

PULSENET STANDARD OPERATING PROCEDURE FOR DNA LIBRARY PREPARATION USING THE ILLUMINA® DNA PCR-FREE PREP KIT

Doc. No. PNL43

Ver. No. 01

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1. **PURPOSE:** This procedure describes a standardized laboratory protocol for DNA library preparation of enteric bacterial organisms using the Illumina® DNA PCR-Free Prep kit, for subsequent sequencing on an Illumina MiSeq, thus ensuring inter-laboratory comparability of sequencing results and optimizing sequencing data output and quality.
2. **SCOPE:** For use by PulseNet WGS certified laboratorians when preparing libraries from DNA from enteric organisms using the Illumina® DNA PCR-Free Prep for sequencing on Illumina MiSeq platform 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-PF:** Bead-Linked Transposomes PCR-Free
 - 3.2. **CD Index:** Combinatorial Dual Index
 - 3.3. **DNA:** Deoxyribonucleic Acid
 - 3.4. **ELM:** Extension Ligation Mix
 - 3.5. **GHS:** Globally Harmonized System
 - 3.6. **HP3:** 2N NaOH
 - 3.7. **HS:** High Sensitivity
 - 3.8. **IEM:** Illumina Experiment Manager
 - 3.9. **IPB:** Illumina Purification Beads
 - 3.10. **LP:** Library Prep
 - 3.11. **LRM:** Local Run Manager
 - 3.12. **Mb:** Megabase
 - 3.13. **nM:** Nanomolar
 - 3.14. **PCR:** Polymerase Chain Reaction
 - 3.15. **PHL:** Public Health Laboratory
 - 3.16. **PPE:** Personal Protective Equipment
 - 3.17. **PulseNet Central:** PulseNet team at CDC comprising of the Outbreak Detection and Surveillance Unit and the Next Generation Subtyping Methods Unit
 - 3.18. **PulseNet/OutbreakNet SharePoint:** A closed, web-based collaboration application used for communication among PulseNet participants.
 - 3.19. **QC:** Quality control
 - 3.20. **RSB:** Resuspension Buffer
 - 3.21. **SDS:** Safety Data Sheet
 - 3.22. **SOP:** Standard Operating Procedure
 - 3.23. **ssDNA:** Single-Stranded DNA
 - 3.24. **ST2:** Stop Tagment Buffer 2
 - 3.25. **TB1:** Tagmentation Buffer 1
 - 3.26. **TWB:** Tagment Wash Buffer
 - 3.27. **UD Index:** Unique Dual Index
 - 3.28. **WGS:** Whole Genome Sequencing
4. **RESPONSIBILITIES:**
 - 4.1. **PulseNet Public Health Laboratory:**
 - 4.1.1. Prepare DNA libraries and QC as necessary, for subsequent WGS
 - 4.1.2. Re-sequence any isolates that do not meet quality thresholds

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4.1.3. Inform PulseNet Central, as necessary, about any complications with laboratory protocols or suspected reagent issues

4.2. PulseNet Central:

4.2.1. Perform additional sequence quality analysis in order to provide feedback and troubleshooting support for PHLs as necessary

4.2.2. Notify PN PHL if any sequences submitted do not meet quality thresholds

4.2.3. Communicate any suspected reagent issues to PHLs as necessary

4.2.4. Maintain and review SOPs on a regular basis and post on SharePoint

5. SAFETY:

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

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

5.2.1. Illumina® DNA PCR-Free Kit:

5.2.1.1. ST2: GHS Category 1 for eye damage/irritant and is harmful to aquatic life

5.2.1.2. TB1: GHS Category 4 for acute toxicity (dust/mist), Category 2A for eye irritant and Category 1B for reproductive toxicity. Contains N, N-Dimethylformamide.

5.2.2. Ethanol is flammable (GHS Flammability Category 2)

6. REAGENTS:

6.1. Illumina® DNA PCR-Free Prep, Tagmentation kit (96 samples, Illumina Cat# 20041795 or 24 samples, Illumina Cat# 20041794) with the following components:

- Beads + Buffers. Store at room temperature.

IPB

ST2

TWB

RSB

- PCR + Buffers. Store at -25 to -15°C.

