4.6.3.3.1. If the machine will be idle for more than 2 weeks a standby wash must be performed.

**NOTE: While the instrument is not in use, the standby wash must be performed monthly.**

- 4.6.3.3.2. Select Standby Wash from the wash options on the screen.
- 4.6.3.3.3. Fill each reservoir of the wash tray with molecular-grade water containing 0.5% Tween 20.
- 4.6.3.3.4. Open the reagent compartment door and reagent chiller door and follow the instructions above for the Post-run wash for proper waste reagent removal. If this is following a post-run wash, empty and refill the wash tray and refill the MiSeq Wash Buffer bottle (also containing the 0.5% Tween 20 solution).
- 4.6.3.3.5. Once the wash tray and MiSeq Wash Buffer bottle have been replaced and the waste appropriately disposed of, close the reagent compartment door.
- 4.6.3.3.6. Select “Next” for the wash to begin. This first step of the wash will take approximately 20 minutes to complete.
- 4.6.3.3.7. After the first step of the wash is complete, remove the wash tray and refill. Refill the Wash Buffer bottle if necessary and discard the waste.

**NOTE: All liquid that accumulates in the MiSeq waste bottle should be disposed of as formamide waste, including liquid from wash cycles. All formamide waste bottles must be clearly labeled with contents, date of first use, and date of final use. Waste must be stored in designated, clearly labeled waste disposal site.**

- 4.6.3.3.8. Return the wash tray and wash bottle, close the reagent compartment door, and select “Next” to begin the second wash.

4.6.3.3.9. The standby wash takes approximately 60 minutes, and once complete, the machine may be left idle for 7 days. The standby wash must be repeated weekly while the instrument is not in use.

**4.6.3.4. Performing a Maintenance Wash**

- 4.6.3.4.1. A maintenance wash should be performed at least once a month.
- 4.6.3.4.2. Under Wash Options select Maintenance Wash and prepare wash tray and Wash Buffer bottle similarly to the standby wash.
- 4.6.3.4.3. This is a series of 3 washes that requires refilling of the wash tray after each wash cycle (30 minutes).
- 4.6.3.4.4. The maintenance wash takes approximately 90 minutes.

**4.6.3.5. MiSeq reboot to maintain virtual memory space**

- 4.6.3.5.1. **A reboot of the MiSeq should be performed once a week after washing the MiSeq and data transfer or deletion.**
- 4.6.3.5.2. On the MiSeq Controller Software select the button Manage Your Instrument then push Reboot.
- 4.6.3.5.3. It will take approximately 10 minutes for the system to reboot and start the MiSeq Control software upon reboot.

**4.6.3.6. Management of Disk Space on D: (Data) Drive of Illumina MiSeq**

- 4.6.3.6.1. The MiSeq requires 100GB of disk space free on the D drive before sequencing on the MiSeq can start. Data should be cleared from the D drive after washing the MiSeq and data transfer of fastq.gz files is complete and assuming no troubleshooting is necessary for that run.
- 4.6.3.6.2. Go to Data (D Drive)/Illumina/MiSeqOutput/(most recent run folder)/
  - 4.6.3.6.2.1. Within this folder select the “Images” and “Thumbnail images” folders and delete.
- 4.6.3.6.3. Go to Data (D Drive)/Illumina/MiSeqAnalysis/
  - 4.6.3.6.3.1. Delete any run folders excluding the most recent folder.

**NOTE: Rebooting the instrument is suggested following deletion of data in order to free up virtual memory in the system.**

**5. FLOW CHART:**

**6. REFERENCES:**

- 6.1. Illumina, Inc. Nextera XT DNALibrary Prep Reference Guide. January 2016.  
[https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry\\_documentation/samplepreps\\_nextera/nextera-xt/nextera-xt-library-prep-guide-15031942-01.pdf](https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/samplepreps_nextera/nextera-xt/nextera-xt-library-prep-guide-15031942-01.pdf).
- 6.2. MiSeq System User Guide. September 2015.  
[http://support.illumina.com/content/dam/illumina-support/documents/documentation/system\\_documentation/miseq/miseq-system-guide-15027617-01.pdf](http://support.illumina.com/content/dam/illumina-support/documents/documentation/system_documentation/miseq/miseq-system-guide-15027617-01.pdf)

<b>VERSION:</b>	<b>REPLACED BY:</b>	<b>AUTHORIZED BY:</b>	<b>Page 29 of 46</b>
-----------------	---------------------	-----------------------	----------------------

**7. CONTACTS:**

- 7.1. Eija Trees, D.V.M., Ph.D.  
 (404) 639-3672  
[EHyytia-Trees@cdc.gov](mailto:EHyytia-Trees@cdc.gov)
  
- 7.2. Ashley Sabol, M.S.  
 (404) 639-2947  
[ASabol@cdc.gov](mailto:ASabol@cdc.gov)
  
- 7.3. Jeniffer Concepcion-Acevedo, Ph.D.  
 (404) 639-2828  
[JConceptionAcevedo@cdc.gov](mailto:JConceptionAcevedo@cdc.gov)
  
- 7.4. PulseNet Central  
[pulsenet@cdc.gov](mailto:pulsenet@cdc.gov)

**8. AMENDMENTS:**

**06/04/2015:** Listed Nextera XT PulseNet kit and catalog number in section 4.3.  
**01/25/2016:**

- Added/updated catalog numbers for various reagents and kits in section 4.3. (i.e. Tris brand recommendations, Illumina reagent kits, etc.)
- Listed optimal prep volume for wash buffer
- Updated MiSeq wash procedures, including frequency and number of various washes
- Added table for suggested index rotation and removed smaller index kit
- Updated bleach wash appendix to current standards/Illumina recommendations
- Adjusted bead drying time from “10-12 minutes” to “10-11 minutes”.
- Added incubation time for ice bath in step 4.6.2.6.5.2
- Updated the Initial Dilution and Normalization and Pooling tabs of the Nextera XT Library Prep workbook (Appendices PNL32-4 and PNL32-5)
- Added Sample Sheet to Nextera XT Library Prep workbook and added instructions for export and transfer to the Illumina Experiment Manager in PNL32-3
- Added 3 nM and 4 nM normalization concentrations and calculations to final sample dilution table
- Changed step at which PhiX is spiked in and recommended percentage of PhiX to spike-in to routine runs in Appendix PNL32-7
- Updated storage conditions to include “± 1” or a range (e.g. 2-8°C instead of 4°C)

