1. **PURPOSE:** This procedure describes a standardized laboratory protocol for whole genome sequencing of enteric bacterial organisms using the Illumina DNA Flex library preparation kit, for subsequent sequencing on an Illumina platform, thus ensuring inter-laboratory comparability of sequencing results.

2. **SCOPE:** For use by PulseNet WGS certified laboratorians when preparing libraries for sequencing on Illumina platforms using the Nextera DNA Flex library preparation from DNA from enteric organisms for submission of sequencing data to PulseNet. Laboratories may amend this procedure as necessary for use within their laboratories after validation per their laboratory's guidelines.

3. **DEFINITIONS:**
   - 3.1. **BLT:** Bead-Linked Transposome
   - 3.2. **BR:** Broad Range
   - 3.3. **BSC:** Biosafety Cabinet
   - 3.4. **DNA:** Deoxyribonucleic Acid
   - 3.5. **DNase:** Deoxyribonuclease
   - 3.6. **dsDNA:** Double-Stranded DNA
   - 3.7. **EPM:** Enhanced PCR Mix
   - 3.8. **GHS:** Globally Harmonized System
   - 3.9. **HS:** High Sensitivity
   - 3.10. **HT1:** Hybridization Buffer
   - 3.11. **IEM:** Illumina Experiment Manager (Illumina Software)
   - 3.12. **MCS:** MiSeq Control Software (Illumina Software)
   - 3.13. **PCR:** Polymerase Chain Reaction
   - 3.14. **PHL:** Public Health Laboratory
   - 3.15. **PPE:** Personal Protective Equipment
   - 3.16. **PR2:** Incorporation Buffer
   - 3.17. **QC:** Quality control
   - 3.18. **RNase:** Ribonuclease
   - 3.19. **RSB:** Resuspension Buffer
   - 3.20. **SDS:** Safety Data Sheet
   - 3.21. **SPB:** Sample Purification Beads
   - 3.22. **TB1:** Tagmentation Buffer 1
   - 3.23. **Tris-HCl:** Tris Hydrochloride
   - 3.24. **TSB:** Tagment Stop Buffer
   - 3.25. **TWB:** Tagment Wash Buffer
   - 3.26. **WGS:** Whole Genome Sequencing

4. **RESPONSIBILITIES:**
   - 4.1. **PulseNet Public Health Laboratory:**
     - 4.1.1. Prepare DNA libraries, and QC as necessary, for subsequent WGS
     - 4.1.2. Communicate with PulseNet Central as necessary about any complications with laboratory protocols, suspected reagent issues, or suspected instrument issues
   - 4.2. **PulseNet Central:**
     - 4.2.1. Perform additional sequence quality analysis in order to provide feedback and troubleshooting support for PHLs as necessary
     - 4.2.2. Communicate any suspected reagent issues to PHLs as necessary
     - 4.2.3. Maintain and review SOPs on a regular basis and post on SharePoint

5. **SAFETY:**

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

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

5.2.1. Illumina DNA Flex Library Preparation Kit: See Illumina SDSs for additional information. Take proper precautions and wear appropriate PPE when handling reagents.

- TSB: GHS Category 1 for eye damage/irritant and is harmful to aquatic life.
- TB1: GHS Category 4 for acute toxicity (dust/mist), Category 2A for eye irritant and Category 1B for reproductive toxicity. Contains N,N=Dimethylformamide.
- EPM: GHS Category 4 for acute oral toxicity and Category 1 for specific organ toxicity. Contains tetramethylammonium chloride.