BLT-PF

TB1

HP3

ELM

6.2. Indexes: choose either 6.2.1. or 6.2.2.

NOTE1: *The index kit(s) needed depends on the sample throughput of the laboratory and the level of multiplexing desired. It is recommended not to use the same index pair in two consequent runs on the same instrument to prevent carry-over from run to run.*

NOTE2: *The CD and UD indexes cannot be mixed on the same run*

6.2.1. Nextera DNA CD Dual Indexes (96 indexes, 96 samples, Illumina Cat# 20018708). Store at -25°C to -15°C.

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

NOTE: *These indexes have TEN base pair adapters; therefore, the number of index read cycles on the instrument must be adjusted to 10 (from 8 for CD indexes).*

6.2.2.1. DNA/RNA UD Indexes, set A, Tagmentation (96 indexes, 96 samples, Illumina Cat# 20027213)

6.2.2.2. DNA/RNA UD Indexes, set B, Tagmentation (96 indexes, 96 samples, Illumina Cat#

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- 20027214)
- 6.2.2.3. DNA/RNA UD Indexes, set C, Tagmentation (96 indexes, 96 samples, Illumina Cat # 20042666)
- 6.2.2.4. DNA/RNA DNA UD Indexes, set D, Tagmentation (96 indexes, 96 samples, Illumina Cat # 20042667)
- 6.3. Ethanol, molecular grade, 95-100% (Fisher Scientific Cat# BP2818-500 or equivalent)
- 6.4. Ethanol, lab-grade, 70% or equivalent for disinfection purposes (Fisher Scientific Cat# 04-355-305 or equivalent)
- 6.5. Water, Molecular grade (Fisher Scientific Cat# BP24701 or equivalent)
- 6.6. Invitrogen™ Qubit™ ssDNA Assay Kit (100 assays, Fisher Scientific Cat# Q10212)
- ssDNA Reagent, Component A (room temperature, protect from light)
 - ssDNA Buffer, Component B (room temperature)
 - ssDNA Standard #1, Component C ($\leq 4^{\circ}\text{C}$)
 - ssDNA Standard #2, Component D ($\leq 4^{\circ}\text{C}$)

7. SUPPLIES:

- 7.1. 96 well PCR plates, skirted, hard shell low profile, thin-wall (BioRad Cat# HSP-9601 or equivalent)
- 7.2. Deepwell storage “MIDI” plates, 96 well (Fisher Scientific Cat# AB-0859 or equivalent) – **Optional**
- 7.3. Ice
- 7.4. Microcentrifuge tubes, 1.5 ml, sterile (Fisher Scientific Cat# 05-408-129 or equivalent)
- 7.5. Microseal A film (BioRad Cat# MSA-5001 or equivalent)
- 7.6. Microseal B adhesive seal (BioRad Cat# MSB-1001 or equivalent)
- 7.7. Microseal F adhesive foil seal (BioRad Cat# MSF-1001 or equivalent) – **Optional**
- 7.8. Pipette tips, sterile, filtered: 20 μl , 200 μl and 1000 μl volumes (Rainin Cat# 30389225, 30389239 & 30389212 or equivalent)
- 7.9. Qubit™ Assay Tubes (Fisher Scientific Cat# Q32856 or equivalent (clear, thin-wall 0.5-ml PCR tubes)).
- 7.10. Solution basins, sterile (Fisher Scientific Cat# 13-681-504 or equivalent)

8. EQUIPMENT:

- 8.1. Ice buckets/containers
- 8.2. Invitrogen™ DynaMag™-96 Side Skirted Magnet (Fisher Scientific Cat# 12-027 or equivalent)
- 8.3. Microcentrifuge for quick spins
- 8.4. Micropipettes, capable of volumes from 1 μl to 1000 μl . Single and multichannel (20 μl and 100 μl volumes)
- NOTE: Two sets of pipettes are suggested, one for working with pre-amplified product and reagents and another set for working with post-PCR amplified product and reagents*
- 8.5. Microplate centrifuge
- 8.6. Pipet-Aid
- 8.7. Qubit™ 4.0 Fluorometer (older versions 2.0 or 3.0), or equivalent for quantification ssDNA
- 8.8. Thermal cycler with heated lid, compatible with 96-well 0.2 ml PCR plate
- 8.9. Vortex

9. PROCEDURE: The Illumina DNA PCR-Free Prep checklist (PNL43.W2) may be used during the procedure if desired.

NOTE1: *The only safe stopping point in the procedure is after clean-up of the indexed libraries (9.5.)*

NOTE2: *Make sure the vortex is turned down once cells have been lysed during DNA extraction and for all steps during library prep*

NOTE3: *It has been documented and observed that cluster density can be negatively affected by the use of*

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ammonium-based cleaning products near sequencing equipment, including lab benches and pipets used for library preparation. Do not use quaternary ammonium compounds or wipes near or on sequencing equipment!