<b>VERSION:</b>	<b>REPLACED BY:</b>	<b>AUTHORIZED BY:</b>	Page 30 of 46
-----------------	---------------------	-----------------------	---------------

- Added appendix (PNL32-9) for reanalysis of a run using MiSeq Reporter or in BaseSpace

**06/23/2016:**

- Updated Sample ID and Run ID format throughout document, and added Sample ID and Run ID format to note under section 4.6.
- Revised the procedure for PNL32-1 to reflect the steps of the replacement kit, Promega Wizard Genomic DNA Purification. Changed all notations of the “ArchivePure kit” to the Promega Wizard kit in the protocol, and updated the supplies list with the catalog information.

**08/19/2016:**

- Updated Sample Name and Sample ID format requirements throughout document, and added Sample ID and Run ID format for PulseNet and GenomeTrakr networks to note under section 4.6 and Appendix 32-3.
- Added v3 600 cycle kit chemistry information to section 4.3.10 and included suggested DNA load threshold and run times.

**10/03/2016:** Updated links to Illumina documents throughout document.

<b>VERSION:</b>	<b>REPLACED BY:</b>	<b>AUTHORIZED BY:</b>	<b>Page 31 of 46</b>
-----------------	---------------------	-----------------------	----------------------

**Appendix PNL32-1**

**DNA Extraction / template preparation using the Promega Wizard Genomic DNA Purification Kit**

**1. Procedure**

**1.1. Day 1**

**1.1.1.** Streak an isolated colony from test cultures to TSA-SB plate (or comparable media). Incubate cultures at 37°C ± 1 for 16 – 24 hours.

**1.2. Day 2**

**1.2.1.** Inoculate Trypticase Soy Broth.

**NOTE:** Culture from a plate can be used as well (see image below step 4.5.1.2.4. for suggested amount of starting culture). If using culture from a plate, continue to step 1.3.1.4. for gram negative bacteria, and 1.3.2.4. for gram positive bacteria.

**1.3. Day 3**

**1.3.1. Cell lysis for Gram negative bacteria**

- 1.3.1.1. Add 1.5 ml of cell suspension (overnight culture) to a 1.5 ml centrifuge tube.
- 1.3.1.2. Centrifuge at 13,000-14,000 x g (10,000-11,000 rpm) for 1 minute to pellet cells.
- 1.3.1.3. Remove the excess broth leaving approximately 50 µl.
- 1.3.1.4. Add 600 µl Nuclei Lysis Solution to the remaining solution and pipette up and down until the cells are re-suspended.  

**NOTE:** If using culture from a plate of overnight growth, add the loopful directly to the 600 µl Nuclei Lysis Solution and vortex for 5-10 seconds.
- 1.3.1.5. Incubate at 80°C ± 1 for 5 minutes.  

**OPTIONAL:** Incubating at 60°C ± 1 for 45 minutes (instead of 80°C ± 1 for 5-10 minutes) may improve cell lysis.
- 1.3.1.6. Add 3 µl RNase A Solution to the cell lysate.
- 1.3.1.7. Mix the sample by inverting the tube 25 times and incubate at 37°C ± 1 for 15 minutes to 1 hour.

**1.3.2. Cell lysis for Gram positive bacteria**

- 1.3.2.1. Add 1.5 ml of cell suspension (overnight culture) to a 1.5ml centrifuge tube.
- 1.3.2.2. Centrifuge at 13,000-14,000 x g (10,000-11,000 rpm) for 1 minute to pellet cells.
- 1.3.2.3. Remove the excess broth leaving approximately 50 µl.
- 1.3.2.4. Add 600µl Lytic Enzyme Solution (see table below) to the cell pellet and gently pipette up and down to lyse the cells.  

**NOTE:** To prepare Lytic Enzyme Solution, add 20 mg/ml of lysozyme to the appropriate amount of enzymatic lysis buffer and use within 24 hours.

Lytic Enzyme Solution	1 Sample
Enzymatic Lysis Buffer	600 µl
Lysozyme	12 mg

<b>VERSION:</b>	<b>REPLACED BY:</b>	<b>AUTHORIZED BY:</b>	<b>Page 32 of 46</b>
-----------------	---------------------	-----------------------	----------------------



**NOTE:** To prepare Enzymatic Lysis Buffer:

- a. 5ml 1M Tris-HCl, pH 8.0
- b. 1ml 0.5M EDTA
- c. 3ml Triton X-100
- d. Bring volume to 250ml with molecular-grade water.
- e. Stock buffer will last 1 year at room temperature.

- 1.3.2.5. Incubate at 37°C ± 1 for 30 minutes to lyse the outer cell wall.
- 1.3.2.6. Centrifuge the sample at 13,000-16,000 x g (10,000-13,000 rpm) for 1 minute. Remove and discard the supernatant.
- 1.3.2.7. Add 600 µl of Nuclei Lysis Solution. Gently pipette up and down until the cells are resuspended.
- 1.3.2.8. Incubate at 80°C ± 1 for 5 minutes to lyse the cells.
- 1.3.2.9. Add 3 µl RNase A Solution to the cell lysate.
- 1.3.2.10. Mix the sample by inverting the tube 25 times and incubate at 37°C ± 1 for 15 minutes to 1 hour.

**1.3.3. Protein precipitation**

- 1.3.3.1. Cool the sample to room temperature by placing on ice for 1 minute.
- 1.3.3.2. Add 200 µl of Protein Precipitation Solution to the RNase-treated cell lysate.
- 1.3.3.3. Vortex vigorously at high speed for 20 seconds to mix the Protein Precipitation Solution uniformly with the cell lysate.
- 1.3.3.4. Incubate the sample on ice for 10 minutes.
- 1.3.3.5. Centrifuge at 13,000-16,000 x g (10,000-13,000 rpm) for 3 minutes. The precipitated protein should form a tight pellet.