6. **REAGENTS:**

6.1. Nextera DNA Flex Library Prep Kit (96 samples, Illumina Cat# 20018705 or 24 samples, Illumina Cat# 20018704)

- Box 1 of 3. Store at room temperature – ***NOTE*** *that one component in the box needs to be refrigerated.*

  SPB **(store at 2-8°C, do not freeze)**
  TSB
  TWB
- Box 2 of 3. Store at -25 to -15°C

  RSB
  TB1
  EPM
- Box 3 of 3. Store at 2-8°C

  BLT

6.2. Nextera DNA CD Indexes (96 samples, Illumina Cat# 20018708 or 24 samples, Illumina Cat# 20018707 or IDT Cat# 20027213). Store at -25°C to -15°C

- 24 Dual Index, tube format: H503, H505, H506, H517, H710, H705, H706, H707, H711, H714

  OR
- 96 Dual Index, plate format: One 96 Dual Adapter Index Plate

  NOTE: *This procedure is written for library preparation using the 96 Dual Index plate format indices.*

  OR
- IDT for Illumina Nextera DNA UD Indexes – Set A (96 indexes, 96 samples; plate format)

  NOTE: These indexes have TEN base pair adapters; if they are utilized for library preparation, the number of Index Read cycles on the instrument must be adjusted to 10 (from 8).

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

6.4. Ethanol, lab-grade, 70% or equivalent for disinfection purposes (Fisher Cat# 04-355-309 or equivalent)

6.5. Water, Molecular grade (Fisher Cat# BP28191 or equivalent)

7. **SUPPLIES:**

7.1. Qubit Assay Tubes (ThermoFisher Cat# Q32856 or equivalent (clear, thin-wall 0.5-ml PCR tubes)).

7.2. Pipette tips, sterile, filtered: 20 µl, 200 µl and 1000 µl volumes (Rainin Cat# 17001865, 17001863 & 17001864 or equivalent)
7.3. Conical tubes, 10 ml and/or 15 ml (Fisher Scientific Cat# 14-959A and/or Fisher Cat# 14-959-53A or equivalent)
7.4. Solution basins, sterile (Fisher Scientific Cat# 13-681-504 or equivalent)
7.5. 96 well PCR plates, skirted, hard shell low profile, thin-wall (BioRad Cat# HSP-9601 or equivalent)
7.6. Microseal A film (BioRad Cat# MSA-5001 or equivalent)
7.7. Microseal B adhesive seal (BioRad Cat# MSB-1001 or equivalent)
7.8. Microseal F adhesive foil seal (BioRad Cat# MSF-1001 or equivalent)
7.9. Microcentrifuge tubes, 1.5 ml, sterile (ThermoFisher Cat# AM12400 or equivalent)
7.10. Serological pipets, 1 ml to 10 ml volumes (various catalog numbers)
7.11. Ice
7.12. **OPTIONAL:** Deepwell storage "MIDI" plates, 96 well (Fisher Cat# AB-0859 or equivalent)

8. **EQUIPMENT:**
8.1. Qubit 2.0 or 3.0 Fluorometer, or equivalent for quantification of dsDNA
8.2. Thermal cycler, capable of accepting a 96-well plate, with heated lid
8.3. Microplate centrifuge
8.4. Vortex
8.5. Magnetic Stand-96 (ThermoFisher Cat# AM10027 or equivalent)
8.6. Micropipettes, capable of volumes from 1 µl to 1000 µl. Single and multichannel (20 µl and 100µl volumes).
NOTE: *Two sets of pipettes are suggested; one for working with pre-amplified product and reagents and one set for working with post-PCR amplified product and reagents.*
8.7. Ice buckets/containers
8.8. Pipet-Aid
8.9. Microcentrifuge for quick spins

9. **PROCEDURE:**
NOTE: *Ensure that DNA going into library preparation has been assessed for quality. The 260/280 value should be between 1.75 and 2.05. See PNL33 for more information.*
9.1. **Preparation of Sequencing Workbook:** The steps for Sample Plate and Sample Sheet setup in the IEM on the instrument can be completed at any point in this protocol, but must be performed prior to the start of the run. However, it is recommend to create the Sample Plate and Sample Sheet at the beginning of library prep.
9.1.1. Prepare the "Library Prep" tab/worksheet of the Nextera DNA Flex Library Prep Workbook (PNL35.W1) as described below:
NOTE: *The workbook is designed with the following color scheme, in general:*
- *White fields should be filled in*
- *Dark gray fields are optional*
- *Blue fields contain formulas, which will auto populate, and should not be altered*