9.1. Prepare the “Library Prep” Tab of the Illumina DNA PCR-Free Prep Workbook

NOTE1: *The workbook PNL43.W1 contains separate tabs for different software versions on the MiSeq: IEM, MiSeq LRM3 and MiSeq LRM versions earlier than 3; ensure that the proper tab is used for the software available on the MiSeq. Do NOT delete rows or columns of the workbooks!*

NOTE2: *The workbook is designed with the following color scheme:*

- *White fields should be filled in*
- *Dark gray fields are optional*
- *Blue fields contain formulas, which will auto populate, and should not be altered*

9.1.1. Enter the Run ID using the following format: PulseNet lab ID-Instrument ID-run start date, i.e., labID-MXXXX-YYMMDD

9.1.2. Enter the Sequencing Date, Sequencing Technician, select the Sequencing Kit Type/Chemistry from the drop-down box, Library Prep Date, and Library Prep Technician

9.1.3. Enter the State Keys (the ID entered in the BioNumerics “Key” field)

NOTE: Important! *Fastq file names will be assigned on the Sample Sheet tab according to this State Key and the Run ID. These fields will be concatenated to create a unique prefix for the resulting fastq files (e.g., Sample1-CDC-M3235-221004)*

9.1.4. Enter the genome size estimate (based on Table 1 below):

Organism	Estimated Genome Size (Mb)
<i>E. coli & Shigella spp.</i>	5
<i>Salmonella spp.</i>	5
<i>Vibrio spp.</i>	5
<i>Listeria monocytogenes</i>	3
<i>Campylobacter spp.</i>	1.6

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

9.1.5. Confirm that the number of isolates on the run is appropriate for the capacity of the MiSeq sequencing kit to be used (Table 2). The sum of the genome sizes (in Mb) for the samples on the run will be displayed on the workbook and is equal to the estimated DNA load of the run. Individual laboratories with experience and consistent library concentrations may be able to exceed the recommended DNA load while still reaching the required minimum coverage levels. **NOTE: PulseNet never accepts, under any circumstances, sequences generated with cycling parameters below 300 cycles (2 x 150).**

MiSeq Sequencing Kit (cycles)	DNA Load (Mb)
v2 300	90
v2 500	100

Table 2. Estimated DNA load capacity (in Mb) for MiSeq reagent kits.

9.1.6. Determine which indexes will be used and enter (or select from the drop-down) the well position (from the index plate) into the Library Prep tab of the workbook

NOTE: *It is recommended not to use the same index pairs within two consecutive runs on the same sequencer to reduce the amount of carryover. The index tracking worksheet, PNL35.W4, may be used to reserve/keep track of used and available indexes for plated Nextera CD Indexes/IDT for Illumina UD Indexes.*

9.1.7. Enter the volume of input DNA to be added to each well at the beginning of the library preparation process (column H)

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NOTE1: PulseNet recommends the starting volume of 10-15 μl , as long as the minimum total quantity (column J) of input DNA is **above 300 ng** (Illumina recommends the range of 300-2000 ng when no quantification of the individual libraries is performed). Individual laboratories may adjust input DNA volumes to ensure the total DNA amount is above the desired minimum.

NOTE2: The minimum volume of input DNA is 2 μl . If DNA is too concentrated, perform a dilution to bring input DNA volume above 2 μl and proceed.

9.2. Dilute and Tagment Input DNA: DNA is fragmented and tagged with the adapter sequences and bound to the BLT-PF during these steps. It is important that beads are well suspended at ALL steps in the 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.

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

NOTE: Ensure that BLT-PF is stored frozen and upright at all times so that the beads always remain submerged in the buffer.