**OPTIONAL:** If the protein did not form a tight pellet, repeat the above steps by pouring the supernatant containing the DNA (leaving behind the precipitated protein pellet) into a clean 1.5ml microcentrifuge tube, incubating the sample on ice for 5 minutes, and centrifuging at 13,000-16,000 x g for 3 minutes.

**1.3.4. DNA Precipitation**

- 1.3.4.1. Transfer the supernatant containing the DNA into a clean 1.5 ml microfuge tube containing 600 µl of 100% Isopropanol (2-propanol).
- 1.3.4.2. Mix the sample by gently inverting 50 times.
- 1.3.4.3. Centrifuge at 13,000-16,000 x g (10,000-13,000 rpm) for 3 minute. The DNA pellet should be visible as a small white pellet.
- 1.3.4.4. Pour off the supernatant. The pellet may be loose so pour slowly so as not to disturb the pellet.
- 1.3.4.5. Add 600 µl 70% Ethanol and invert the tube several times to wash the pellet.
- 1.3.4.6. Centrifuge at 13,000-16,000 x g (10,000-13,000 rpm) for 1 minute. Carefully pour off the ethanol. The pellet may be loose so pour slowly so as not to disturb the pellet.
- 1.3.4.7. Briefly spin down the tube to bring the remaining ethanol to the bottom of the tube. Remove the remaining ethanol using a 100 µl pipette.

<b>VERSION:</b>	<b>REPLACED BY:</b>	<b>AUTHORIZED BY:</b>	<b>Page 33 of 46</b>
-----------------	---------------------	-----------------------	----------------------

<b>LABORATORY STANDARD OPERATING PROCEDURE FOR PULSENET  NEXTERA XT LIBRARY PREP AND RUN SETUP FOR THE ILLUMINA  MISEQ</b>	<b>CODE: PNL32</b>				
	Effective Date: <table border="1"> <tr> <td>1</td> <td>27</td> <td>16</td> </tr> </table>			1	27
1	27	16			

1.3.4.8. Invert and drain the tube on clean absorbent paper. Allow the tube to air dry for 10-15 minutes.

**1.3.5. DNA Hydration**

1.3.5.1. Add 50-100 µl of 10mM Tris-HCl, pH 8.0. Pipette up and down to dislodge the DNA pellet from the bottom and/or wall of the tube. The DNA may form a diffuse pellet along the tube wall.

**OPTIONAL:** An aliquot of 10 mM Tris-HCl can be warmed briefly (approx. 1-2 minutes) in a 65°C ± 1 water bath.

**NOTE:** A 10mM stock of Tris-HCl, pH 8.0 can be obtained by adding 10 ml of 1M stock Tris-HCl to 900ml of molecular grade water. All stocks of Tris-HCl should be stored at 2 – 8°C.

1.3.5.2. Rehydrate the DNA by incubating the sample for 1 - 1.5 hour(s) at 65°C ± 1 and/or overnight at room temperature.

**OPTIONAL:** Pulse spin briefly to collect sample at the bottom of the tube.

**NOTE:** Samples can be left overnight at room temperature after the 65°C incubation to facilitate rehydration. Store DNA extracts at 2-8°C for short-term storage, or at -15°C to -80°C for long-term storage.

<b>VERSION:</b>	<b>REPLACED BY:</b>	<b>AUTHORIZED BY:</b>	<b>Page 34 of 46</b>
-----------------	---------------------	-----------------------	----------------------

**Appendix PNL32-2**

**DNA Clean & Concentrator-5 Kit Procedure**

**1. Before starting:**

- 1.1.** For the 25 prep kit: Add 24 ml 100% ethanol (26 ml 95% ethanol) to the 6 ml DNA Wash Buffer concentrate.
- 1.2.** For the 100 prep kit: Add 96 ml 100% ethanol (104 ml 95% ethanol) to the 24 ml DNA Wash Buffer concentrate.

**2. Procedure:**

**NOTE:** All centrifugation steps should be performed between 10,000 - 16,000 x g (9,000 – 11,500 rpm).

- 2.1. In a 1.5 ml microcentrifuge tube, add 2-5 volumes of ChIP DNA Binding Buffer to each volume of DNA sample1 (see table below). Mix thoroughly.

<b>Application</b>	<b>DNA Binding Buffer : Sample</b>	<b>Example</b>
Plasmid, genomic DNA (> 2 kb)	2 : 1	200 µl : 100 µl
PCR product, DNA fragment	5 : 1	500 µl : 100 µl

- 2.2. Transfer this mixture to a provided Zymo-Spin Column<sup>1</sup> in a collection tube.
- 2.3. Centrifuge for 30 seconds and discard the flow-through.
- 2.4. Add 200 µl DNA Wash Buffer to the column. Centrifuge for 1 minute and repeat the wash step.
- 2.5. Add 20 µl of 10 mM Tris-HCl pH 8.0 directly to the column matrix (do not touch the filter with the pipette tip).
- 2.6. Incubate at room temperature for one minute.
- 2.7. Transfer the column to a 1.5 ml microcentrifuge tube and centrifuge for 30 seconds to elute the DNA.
- 2.8. Repeat steps 2.5 - 2.7 eluting into the same microcentrifuge to obtain a final DNA sample volume of 40 µl.
- 2.9. The DNA is now ready for library preparation.

<sup>1</sup>The sample capacity of the column is 800 µl. It may be necessary to load and spin a column multiple times if a sample has a volume larger than 800 µl.