9.1.2. Enter the Run ID in C2; in the following format: labID-MXXXX-YYMMDD
NOTE: *The date should be the date the sequencing run is started.*
9.1.3. Enter the Sequencing Date (C3), Sequencing Technician (C4), select the Sequencing Kit Type/Chemistry from the drop down box (C5), Library Prep Date (C6), and Library Prep Technician (C7).

9.1.4. Enter the State Key (the ID entered in the BioNumerics "Key" field) in appropriate location (Column B).

NOTE: ***Effective after PFGE is dropped. Until then, use the WGS ID (PNUSA#) assigned by the PulseNet Database Team in Column B.***

9.1.5. Enter the Genome Size Estimate (based on Table 1 below) into Column D.

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

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

9.1.6. Confirm that the number of isolates on the run is appropriate for the capacity of the instrument reagent kit to be used. The sum of the genome sizes (in Mb) for the samples on the run (from column D, found in cell D41 of the workbook) will give the estimated DNA load of the run. This cannot exceed the DNA load allowance of the reagent kit to be used. The DNA load for MiSeq cartridges can be found in Table 2 below.

MiSeq Cartridge	DNA Load (Mb)
***v2 300**	**60**
v2 500	**100**
***v3 600, mixed runs**	**200**
***v3 600, E. coli/Shigella**	**175**
***Micro (300)**	**35**
***Nano (500)**	**13**

Table 2. Estimated DNA Load (in Mb) Capacity for Illumina Reagent Kits.
*DNA capacity for v2 300, V3 *E. coli*, Micro and Nano kits are still being evaluated. Current recommendations are based on Nextera XT and can be likely slightly increased.

9.1.7. Determine which set of indices will be used and enter (or select from the dropdown) the well position (from the index plate), into column J of the Library Prep tab of the workbook.
NOTE: *It is recommended not to use the same index pairs within 2 consecutive runs on the same sequencer to reduce the amount of carryover. See Appendix PNL35-1 for Illumina DNA CD Indices Tracking Worksheet.*

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

9.2. **Dilute and Tagment Input DNA: DNA is fragmented and tagged with the adapter sequences by the BLT during this step.**

9.2.1. Bring BLT (from refrigerator) and TB1 (from freezer) to room temperature.

NOTE: *Ensure that BLT is stored upright at all times, so that the beads remain submerged in the buffer and that it is never frozen.*

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

9.2.3. Add molecular-grade water to each sample well (automatically calculated in Column H of the Workbook).

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

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

9.2.6. Vortex TB1 to mix and quick spin.

9.2.7. Prepare tagmentation master mix:

Reagent	Volume per Sample
TB1	10 µl
BLT	10 µl

Table 3: Reagent volumes per sample for tagmentation master mix

NOTE: *It is recommended to increase number of samples by 1-2 to ensure sufficient master mix volume.*

9.2.8. Vortex the tagmentation master mix well.

9.2.9. Add 20µl of tagmentation master mix to each sample well.
NOTE: *Alternatively, the tagmentation master mix may be divided into an 8-tube strip (or a new column of wells on the plate) and a multichannel pipet then used to add the 20 µl of master mix to each sample well, if preferred.*

9.2.10. Mix well, resuspending the beads.

9.2.11. Seal the plate with Microseal B (or equivalent) and incubate the plate at 55°C for 15 minutes, followed by a 10°C hold (volume is 50 µl) on a thermal cycler with the lid heated at 100°C.
NOTE1: *It is recommended to pre-program a thermal cycler for this purpose.*
NOTE2: *This is not a recommended stopping point in the procedure and post tagmentation clean up should be commenced once the samples have reached 10°C.*

9.3. **Post Tagmentation Clean Up: DNA (now tagged with adapters and bound to the BLT) will be washed prior to amplification.**

9.3.1. Check TSB for precipitate (if present, warm at 37°C for up to 10 minutes and vortex) and ensure it is at room temperature.