9.2.1. Label a 96-well PCR plate (LP1) with Run ID

9.2.2. Add RSB to each sample well (typically 10-15 μl ; volume is automatically calculated and displayed on the Workbook)

9.2.3. Add DNA to each sample well (per volume in the Workbook, typically 10-15 μl) and mix well by gently pipetting approximately 5-10 times

9.2.4. Vortex BLT-PF for a **minimum** of 1 minute and verify proper suspension of beads; repeat if necessary. Do not centrifuge.

9.2.5. Add 15 μl BLT-PF to each well, set pipet to volume 35 μl , and pipette to mix

9.2.6. Vortex TB1 to mix and perform a quick spin

9.2.7. Add 10 μl TB1 to each well.

9.2.8. Set a pipet to 40 μl , pipet gently to mix well, resuspending the beads (using a multichannel pipette for mixing is recommended). Inspect the wells to ensure that samples and master mix are homogeneous before incubation. See Figure 1 below for additional information.

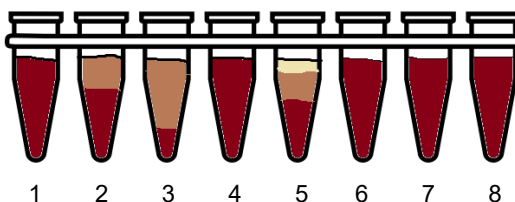


Figure 1. Example of homogeneous and non-homogeneous sample wells. Wells 1, 4, 6, 7 and 8 are well mixed. There should be no color striations (“sunsets”) from the bottom to the top of the wells. Wells 2, 3 and 5 display this settling and should be re-mixed prior to incubation.

9.2.9. Seal the plate with Microseal A (or equivalent) and incubate the plate at 41°C for 5 minutes, followed by a 10°C hold on a thermal cycler with the lid heated at 100°C (volume is 50 μl).

NOTE: It is recommended to pre-program a thermal cycler for this purpose.

9.3. Clean Up Tagmented DNA: The tagmentation reaction will be stopped and the DNA (now tagged with adapters and bound to the BLT-PF) will be washed prior to index ligation

NOTE: A deep-well MIDI plate may be used for the washing steps below if desired. This may improve formation of the pellet and decrease cross-contamination risks. The samples will need to be transferred to a 96-well skirted PCR plate for thermal cycler steps.

9.3.1. Place ELM on ice. Check ST2 for precipitate (if present, warm at 37°C for up to 10 minutes and vortex).

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- 9.3.2. Add 10 µl ST2 to each well and pipette to mix
- 9.3.3. Incubate at room temperature for 2 minutes
- 9.3.4. Place the plate on the magnetic stand and wait until the liquid is clear (2 minutes)
- 9.3.5. After the 2 minutes, remove and discard all supernatant from each well (volume set to 50 µl)
- 9.3.6. Remove the plate from the magnetic stand
- 9.3.7. Add 150 µl TWB to each well, and pipette gently (to avoid foaming) to mix ten times or until beads are fully resuspended
NOTE1: *A solution basin may be used for TWB if desired*
NOTE2: *Pipet with care. Some foaming may occur, but excessive foaming of TWB should be avoided, as foaming can lead to sample loss.*
- 9.3.8. Place back on the magnetic stand and wait until the liquid is clear (2 minutes)
- 9.3.9. Remove TWB from wells; using a small volume pipette is recommended to ensure removal of residual TWB before proceeding

9.4. Add the Indexes to the Tagmented DNA: In this step the indexes are ligated to the tagmented DNA but no library amplification (PCR) occurs

- 9.4.1. Remove the plate from the magnetic stand
- 9.4.2. Add 45 µl of ELM to each well
NOTE: *It is recommended to ensure that each pellet is submerged in master mix prior to moving on to the next well and to add master mix to all wells prior to mixing in order to avoid beads drying*
- 9.4.3. Pipette gently to mix until beads are fully resuspended and not on the side of the wells
- 9.4.4. Add 5 µl of appropriate index pair from the Nextera CD/IDT for Illumina UD Index plate to each sample well as indicated by the workbook (PNL43.W1)
NOTE: *Each well contains a unique index pair in a single-use volume. It is recommended to pierce the foil of the desired well on the index plate with a new pipette tip, then to use a fresh pipette tip to remove the indexes from the wells, followed by resealing the open wells (Microseal F or equivalent) on the index plate after use.*
- 9.4.5. Pipet a minimum of 10 times to mix. Inspect the wells to ensure that samples and master mix are homogeneous prior to incubation. Refer to Figure 1.
- 9.4.6. Seal the plate with Microseal A or equivalent and place onto a thermal cycler. Incubate at 37°C for 5 minutes and then 50°C for 5 minutes. Hold at 10°C on a thermal cycler with a lid pre-heated to 100°C.
NOTE: *It is recommended to pre-program a thermal cycler for this purpose*
- 9.4.7. Prepare diluted HP3:

Reagent	Volume per Sample	Example: 20 samples
HP3	6 µl	120 µl
Molecular grade water	54 µl	1080 µl

Table 3: Reagent volumes per sample for diluted HP3.

- NOTE1:** *These volumes produce 60 µl diluted HP3 per reaction, including overage*
- NOTE2:** *The number of samples being prepared may be entered into the Illumina DNA PCR-Free checklist, PNL43.W2 (“checklist”) and master mix volumes will be auto-calculated*
- 9.4.8. Remove the plate from thermal cycler and place on the magnetic stand for 2 minutes (wait until the liquid is clear)
- 9.4.9. While on the magnetic stand, remove and discard all supernatant from each well
- 9.4.10. Remove the plate from the magnetic stand and add 75 µl of TWB onto the beads in each well
- 9.4.11. Pipette gently to mix until beads are fully resuspended and not on the side of the wells

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- 9.4.12. Place the plate back on the magnetic stand and wait until the liquid is completely clear (2 minutes)
- 9.4.13. Remove and discard all supernatant from each well
- 9.4.14. Seal the plate with Microsoft A, and then centrifuge at 280 x g for 10 seconds
- 9.4.15. Place on the magnetic stand, without disturbing the bead pellet, and remove and discard residual supernatant from each well
- 9.4.16. Remove the plate from the magnetic stand
- 9.4.17. Add 45 µl of diluted HP3 to each sample
- 9.4.18. Pipette gently to mix until beads are fully resuspended and not on the side of the well
- 9.4.19. Incubate at room temperature for 2 minutes, while proceeding immediately to the next step, *Clean up Indexed Libraries*

9.5. Clean Up and Size Select Indexed Libraries: This dual bead clean-up procedure is for purification and size selection of libraries

NOTE: *The steps listed below are critical for efficient size selection, product recovery, and thus cluster generation and sequencing. Always check pipette tips for correct volumes and ensure that no beads have accidentally been aspirated on steps where supernatant is being removed. If beads have accidentally been aspirated or the bead pellet has been disturbed, allow the pellet to re-form and repeat the step. It is also important to ensure that beads are well suspended at all “pipet to mix steps” as described below.*

- 9.5.1. Dilute enough fresh 80% ethanol for all samples:

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

Table 4: Reagent volumes per sample for 80% ethanol

NOTE1: *It is recommended to increase the number of samples by 3-4 to ensure sufficient volume of master mix*

NOTE2: *The number of samples being prepared may be entered into the Illumina DNA PCR-Free checklist, PNL43.W2 (“checklist”) and master mix volumes will be auto-calculated*

- 9.5.2. Vortex and invert IPB several times to fully resuspend the beads
- 9.5.3. Add 36 µl IPB to each well
- 9.5.4. Pipet to mix a minimum of 10 times; using a multichannel pipet is recommended
- 9.5.5. Incubate at room temperature for 2 minutes
- 9.5.6. Place on the magnet for 5 minutes (or until beads form a tight pellet)
- 9.5.7. Label a second PCR-Plate (LP2)
 - NOTE1:** *Alternatively, the clean wells of the LP1 plate can be used*
 - NOTE2:** *A MIDI plate may be used for steps 9.5.9. through 9.5.24. if desired. This may improve pelleting of the beads and reduce the tendency of the beads to be pulled off of the magnet during removal of the final product.*
- 9.5.8. Vortex and invert IPB several times to fully resuspend the beads
- 9.5.9. Transfer 76 µl supernatant from each well of LP1 to the corresponding well of the LP2
- 9.5.10. Add 42µl IPB to each well with the supernatant
- 9.5.11. Gently pipet a minimum of 10 times to mix
- 9.5.12. Remove LP1 from the magnetic stand, and then discard
- 9.5.13. Incubate LP2 at room temperature for 2 minutes
- 9.5.14. Place on magnet for 5 minutes (or until beads form a tight pellet and supernatant clears)
- 9.5.15. Remove and discard supernatant
- 9.5.16. Perform the steps below (9.5.16.1. through 9.5.16.3.) twice, for a total of two washes:
 - 9.5.16.1. While the plate is on the magnet, add 180 µl of fresh 80% ethanol