**Appendix PNL32-3**

**MiSeq Sample Plate and Sample Sheet Setup**

**1. Creating a MiSeq Sample Plate**

- 1.1. Open Illumina Experiment Manager (IEM icon on the MiSeq screen.)
- 1.2. Choose “Create Sample Plate”
- 1.3. Select the appropriate Sample Prep Kit and push “Next” (e.g. NexteraXT)
- 1.4. Name the plate in the following format:  
**Lab ID, Machine ID, Run Date.** For example, CDC-MXXXX-YYMMDD.
- 1.5. Select ‘2’ for the number of index reads and push “Next”.
- 1.6. Under the ‘Plate’ tab, start in position A01 and enter the first Sample ID. The ID used will be the anonymized code for each isolate provided by PulseNet CDC database managers, it will be a WGS ID that contains a letter code PNUSA, which signifies the organism (*Salmonella* (S), *E. coli* & *Shigella* (E), *Campylobacter* (C), *Listeria* (L), *Vibrio* (V)) and then a 6 digit number (e.g. PNUSAS123456).  
**NOTE: If also sequencing isolates as part of the GenomeTrakr network, please include the GenomeTrakr ID only in the Sample ID field.**
- 1.7. In position A02, enter the first Sample Name which should be the WGS ID plus the Run ID (<PNUSA Id> - <Run Id>), e.g. PNUSAS123456- CDC-M12345-160601 for a Salmonella isolate run at CDC.
- 1.8. Repeat step 1.6 – 1.7 for all samples, entering the information in the wells corresponding to their positions in the Initial DNA Dilution worksheet.
- 1.9. Assign indices for all samples by selecting “Indices” from the drop-down menu at the top left of the screen. For each sample, select the appropriate index from the drop-down menu in each cell. Repeat these steps for index 2.  
**NOTE: Refer to the Dual Indexing Principles and Pooling Guidelines for troubleshooting in the Illumina Nextera XT DNA Sample Preparation Guide (pg. 44).**
- 1.10. Verify the layout by clicking on the “Table” tab.
- 1.11. Click Finish.
- 1.12. Save the Plate file as the Plate ID (e.g. CDC-MXXXX-YYMMDD).

**2. Creating a MiSeq Sample Sheet**

- 2.1. Open the Illumina experiment Manager (IEM icon on the MiSeq screen.)
- 2.2. Choose Create Sample Sheet.
- 2.3. Choose MiSeq and press “Next”.
- 2.4. Select the appropriate category and application and then press the “Next” button
  - 2.4.1. Category: Other.
  - 2.4.2. Application: FastQ Only
- 2.5. On the Run Settings page, enter the following:
  - 2.5.1. Reagent Cartridge Barcode: provide plate ID (e.g. CDC-MXXXX-YYMMDD)
  - 2.5.2. Sample Prep Kit: NexteraXT
  - 2.5.3. Index Reads: 2
  - 2.5.4. Experiment Name: Plate ID
  - 2.5.5. Investigator Name: Initials of technician(s) performing the run

<b>VERSION:</b>	<b>REPLACED BY:</b>	<b>AUTHORIZED BY:</b>	<b>Page 36 of 46</b>
-----------------	---------------------	-----------------------	----------------------

- 2.5.6. Description: leave blank if desired (not required)
- 2.5.7. Cycle Reads:
  - 2.5.7.1. For a 300 cycle kit, set at 151 x 151
  - 2.5.7.2. For a v2 500 cycle kit, set at 251 x 251
  - 2.5.7.3. For a v3 600 cycle kit, set at 251 x 251 or 301 x 301 depending on desired read length
- 2.5.8. Leave all other settings as default.
- 2.6. Verify that the “use adapter trimming” box is checked (right side of screen) and push “Next”.
- 2.7. On the Sample Selection Page, click on “Select Plate”.
 

**NOTE:** If this option is not displayed, uncheck the “Maximize” box at the top right-hand side of the screen.
- 2.8. Find the plate ID from the directory in the new pop-up window.
- 2.9. Highlight the correct plate and select “Open”.
- 2.10. Click the “Select All” button at the bottom left-hand side of screen.
- 2.11. Click “Add Selected Samples”.
- 2.12. Verify the successful addition of all desired samples to the Sample Sheet in the Samples “To Include in the Sample Sheet Field” window on the right-hand side of the screen.
- 2.13. Verify the Sample Sheet is ‘valid’ by checking for the green “Valid” status in the Sample Sheet Status field on the right-hand side of the screen.
- 2.14. Click “Finish”.
- 2.15. Save the Sample Sheet using the Plate ID (e.g. CDC-MXXXX-YYMMDD). Select No when prompted to view the sheet in Excel.

**3. Exporting a MiSeq Sample Sheet from the Nextera XT Library Prep Workbook**

- 3.1. After completing the Initial Dilution Page, select the Sample Sheet tab in the Nextera XT Library Prep Workbook.
- 3.2. Check each field in the Sample Sheet to ensure that all of the data has been accurately populated. Only the Description (following Assay) and Sample\_Project (if not using BaseSpace) fields will not be filled.
 

**NOTE: The index sequences are populated from the Indices tab. Do not modify or delete this tab.**
- 3.3. To export the Sample Sheet, open the File menu option and select Export.
- 3.4. Select the Change File Type option
- 3.5. Select CSV (Comma delimited) under Other File Types submenu.
- 3.6. Click Save As and save to desired file folder location using the Plate ID as the filename (e.g. CDC-MXXXX-YYMMDD).
- 3.7. Click OK to save only the active sheet, and Yes in the following window to keep using the CSV format.
- 3.8. Copy the CSV file to a flash drive.
- 3.9. Transfer this file to the Illumina Experiment Manager (IEM) by opening the software and selecting Edit Sample Sheet or copying it to the appropriate Sample Sheet folder on the D drive.
- 3.10. In the new window copy and paste the CSV file among the list of other sample sheets and select Open.
- 3.11. In the Run Settings window, verify that all information is correct. Enter the Plate ID as the Experiment Name and check the “Use adapter trimming” box (right side of screen). Click Next.