9.3.2. Add 10 µl of TSB to each sample.

9.3.3. Pipet gently to mix and re-suspend the beads (using a multichannel pipette for mixing is recommended).

9.3.4. Seal the plate with Microseal A or equivalent.

9.3.5. Incubate at 37°C for 15 minutes and hold at 10°C on a thermal cycler with a lid pre-heated to 100°C.
NOTE1: *It is recommended to pre-program a thermal cycler for this purpose.*
NOTE2: *This is not a recommended safe stopping point, and it is recommended to proceed to the washing steps after samples have reached 10°C.*

9.3.6. While samples are incubating, thaw EPM on ice and thaw indices at room temperature.

9.3.7. After incubation, remove from thermal cycler, quick spin the plate and place on a magnet for 3 minutes (or until beads form a tight pellet).

9.3.8. Remove and discard supernatant.
9.3.9. Remove the plate from the magnet and add 100 µl TWB directly to the pellet.
9.3.10. Pipet gently (to avoid foaming) to mix until beads are fully re-suspended.
NOTE: *Avoiding foaming of TWB is important, as foaming can lead to sample loss.*
9.3.11. Place back on the magnet for 3 minutes (or until beads form a tight pellet).
9.3.12. Remove and discard the supernatant.
9.3.13. Remove from the magnet and add 100 µl of TWB directly to the pellet.
9.3.14. Pipet gently to mix (avoiding foaming) until beads are fully re-suspended.
9.3.15. Place back on the magnet for 3 minutes (or until beads form a tight pellet).
9.3.16. Remove and discard supernatant
9.3.17. Add 100µl of TWB directly to the beads and gently pipet to re-suspend.
9.3.18. Place back on the magnet for 3 minutes, allow TWB to remain in the wells (to prevent drying of beads) and proceed to amplification steps.

9.4. **Amplify Tagmented DNA: The index adapters are added to the tagmented DNA, and amplification occurs during this step.**
9.4.1. Vortex briefly the thawed EPM prior to use.
9.4.2. Prepare PCR master mix:

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

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

NOTE: *It is recommended to increase number of samples during master mix calculation by 1-2 to ensure sufficient master mix volume.*

9.4.3. Vortex and quick spin the PCR master mix.
9.4.4. Remove TWB from beads, recommended to use a small volume pipette to ensure removal of residual TWB before proceeding.
NOTE: *Removal of TWB is crucial, as it can impede PCR. However, any foam remaining on the wells will not negatively impact the library.*
9.4.5. Remove from the magnet and immediately add 40 µl of PCR master mix to each sample.
9.4.6. Gently pipet to mix, re-suspending the pellet.
9.4.7. Add 10 µl of appropriate index pair from indices plate to each sample well.
NOTE: *It is recommended to pierce the foil of the desired well on the index plate with a new pipet tip, then to use a fresh pipette tip to withdraw the indices from the wells, followed by re-sealing the index plate with a new foil cover (i.e. Microseal F) after each use.*
9.4.8. Mix by pipetting a minimum of 10 times.
9.4.9. Seal the plate with Microseal A or equivalent, and run the following pre-programmed settings on a thermal cycler with a heated lid (100°C):

Step 1: 68°C for 3 minutes
Step 2: 98°C for 3 minutes
Step 3: 5 cycles
98°C for 45 seconds
62°C for 30 seconds
68°C for 2 minutes

Step 4: 68°C for 1 minute
Step 5: Hold at 10°C
Total volume: 50 µl

9.4.10. Centrifuge plate for at 280 x g for 1 minute.

NOTE: ***This is a safe stopping point. The plate may be sealed with Microseal B or equivalent, and stored at 2°C to 8°C for up to 3 days.***