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NOTE1: *Do not add directly to the bead, do not mix, and do not remove from the magnet during the wash steps*

NOTE2: *A solution basin may be used for the ethanol if desired*

9.5.16.2. Incubate for 30 seconds

9.5.16.3. Remove and discard supernatant

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

9.5.18. While on magnet, allow beads to air dry for 2-3 minutes

NOTE: *Do not allow beads to over-dry or crack. If cracking is observed, immediately resuspend beads as described below regardless of drying time.*

9.5.19. Remove from magnet and add 23 µl of RSB

9.5.20. Pipet gently and thoroughly to mix

9.5.21. Incubate at room temperature for 2-5 minutes

9.5.22. Centrifuge 280 x g for 10 seconds

9.5.23. Place on magnet for 2 minutes (or until supernatant is clear)

9.5.24. Transfer 20 µl of the supernatant into new wells (or into wells on a new plate) – this is the final product

NOTE1: *A quantification step (Qubit or equivalent) using a kit meant for measuring single-stranded DNA may be performed at this point on the individual libraries and results may be recorded in the Workbook if desired. Checking post-clean up library concentrations is recommended for new users of the Illumina DNA PCR-Free kit and for troubleshooting purposes. See PNL33 for specific instructions on operating the Qubit. Ideal library yield is >1 ng/µl. Sequencing libraries successfully with yields as low as 0.7 ng/µl may be possible but indicates inefficient library preparation.*

NOTE2: *Due to the single-stranded nature of the library DNA, determination of the fragment size distribution by fragment analysis on a capillary electrophoresis instrument is not possible*

NOTE3: *This is a safe stopping point. The plate may be sealed with Microseal B or equivalent and stored at -20°C for up to 30 days per Illumina recommendation*

9.6. Pool Libraries: In this step the individual libraries are combined into a single pool and the pool is quantified and diluted to be sequenced with a v2 MiSeq cartridge (300 or 500 cycles)

9.6.1. Using the following volumes, pool each library into a new well, and pipet thoroughly to mix:

9.6.1.1. *Escherichia/Shigella, Salmonella, Vibrio:* 4 µl

9.6.1.1. *Campylobacter, Listeria:* 1.5 µl

9.6.2. Quantify the pool using the Qubit (or equivalent) instrument and the ssDNA kit. See SOP PNL33 for instructions on operating the Qubit.

9.6.3. Enter the concentration for the pool in the appropriate cell of the Workbook

9.6.4. Calculate the molarity (nM) of the pool:

ssDNA Qubit reading (ng/µl) x 3.36

NOTE1: *The workbook will automatically calculate and display this value*

NOTE2: *The formula in the workbook is based on an assumed average fragment size of 450 base pairs*