<b>VERSION:</b>	<b>REPLACED BY:</b>	<b>AUTHORIZED BY:</b>	<b>Page 37 of 46</b>
-----------------	---------------------	-----------------------	----------------------

<b>LABORATORY STANDARD OPERATING PROCEDURE FOR PULSENET          NEXTERA XT LIBRARY PREP AND RUN SETUP FOR THE ILLUMINA          MISEQ</b>	<b>CODE: PNL32</b>		
	Effective Date:		
	1	27	16

- 3.12. Verify the Sample Sheet is valid by checking for the green “Valid” status in the Sample Sheet Status field on the left-hand side of the screen.
- 3.13. Click Finish.
- 3.14. Save the Sample Sheet using the same Plate ID (e.g. CDC-MXXXX-YYMMDD).
- 3.15. Click Yes to overwrite the previous file.
- 3.16. Select No when prompted to view the sheet in Excel.

<b>VERSION:</b>	<b>REPLACED BY:</b>	<b>AUTHORIZED BY:</b>	<b>Page 38 of 46</b>
-----------------	---------------------	-----------------------	----------------------

**LABORATORY STANDARD OPERATING PROCEDURE FOR PULSENET  
NEXTERA XT LIBRARY PREP AND RUN SETUP FOR THE ILLUMINA  
MISEQ**

**CODE: PNL32**

Effective Date:

1 27 16

**Appendix PNL32-4**

**Initial Dilution Worksheet**

Run Name	LabID-MXXXX-YYMMDD
Sample Plate Name	LabID-MXXXX-YYMMDD
Sample Sheet Name	LabID-MXXXX-YYMMDD
Library Prep Date	10/5/2015
Technician	XXX

**Instructions**

Starting with this sheet, and moving from top to bottom, fill in all tan shaded cells.

Dilution calculation:  $V1 = (V2C2)/C1$

Where V1 = volume of stock needed for dilution

C1 = stock concentration

V2 = 20 ul

C2 = 10 ng/ul

Serial Dilutions

Well position	Sample ID	Project ID	Organism	RUN1		Nanodrop Purity (260/280 ratio)	Genome Size Estimate (Mbp)	Qubit Input DNA Stock Conc (ng/ul)	Dilution for Nextera normalization 10 ng/ul (20 ul total volume)		1 ng/ul		0.2 ng/ul	
				Index 1	Index 2				DNA	Diluent (dH2O)	DNA	Diluent (dH2O)	DNA	Diluent (dH2O)
A01	PNUSAS123456		<i>Salmonella enterica serovar Typhimurium</i>	N701	S517	1.85	5	30	6.7	13.3	2	18	2	8
B01	PNUSAV123456		<i>Vibrio cholerae</i>	N702	S517	1.85	5	30	6.7	13.3	2	18	2	8
C01	Sample Name_3		<i>Genus species serovar Serotype</i>	N703	S517	1.85	5	30	6.7	13.3	2	18	2	8
D01	Sample Name_4		<i>Genus species serovar Serotype</i>	N704	S517	1.85	5	30	6.7	13.3	2	18	2	8
E01	Sample Name_5		<i>Genus species serovar Serotype</i>	N705	S517	1.85	5	30	6.7	13.3	2	18	2	8
F01	Sample Name_6		<i>Genus species serovar Serotype</i>	N706	S517	1.85	5	30	6.7	13.3	2	18	2	8
G01	Sample Name_7		<i>Genus species serovar Serotype</i>	N707	S517	1.85	5	30	6.7	13.3	2	18	2	8
H01	Sample Name_8		<i>Genus species serovar Serotype</i>	N708	S517	1.85	5	30	6.7	13.3	2	18	2	8
A02	Sample Name_9		<i>Genus species serovar Serotype</i>	N701	S502	1.85	5	30	6.7	13.3	2	18	2	8
B02	Sample Name_10		<i>Genus species serovar Serotype</i>	N702	S502	1.85	5	30	6.7	13.3	2	18	2	8
C02	Sample Name_11		<i>Genus species serovar Serotype</i>	N703	S502	1.85	5	30	6.7	13.3	2	18	2	8
D02	Sample Name_12		<i>Genus species serovar Serotype</i>	N704	S502	1.85	5	30	6.7	13.3	2	18	2	8
E02				N705	S502				#DIV/0!	#DIV/0!	2	18	2	8
F02				N706	S502				#DIV/0!	#DIV/0!	2	18	2	8
G02				N707	S502				#DIV/0!	#DIV/0!	2	18	2	8
H02				N708	S502				#DIV/0!	#DIV/0!	2	18	2	8
A02									#DIV/0!	#DIV/0!	2	18	2	8
B02									#DIV/0!	#DIV/0!	2	18	2	8
C02									#DIV/0!	#DIV/0!	2	18	2	8
D02									#DIV/0!	#DIV/0!	2	18	2	8

**Notes:**

Reagents	Lot no.	Exp. Date
TD Buffer		
ATM		
NT Buffer		
NPM		
Indices		
Beads		
RSB		
EBT		

<b>VERSION:</b>	<b>REPLACED BY:</b>	<b>AUTHORIZED BY:</b>	<b>Page 39 of 46</b>
-----------------	---------------------	-----------------------	----------------------

**LABORATORY STANDARD OPERATING PROCEDURE FOR PULSENET  
NEXTERA XT LIBRARY PREP AND RUN SETUP FOR THE ILLUMINA  
MISEQ**

**CODE: PNL32**

Effective Date:

1	27	16
---	----	----

**Appendix PNL32-5**

**Normalization and Pooling Worksheet**

Sample Plate Name	LabID-MXXXX-YYMMDD
Sample Sheet Name	LabID-MXXXX-YYMMDD
Date	10/5/2015
Technician	XXX

NOTE: All sample names should end with Plate ID (eg. 2012K-0644\_1-M947-13-001)