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

NOTE: *The steps listed below are critical for efficient size selection, product recovery and thus cluster generation and sequencing. Always check pipette tips for correct volumes and ensure that no beads have accidentally been aspirated. If beads have been aspirated or the bead pellet is disturbed, allow the pellet to reform (3-5 minutes on the magnet) and repeat the step.*

9.5.1. Before starting, prepare reagents:

9.5.1.1. Dilute fresh 80% ethanol sufficient for all samples:

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

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

9.5.1.2. Bring SPB to room temperature (at least 30 minutes) from refrigerator.
9.5.1.3. Bring RSB to room temperature (from the freezer) and vortex to mix.
9.5.1.4. Vortex and invert SPB several times to fully re-suspend the beads.

9.5.2. Prepare SPB master mix:

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

Table 6: Reagent volumes per sample for SPB master mix

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

9.5.3. If plate was retrieved from cold storage, centrifuge plate at 280 x g for 1 minute.
9.5.4. Place sample plate on the magnet for 5 minutes (or until beads have formed a tight pellet).
9.5.5. Transfer 45 µl of supernatant (now containing the DNA) to new wells.
9.5.6. Remove sample plate from the magnet.
9.5.7. Vortex SPB master mix thoroughly and add 85µl to each sample.
9.5.8. Pipet to mix a minimum of 10 times; using a multichannel pipet is recommended.
NOTE*: Use caution when mixing as the volume will be > 100 µl.*
9.5.9. Incubate at room temperature for 5 minutes.
9.5.10. Place on the magnet for 3-5 minutes (or until beads form a tight pellet).
9.5.11. During incubation re-vortex the stock SPB.

9.5.12. After incubation, with the plate still on the magnet, transfer 125 µl of supernatant (containing DNA) to new wells.
9.5.13. Remove the plate from the magnet, and add 15 µl of stock SPB solution to the supernatant.
9.5.14. Gently pipet a minimum of 10 times to mix.
9.5.15. Incubate at room temperature for 5 minutes.
9.5.16. Place on magnet for 3-5 minutes (or until beads form a tight pellet and supernatant clears).
9.5.17. Remove and discard supernatant (DNA is now bound to the beads).
9.5.18. Perform the steps below twice (for a total of two washes):
9.5.18.1. While the plate is on the magnet, add 170 µl of fresh 80% ethanol.
NOTE*: Do not add directly to the bead, and do not mix.*
9.5.18.2. Incubate for 30 seconds.
9.5.18.3. Remove and discard supernatant.
9.5.19. Use a small volume pipet to remove excess ethanol, if necessary.
9.5.20. Allow beads to air dry for 3-5 minutes.
NOTE: *Do not allow beads to over-dry. If cracking is observed, immediately re-suspend beads as described below regardless of drying time.*
9.5.21. Remove from magnet and add 32 µl of RSB.
9.5.22. Pipet to thoroughly mix.
9.5.23. Incubate at room temperature for 2-5 minutes.
9.5.24. Place on magnet for 3 minutes (or until supernatant is clear).
9.5.25. Transfer 30 µl of the supernatant into new well (or into wells on a new plate) – this is the final product.
NOTE: *A Qubit step may be performed at this point and results recorded in Column K of the Workbook) if desired.*

NOTE: ***This is a safe stopping point. The plate may be sealed with Microseal B or equivalent and stored at -20°C until use, or for long-term storage.***