$10^6 / (660 \text{g/mol} \times 450 \text{bp}) = 3.36$

9.6.5. Calculate the volume (µl) of pool necessary to generate 50µl of a 2 nM pool:

$(100 / \text{Molarity of pool}) = \text{volume of pool for dilution}$

NOTE: *The workbook will automatically calculate and display this value*

9.6.6. Calculate the volume (µl) of RSB diluent required:

$50 - \text{volume of pool} = \text{volume of RSB required}$

NOTE: *The workbook will automatically calculate and display this value*

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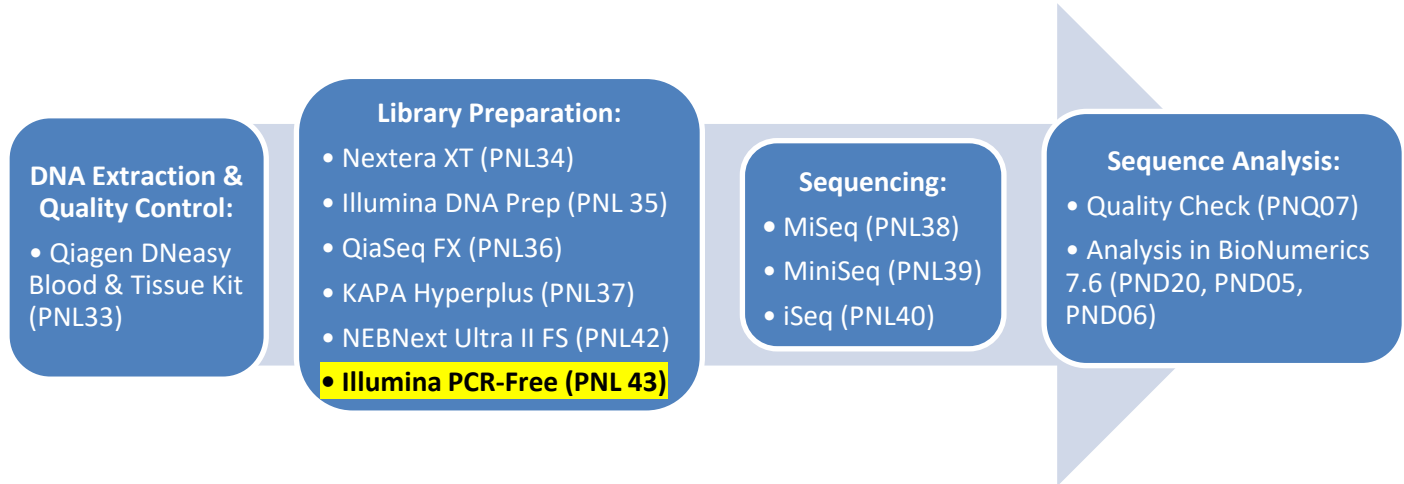
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9.6.7. In a new well, dilute the pool to 2 nM by adding the calculated volume of library pool to the calculated volume of RSB

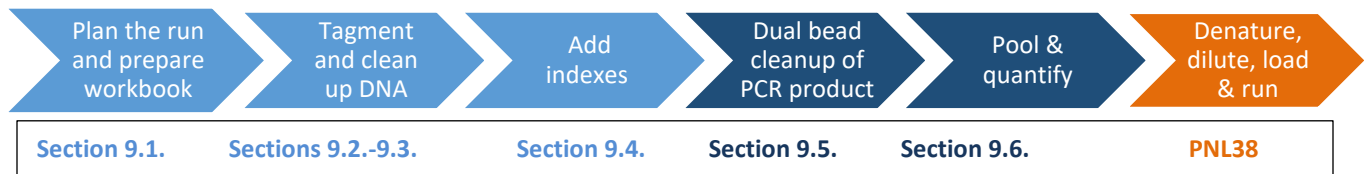
NOTE: The pooled libraries are now ready for sequencing. Proceed to the SOP PNL38 for instructions to denature and/or dilute the pool to the proper loading concentration.

10. FLOW CHARTS:

10.1. PulseNet WGS Workflow:



10.2. Illumina PCR-Free Library Preparation Workflow:



11. RELATED DOCUMENTS:

Document Number	Title
PNL33	DNA Extraction and QC SOP
PNL38	Sequencing on the MiSeq SOP
PNQ07	Illumina Sequence Data QC SOP
PND20	BioNumerics RefID Database Workflow SOP
PND05	BioNumerics Organism-Specific Database Workflow SOP
PNL43.W1	Illumina DNA Prep Workbook, 96 CD/UD Indexes, with separate sample sheets for MiSeq IEM, MiSeq LRM and MiSeq LRM3
PNL43.W2	Illumina DNA PCR-Free Library Prep Checklist
PNL35.W4	Illumina DNA Prep Index Tracking Template Worksheet

12. REFERENCES:

12.1. Illumina, Inc. Index Adapters Pooling Guide. (Doc.# 1000000041074 v10). July 2020.
<https://support.illumina.com/content/dam/illumina->

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[support/documents/documentation/chemistry_documentation/experiment-design/index-adapters-pooling-guide-1000000041074-10.pdf](https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/experiment-design/index-adapters-pooling-guide-1000000041074-10.pdf)

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13. CONTACTS:

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

14. AMENDMENTS:

14.1. **03/14/2023:** New Document

15. APPROVAL SIGNATURES:

Approved By: _____ Date: _____
QA/QC Personnel

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

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

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