Pooling Factor: Proportion of sample genome size relative to total DNA load

Pooling Volume: Pooling Factor \* 50 ul pool

ng/uL to nM conversion factor (nm\*uL/ng): 1.5

Dilution conc (nM):	2
---------------------	---

Dilution volume (uL): 50

Pooling volume (uL): 50

Available dilution conc (nM): 2 3 4

Sample ID	Project ID	Organism	Genome Size	Optional pre-clean up Qubit (ng/uL)	Qubit Output		For 50 ul of 2nM, 3nM, or 4nM		Pooling Factor	Pooling Volume (50ul)	Comments
					(ng/uL)	nM	Sample	Diluent (EBT)			
PNUSAS123456	0	Salmonella enterica serovar Typhimurium	5		4	6	16.7	33.3	0.08	4.2	
PNUSAV123456	0	Vibrio cholerae	5		4	6	16.7	33.3	0.08	4.2	
Sample Name_3	0	Genus species serovar Serotype	5		4	6	16.7	33.3	0.08	4.2	
Sample Name_4	0	Genus species serovar Serotype	5		4	6	16.7	33.3	0.08	4.2	
Sample Name_5	0	Genus species serovar Serotype	5		4	6	16.7	33.3	0.08	4.2	
Sample Name_6	0	Genus species serovar Serotype	5		4	6	16.7	33.3	0.08	4.2	
Sample Name_7	0	Genus species serovar Serotype	5		4	6	16.7	33.3	0.08	4.2	
Sample Name_8	0	Genus species serovar Serotype	5		4	6	16.7	33.3	0.08	4.2	
Sample Name_9	0	Genus species serovar Serotype	5		4	6	16.7	33.3	0.08	4.2	
Sample Name_10	0	Genus species serovar Serotype	5		4	6	16.7	33.3	0.08	4.2	
Sample Name_11	0	Genus species serovar Serotype	5		4	6	16.7	33.3	0.08	4.2	
Sample Name_12	0	Genus species serovar Serotype	5		4	6	16.7	33.3	0.08	4.2	
0	0	0	0		0	0	#DIV/0!	#DIV/0!	0.00	0.0	
0	0	0	0		0	0	#DIV/0!	#DIV/0!	0.00	0.0	
0	0	0	0		0	0	#DIV/0!	#DIV/0!	0.00	0.0	
0	0	0	0		0	0	#DIV/0!	#DIV/0!	0.00	0.0	
0	0	0	0		0	0	#DIV/0!	#DIV/0!	0.00	0.0	
0	0	0	0		0	0	#DIV/0!	#DIV/0!	0.00	0.0	
0	0	0	0		0	0	#DIV/0!	#DIV/0!	0.00	0.0	
0	0	0	0		0	0	#DIV/0!	#DIV/0!	0.00	0.0	

Total DNA load 60

NOTE: pooling volume factors in genome size contribution to overall DNA load

Notes:

	Lot #	Exp date
Incorporation buffer		
MiSeq cartridge		
Flow cell		

Post-Run Metrics	
Cluster Density	
Clusters Passing Filter	
Q30	
Estimated Yield	

<b>VERSION:</b>	<b>REPLACED BY:</b>	<b>AUTHORIZED BY:</b>	<b>Page 40 of 46</b>
-----------------	---------------------	-----------------------	----------------------



**Appendix PNL32-6**

**Thawing and Storing Thawed MiSeq cartridges**

**1. Overnight thawing method (preferred)**

- 1.1. Remove cartridge from freezer and place at 2 – 8 °C in original packaging.
- 1.2. Just before use, invert the cartridge several times and gently tap it on the bench to mix and reduce air bubbles in the reagents.  
**NOTE:** The cartridge can be kept thawed for up to seven days at 2 – 8 °C, though it is recommended to use the thawed cartridge within 24 hours.

**NOTE2: Once the cartridge is thawed it cannot be refrozen.**

**2. Rapid thawing method**

- 2.1. Place the reagent cartridge in a large ice bucket containing enough room temperature deionized water to submerge the base of the reagent cartridge up to the water fill line printed on the reagent cartridge.  
**NOTE: Do not allow the water to exceed the maximum water line or allow the top of the cartridge to get wet.**

- 2.2. Allow the reagent cartridge to thaw in the room temperature water bath until completely thawed.  
**NOTE:** Water can be changed every 15 – 20 minutes to expedite thawing. The cartridge takes approximately 1 hour to thaw at room temperature and once thawed can be stored on ice or at 2 – 8 °C for up to 24 hours.

**NOTE: Once the cartridge is thawed it cannot be refrozen.**

- 3. Just before use, invert the cartridge several times and gently tap it on the bench to mix and reduce air bubbles in the reagents.

<b>VERSION:</b>	<b>REPLACED BY:</b>	<b>AUTHORIZED BY:</b>	<b>Page 41 of 46</b>
-----------------	---------------------	-----------------------	----------------------

**Appendix PNL32-7**

Addition of PhiX Control

**1. Procedure**

**NOTE:** If using a previously prepared PhiX denatured dilution, proceed to Step 8.

- 1.1. Combine the following volumes to dilute the PhiX library to 2 nM:
  - 1.1.1. 10 nM PhiX library (1 µl)
  - 1.1.2. EBT (4 µl)
- 1.2. Add 400 ml of molecular-grade water to a 100 µl aliquot of 1 N NaOH to obtain a 0.2 N solution.
- 1.3. Combine the following volumes of 2 nM PhiX library and 0.2 N NaOH in a microcentrifuge tube to result in a 1 nM PhiX library:
  - 1.3.1. 2 nM PhiX library (5 µl)
  - 1.3.2. 0.2 N NaOH (5 µl)
- 1.4. Discard the remaining dilution of 0.2 N NaOH.
- 1.5. Vortex briefly to mix the 1 nM PhiX library solution.
- 1.6. Centrifuge the PhiX solution briefly at 200-280 x g.
- 1.7. Incubate for 5 minutes at room temperature to denature the PhiX library into single strands.
- 1.8. **TIME SENSITIVE:** Add the following volume of pre-chilled HT1 to the tube containing denatured PhiX library to result in a 20 pM PhiX library:
  - 1.8.1. Denatured PhiX library (10 µl)
  - 1.8.2. Pre-chilled HT1 (490 µl)
- 1.9. Label, date and initial the microcentrifuge tube. Denatured 20 pM PhiX libraries may be stored up to three weeks at -15° to -25°C.
- 1.10. Dilute the denatured 20 pM PhiX library to 8 pM as follows:
  - 1.10.1. 20 pM denatured PhiX library (40 µl)
  - 1.10.2. Pre-chilled HT1 (60 µl)
- 1.11. Determine percentage PhiX (N%) to add to run. If troubleshooting a library preparation, the 1-10% is appropriate, if troubleshooting a MiSeq instrument issue, then 5% or above (also depending on Illumina tech support guidance) is appropriate. If using PhiX routinely in every run, then 1% is recommended.
- 1.12. Combine N x 10µl of 8 pM PhiX with 1000 – (N x 10) µl of the diluted denatured 10 pM sample library.
  - 1.12.1. For example, for a 1% PhiX addition, add 10 µl of 8 pM PhiX to 990 µl of denatured 10 pM sample library.
- 1.13. Set PhiX-spiked pooled library aside on ice until you are ready to load it onto the MiSeq reagent cartridge.