9.6. **Pooling of Libraries**
9.6.1. Pool 5 µl of each library into a new well, and pipet well to mix.
NOTE: ***EXCEPT for Campylobacter. Use a pooling volume of 2.5 µl for Campylobacter ssp.***
9.6.2. Quantify the pool, using the Qubit or equivalent. See SOP PNL33 for instructions on operating the Qubit.
9.6.3. Enter the Qubit value for the pool in cell D43 of the Workbook.
9.6.4. Calculate the molarity (nM) of the pool:
(Qubit reading ng/µl x 10^6)/(660g/mol x 1000bp)
NOTE1: *The workbook will automatically calculate this value and display it in cell D44.*
NOTE2: *The formula in the workbook is based off of an assumed average fragment size of 1000 base pairs (in blue above). If laboratories generate fragment sizes after library prep which vary greatly from 1000 bp in length, they may adjust this formula accordingly, as this will make the determination of the library concentration and dilution to loading concentration more accurate.*
- *For example, if fragment analysis reveals average library lengths around 800 bp, the formula would be the following: Qubit reading ng/µl x 10^6)/(660g/mol x 800bp) and therefore cell D44 would need to be changed to "=Pool Concentration*1000/528". Contact PulseNetNGSLab@cdc.gov for assistance if necessary.*

9.6.5. Calculate the volume (µl) of pool necessary to generate 50µl of a 4nM pool:

(200/Molarity of pool) = volume of pool for dilution
NOTE: *The workbook will automatically calculate this value and display it in cell D45 of the Workbook.*

9.6.6. Calculate the volume (µl) of RSB diluent required:
50 – volume of pool = volume of RSB required
NOTE: *The workbook will automatically calculate this value and display it in cell D46 of the Workbook.*

9.6.7. In a new well, dilute the pool to 4 nM by adding calculated volume of library pool (cell D45) to calculated volume of RSB (cell D46).

NOTE: The libraries are now ready for sequencing. Please proceed to the appropriate instrument sequencing SOP for instructions to denature and dilute the pool to the proper loading concentration. A checklist for the Nextera DNA Flex library prep is provided in Appendix PNL35-2.

10. **FLOW CHART:** N/A

11. **RELATED DOCUMENTS:**

Document Number	
PNL33	**DNA Extraction and QC SOP**
PNL38	**Sequencing on the MiSeq SOP**
PNQ07	**Illumina MiSeq Data QC**
PNL35.W1	**DNA Flex Workbook**

12. **REFERENCES:**

12.1. Illumina, Inc. Nextera DNA Flex Library Prep Checklist (Doc.# 1000000033561 v01). October 2018. https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/samplepreps_nextera/nextera_dna_flex/nextera-dna-flex-checklist-1000000033561-01.pdf

12.2. Illumina, Inc. Nextera DNA Flex Library Prep Reference Guide (Doc.# 1000000025416 v03). October 2018. https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/samplepreps_nextera/nextera_dna_flex/nextera-dna-flex-library-prep-reference-guide-1000000025416-03.pdf

13. **CONTACTS:**

13.1. PulseNet NGS Lab troubleshooting account:
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14. **APPENDICES:**

14.1. PNL35-1: PN Ilumina Nextera DNA CD Indices Tracing_v02
14.2. PNL32-2: PN Illumina Nextera DNA Flex Checklist_v3

15. **AMENDMENTS:**

15.1. **01/28/19:** New Document

16. **APPROVAL SIGNATURES:**

Approved By: _____ Date: _____
Author

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

Approved By: _____ Date: _____
PulseNet Outbreak Detection and Surveillance Unit Chief

Approved By: _____ Date: _____
PulseNet PFGE Reference Unit Chief

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

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

Appendix PNL35-1:
Example of PN Illumina Nextera DNA CD Indices Tracking

Illumina Nextera DNA CD Indices Tracking

	1	2	3	4	5	6	7	8	9	10	11	12
A	H505- H701	H506- H702	H517- H703	H505- H705	H506- H707	H517- H723	H505- H706	H506- H712	H517- H720	H505- H710	H506- H711	H517- H714
B	H517- H702	H505- H703	H506- H701	H517- H707	H505- H723	H506- H705	H517- H712	H505- H720	H506- H706	H517- H711	H505- H714	H506- H710
C	H506- H703	H517- H701	H505- H702	H506- H723	H517- H705	H505- H707	H506- H720	H517- H706	H505- H712	H506- H714	H517- H710	H505- H711
D	H503- H705	H503- H707	H503- H723	H503- H706	H503- H712	H503- H720	H503- H710	H503- H711	H503- H714	H503- H701	H503- H702	H503- H703
E	H516- H706	H516- H712	H516- H720	H516- H710	H516- H711	H516- H714	H516- H701	H516- H702	H516- H703	H516- H705	H516- H707	H516- H723
F	H522- H710	H510- H711	H513- H714	H522- H701	H510- H702	H513- H703	H522- H705	H510- H707	H513- H723	H522- H706	H510- H712	H513- H720
G	H513- H711	H522- H714	H510- H710	H513- H702	H522- H703	H510- H701	H513- H707	H522- H723	H510- H705	H513- H712	H522- H720	H510- H706
H	H510- H714	H513- H710	H522- H711	H510- H703	H513- H701	H522- H702	H510- H723	H513- H705	H522- H707	H510- H720	H513- H706	H522- H712

Lot Number	
Expiration Date	
Rec'd Date	
Open Date	

Runs

Appendix PNL35-2: PN Illumina Nextera DNA Flex Checklist

		Library Preparation Using Illumina DNA Flex (1 of 2)	
		Tagmentation	
	1	Quantify DNA on Qubit and record on worksheet	
	2	Bring BLT & TB1 to room temperature (BLT from fridge & TB1 from freezer)	
	3	Add H2O to 96 well plate (per Column G on workbook, typically 20 µl)	
	4	Add DNA to sample wells (per Column H on workbook, typically 10 µl)	
	5	Vortex BLT for 10s (ensure suspension)	
	6	Prepare tagmentation master mix: 10µl BLT + 10µl TB1	**Volume (µl)**
		# of samples (plus 2):	7
		Volume of BLT	70
		Volume of TB1	70
	7	Vortex master mix well	
	8	Add 20µl of tagmentation master mix to each sample well	
	9	Pipette to mix (50µl total volume)	
	10	Seal the plate with Microseal B or equivalent	
	11	Incubate the plate at 55°C for 15 minutes, followed by 10°C hold ("Flex Tagment")	
		Post Tagmentation Clean Up	
	12	Check TSB for precipitates (if present, warm at 37°C for 10 minutes & vortex)	
	13	Add 10µl of TSB to sample wells	
	14	Pipette gently to mix (60µl total volume)	
	15	Incubate at 37°C for 15 minutes then hold at 10°C ("Flex Post Tag")	
	16	Place on magnet for 3 minutes (or until clear)	
	17	Use multichannel pipet to remove and discard supernatant	
	18	Remove from magnet & add 100µl TWB	
	19	Pipette gently until fully suspended	
	20	Place back on the magnet for 3 min (or until clear)	
	21	Use multichannel pipet to remove and discard supernatant	
	22	Remove from magnet & add 100µl TWB	
	23	Pipette gently until fully suspended	
	24	Place back on the magnet for 3 min (or until clear)	
	25	Use multichannel pipet to remove and discard supernatant	
	26	Remove from magnet & add 100µl TWB	
	27	Place back on the magnet for 3 min (or until clear)	
		Amplify Tagmented DNA	
	28	Thaw EPM on ice	
	29	Thaw Nextera DNA Flex Indexes at room temperature (spin briefly before use)	
	30	Prepare PCR master mix (20µl EPM + 20µl H2O for each sample)	**Volume (µl)**
		# of samples (plus 2):	6
		Volume of EPM	120
		Volume of H2O	120
	31	Vortex & spin the master mix	
	32	Remove the TWB from the samples (still on the magnet)	
	33	Remove excess TWB w/ pipet	
	34	Remove from magnet and immediately add 40µl of PCR master mix	
	35	Gently pipet to mix	
	36	Add index adapters to each sample (96 well plate, each well contains 10µl of primer mix for one time use)	
	37	Mix a minimum of 10x (set to 40µl)	
	38	Seal the plate and run the "Flex Amplify" program on the thermal cycler	
	39	Remove the plate and centrifuge for 1 min at 280xg	
		May stop here and store at 2-8C for up to 3 days	