**Appendix PNL32-8**

**Performing a Maintenance Bleach Wash**

1. When performing a weekly post-run bleach (Sodium hypochlorite) wash prepare a fresh dilution of bleach solution using molecular grade water and sodium hypochlorite (there are several options for bleach solutions with varying percentages of chlorine, see the appropriate dilutions for 4%, 6%, and 8.25% below). Bleach may be used for specific applications to reduce carry-over of nucleic acid from previous runs.

1.1. Refer to the table below to make the serially diluted bleach solutions. The second dilution (0.01%) should be made in a MiSeq wash tube/wash reservoir (Figure 1, Item A).

**NOTE:** Contact Illumina Tech Support for information on obtaining wash tubes.

**NOTE2:** A fresh dilution of bleach should be prepared before each wash.

<b><u>Final dilution</u></b>	<b><u>Starting Bleach concentration</u></b>	<b><u>Bleach volume (µl)</u></b>	<b><u>Water volume (µl)</u></b>
<b>DILUTION 1:</b> (0.2%)	4%	30	570
	6%	20	580
	8.25%	15	585
<b>DILUTION 2:</b> (0.01%)	0.2%	50	950

1.2. Perform this wash as weekly maintenance by accessing the Post-run Wash option from the Illumina MiSeq home screen > Perform Wash or as the post-run wash following a sequencing run.

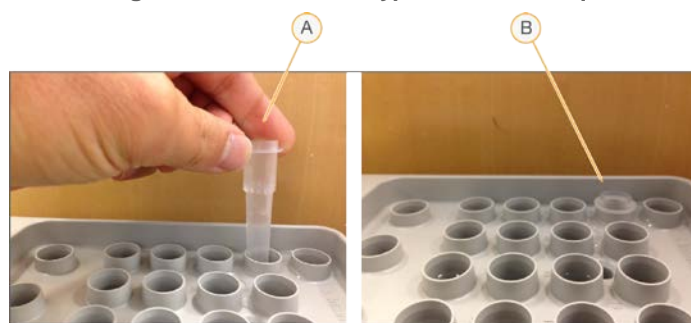
1.2.1. In the following screen select the Perform Post-Run Wash option, and then select Start Wash on the next screen.

1.2.2. Open the reagent compartment door and then chiller door to access the wash tray.

1.2.3. Remove the wash tray and empty the left over wash solution (0.5% Tween 20) into the sink.

1.2.4. Insert the wash tube into position 17 in the wash tray until the neck is flush with the tray (Figure 1, Item B), and fill the remaining reservoirs with 0.5% Tween 20 wash solution.

**Figure 1. Inserting the 0.01% Sodium Hypochlorite MiSeq Tube**



A. Insert the tube into position 17 of the MiSeq wash cartridge.

B. Make sure the neck of the MiSeq tube is flush with the wash cartridge.

<b>VERSION:</b>	<b>REPLACED BY:</b>	<b>AUTHORIZED BY:</b>	<b>Page 43 of 46</b>
-----------------	---------------------	-----------------------	----------------------

- 1.2.5. Slide the wash cartridge into the reagent chiller until it stops and close the reagent chiller door.
- 1.2.6. Make sure to select the box to “Perform optional template line wash”.
- 1.2.7. In the reagent compartment, raise the sipper handle, remove the PR2 bottle and replace with the MiSeq wash bottle containing 0.5% Tween 20.
- 1.2.8. Remove the liquid waste receptacle and dispose of contents in the formamide waste bottle.
  - 1.2.8.1. NOTE: All liquid that accumulates in the MiSeq waste bottle should be disposed of as formamide waste, including liquid from wash cycles. All formamide waste bottles must be clearly labeled with contents, date of first use, and date of final use. Waste must be stored in designated, clearly labeled waste disposal site.**
- 1.2.9. Return the liquid waste receptacle to the reagent compartment, lower the sipper arm, and close the reagent compartment door.
- 1.2.10. Select Next to begin the wash.
- 1.2.11. Once the 20 minute Post-run wash is complete, select “Done” on the screen to return to the MiSeq Control software home screen.
  - 1.2.11.1. OPTIONAL:** A post-run wash with 0.5% Tween 20 wash solution should be performed directly following the post-run bleach wash to ensure all bleach is removed from the template line.
  - 1.2.11.2. To perform a post-run wash following a bleach wash:
    - 1.2.11.2.1. From the home screen select Perform Wash > Perform Post-Run Wash.
    - 1.2.11.2.2. Select Start Wash.
    - 1.2.11.2.3. Remove the wash tray from the reagent chiller and the wash tube from Position 17.
    - 1.2.11.2.4. Refill all reservoirs, including Position 17, in the tray with 0.5% Tween 20 wash solution.
    - 1.2.11.2.5. Raise the sipper arm and remove the waste receptacle. Dispose of the contents in the waste bottle, return the waste receptacle to the reagent compartment, and lower the sipper arm.
    - 1.2.11.2.6. Select Next to begin the wash.
    - 1.2.11.2.7. Once the wash is complete, the flow cell, wash bottle, and liquid waste receptacle should remain in place until the next sequencing run is to be performed.