Library Preparation Using Illumina DNA Flex (2 of 2)			
Clean up Libraries			
	40	Bring SPB to room temperature (30min) from fridge	
	41	Bring RSB to room temperature (from freezer) & vortex to mix	
	42	Place on magnet for 5 minutes (or until clear)	
	43	Transfer 45µl of supernatant to new wells	
	44	Remove from magnet	
	45	Vortex & invert SPB several times	
	46	Prepare SPB master mix (40.8µl SPB + 44.2µl H2O (.48x) per sample)	**Volume (µl)**
		# of samples (plus 3):	6
		Volume of SPB	244.8
		Volume of H2O	265.2
	47	Vortex SPB master mix well & add 85µl to each sample well	
	48	Pipet to mix a minimum of 10x (complete mixing is critical)	
	49	Incubate at room temperature for 5 minutes	
	50	Place on the magnet for 3 minutes (or until clear)	
	51	During the incubation, vortex the stock SPB thoroughly	
	52	While on the magnet transfer 125µl of supernatant into new wells.	
	53	Remove from the magnet and add 15µl of stock SPB into wells containing supernatant	
	54	Pipet to mix a minimum of 10x (complete mixing is critical)	
	55	Incubate at room temperature for 5 minutes	
	56	Dilute fresh 80% EtOH (e.g. for 16 samples, 6.4 ml EtOH + 1.6 ml H2O. For 20 samples, 8 ml EtOH + 2 ml H20)	
	57	Place on magnet for 3 minutes (or until clear)	
	58	Remove & discard supernatant	
	59	Add 170µl of 80% EtOH and incubate for 30 seconds	
	60	Pipet to remove the EtOH	
	61	Add 170µl of 80% EtOH and incubate for 30 seconds	
	62	Pipet to remove the EtOH	
	63	Remove excess EtOH with pipet if present	
	64	Allow beads to air dry for 5 minutes	
	65	Remove from magnet & add 32µl of RSB	
	66	Pipet to mix thoroughly	
	67	Incubate at room temperature for 2-5 minutes	
	68	Place back on the magnet for 3 minutes (or until clear)	
	69	Transfer 30µl of the supernatant to new wells	
		May stop here, store at -25C to -15C for up to 30 days	
Pooling Libraries			
	70	Remove 100µl of 1M NaOH from the freezer and add 400µl molecular grade water to dilute to 0.2N working solution. Invert or vortex to mix	
	71	Remove HT1 from the freezer and thaw on ice	
	72	Ensure heat block is at 96°C	
	73	Pool 5µl of each sample into a single well of the plate & pipet well to mix	
	74	Quantify the pool on Qubit - record value on Workbook	
	75	Calculate Molarity(nM): (pool concentration x 10^6)/(660 g/mol x 1000 bp) - record on workbook	
	76	Calculate volume of pool required for 50µl of 4nM: (200/molarity of pool) (workbook will calculate)	
	77	Determine volume of RSB needed (50µl minus volume above) (workbook will calculate)	
	78	Dilute appropriately (now have 50µl of 4nM pool)	
	79	Add 5µl of pool to microcentrifuge tube	
	80	Add 5µl of diluted NaOH to the tube, vortex briefly & quick spin	
	81	*ncubate at room temp for 5 minutes	
	82	Add 990µl of chilled HT1 (now 20pM)	
	83	Dilute library to desired loading concentration (e.g. for 15pM, add 750 µl of HT1 buffer to 250 µl of library pool, see Cells D51-53 in workbook)	
	84	Incubate at 96°C for 2 minutes	
	85	Immediately place in ice bath for a minimum of 5 minutes, until ready to load	
		Version 3	
		Jan-19	
		Steps 79 - 85 (shaded) can be found in appropriate instrument sequencing SOPs	