**Appendix PNL32-9**

**Requeueing a Run for Analysis**

There are detailed instructions on how to requeue analyses from the MiSeq Reporter (MSR) interface and in BaseSpace in the respective software guides:

- Miseq Reporter Software Guide on page 10: [http://support.illumina.com/content/dam/illumina-support/documents/documentation/software\\_documentation/miseqreporter/miseq-reporter-software-guide-15042295-04.pdf](http://support.illumina.com/content/dam/illumina-support/documents/documentation/software_documentation/miseqreporter/miseq-reporter-software-guide-15042295-04.pdf)
- BaseSpace User Guide on page 55: <https://dals119xsxmu0.cloudfront.net/sites/knowledgebase/userguide/07112014/UserGuide%20PDF/bas-espace-user-guide-15044182-d.pdf>

**1. Procedure**

**1.1. Requeue a run Using MiSeq Reporter**

- 1.1.1. In order to apply the corrected or new analysis parameters to a run, a new sample sheet must first be created. To select a run to be re-queued with a new sample sheet, it is important that the input files are present in the analysis run folder. SampleSheet.csv, RTAComplete.txt, and RunInfo.xml must all be present.
- 1.1.2. In the analysis run folder (D:Illumina/MiSeq Analysis/"Recent run") delete the "QueueForAnalysis.txt" file.
- 1.1.3. Rename the old sample sheet (for example, "SampleSheet.csv.old") and paste the newly revised sample sheet in the analysis folder under the name "SampleSheet.csv".
  - 1.1.3.1. If correcting index assignment, the new sample sheet should have the corrected index pair assignments and corresponding index sequences.
- 1.1.4. Connect to MiSeq Reporter on the local computer by opening Internet Explorer. If MiSeq Reporter does not open with the launch of Internet Explorer, type in the search bar: <http://localhost:8042/>
- 1.1.5. Check the checkbox next to the run and Click "Requeue". The reanalysis will take roughly an hour.

**NOTE: MiSeq Reporter requires the following files which are generated during the sequencing run to perform secondary analysis or to requeue analysis: all \*.bcl, \*.filter, and \*.locs.**

- 1.1.6. The reanalyzed data (i.e. fastq files) will be stored in the "Basecalls" folder of the run analysis and output folders.

**1.2. Fix Sample Sheet / Re-Run Workflow in BaseSpace**

**NOTE:** In order to requeue the analysis within BaseSpace, you must be the owner of the run. Additionally, **a corrected sample sheet and requeued run can only be submitted one time.**

- 1.2.1. The Fix Sample Sheet page can be reached in one of two ways:

<b>VERSION:</b>	<b>REPLACED BY:</b>	<b>AUTHORIZED BY:</b>	<b>Page 45 of 46</b>
-----------------	---------------------	-----------------------	----------------------

<b>LABORATORY STANDARD OPERATING PROCEDURE FOR PULSENET  NEXTERA XT LIBRARY PREP AND RUN SETUP FOR THE ILLUMINA  MISEQ</b>	<b>CODE: PNL32</b>				
	Effective Date: <table border="1"> <tr> <td>1</td> <td>27</td> <td>16</td> </tr> </table>			1	27
1	27	16			

- 1.2.1.1. If the run is in a “Needs Attention” state, then the run can be opened and the Fix Sample Sheet option selected.
- 1.2.1.2. Or you can navigate to a run, select the More drop-down list, and select Fix Sample Sheet.
- 1.2.2. Once the **Fix Sample Sheet** page opens, any errors BaseSpace has detected should be shown above the black sample sheet editor.
- 1.2.3. There are two options depending on the complexity of the change:
  - 1) The easier fix is editing the sample sheet in the Sample Sheet Editor. BaseSpace will validate the sample in real-time as you edit.
  - 2) The more complex option is using the Illumina Experiment Manager (IEM).
    - a. The original sample sheet can be imported and edited or a new sample sheet can be generated.
    - b. Copy and paste the updated or new sample sheet into the Sample Sheet Editor in BaseSpace.
    - c. BaseSpace will validate the sample sheet and display any issues above the sample sheet editor.
- 1.2.4. When the new or revised Sample Sheet is valid, click the Queue Analysis button. BaseSpace will start analyzing the run using the new sample sheet.

**NOTE:** Only valid sample sheets will be used for reanalysis. If the sample sheet is invalid, the Queue Analysis button will not be available.
- 1.2.5. If **fixing index assignment, the Prep Tab** can be used to change indices and regenerate fastq files for samples that have already been sequenced.
  - 1.2.5.1. Proceed to the run with the incorrect index assignments and click the Run Settings button in the navigation task pane.
  - 1.2.5.2. At the bottom of the page click the pool.
  - 1.2.5.3. Click the Plate ID of the plate, then click Edit.
  - 1.2.5.4. Correct the index in the dropdown menu.
  - 1.2.5.5. Navigate to the affected run and select Generate FASTQ Files from the More dropdown menu.
  - 1.2.5.6. BaseSpace will start to regenerate the fastq files with the corrected indices. The new fastq files will be added to the sample list and can be identified by the date.

**NOTE:** This option cannot be used for runs that are not set up with the Prep tab or where the wrong library prep kit was selected in the Prep tab.

**ATTENTION:** MSR and BaseSpace will requeue a new sample sheet independent of each other. If a new sample sheet is requeued with MSR, it will not update the information on BaseSpace. If the one reanalysis attempt has already been used and you want to update the run data on BaseSpace, a new sample sheet can be sent to Illumina Technical Support and they will make the changes on BaseSpace. Along with the request, send the Run ID information, which is the name of the folder for that run. The request file is only valid for 7 days.

<b>VERSION:</b>	<b>REPLACED BY:</b>	<b>AUTHORIZED BY:</b>	<b>Page 46 of 46</b>
-----------------	---------------------	-----------------------	----------------------