1. **PURPOSE:** This SOP describes the standardized laboratory protocol for whole genome sequencing of bacterial organisms on the MiSeq, including instrument requirements and maintenance.

2. **SCOPE:** This document applies to all PulseNet WGS certified laboratories performing whole genome sequencing on enteric bacteria using the Illumina MiSeq platform and libraries generated with the library preparation kits accepted by PulseNet (Table 2) for submission of sequence data to PulseNet. Participating PulseNet laboratories may amend this procedure as necessary for use within their laboratories after validation per their laboratory's guidelines.

3. **DEFINITIONS**

3.1. **BaseSpace:** Illumina cloud-based computing environment for next generation sequencing data analysis, management, and storage, including data sharing.
3.2. **CD:** Cluster Density; indicates the quantity of clusters that are generated per flow cell surface area during the cluster generation stage.
3.3. **CDC:** Centers for Disease Control and Prevention
3.4. **CPF**: Clusters Passing Filter; percentage of generated clusters that pass an internal quality filtering procedure.
3.5. **CSV:** Comma-Separated Values (file) or Comma Delimited (file)
3.6. **DNA:** Deoxyribonucleic acid
3.7. **dsDNA:** double-stranded DNA
3.8. **EBT:** Elution Buffer with Tween
3.9. **Fastq:** A text-based file format for storing both a sequence and its corresponding quality scores.
3.10. **GB:** Giga byte
3.11. **GHS:** Globally Harmonized System
3.12. **HT1:** Hybridization buffer
3.13. **LRM:** Local Run Manager
3.14. **MCS:** MiSeq Control Software
3.15. **NaOCl:** Sodium Hypochlorite
3.16. **NaOH:** Sodium Hydroxide
3.17. **nM:** Nanomolar
3.18. **PhiX**: a control library that is derived from a well characterized bacteriophage genome. It has average size of 500 bp and balanced base composition.
3.19. **PHL:** Public Health Laboratory
3.20. **pM:** Picomolar
3.21. **PN:** PulseNet
3.22. **PPE:** Personal Protective Equipment
3.23. **PR2:** Incorporation Buffer
3.24. **PulseNet Central:** PulseNet team at CDC comprising of the PulseNet Response and Outbreak Management Team (PulseNet@cdc.gov) and the WGS Core Laboratory Activity (PulseNetNGSlab@cdc.gov).
3.25. **PulseNet/OutbreakNet SharePoint**: A closed, web-based collaboration application used for communication among PulseNet participants.
3.26. **Q30:** The percentage of reads within the entire run that have Q scores >30.0.

3.27. **QC:** Quality Control
3.28. **Q Score:** The sequence quality score for each individual base position in a sequence, indicating the accuracy of the base call. Phred scores are used, where Q = -10log (Error Probability). The higher the quality score, the more reliable the base call. A Q30 means a 1 in 1000 likelihood of an incorrect base call at that position.
3.29. **RFID:** Radio-Frequency Identification
3.30. **RSB:** Resuspension Buffer
3.31. **SAV:** Sequencing Analysis Viewer; an application software that allows real-time viewing of quality metrics generated by the real-time analysis (RTA) software on the Illumina sequencing systems.
3.32. **SDS:** Safety Data Sheet
3.33. **SOP:** Standard Operating Procedure
3.34. **Tris-HCl:** Tris Hydrochloride
3.35. **UPS:** Uninterrupted Power Supply
3.36. **VP10:** Custom Read 1 Primer for sequencing Illumina DNA PCR-Free Prep libraries.
3.37. **WGS**: Whole Genome Sequencing

4. RESPONSIBILITIES

4.1. **PulseNet Public Health Laboratories:**
4.1.1. Sequence isolates and perform quality check of the sequencing run and subsequent sequence data.
4.1.2. Re-sequence any isolates that do not meet quality thresholds.
4.1.3. Inform PulseNet Central, as necessary, about any complications with laboratory protocols or suspected instrument or 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. The MiSeq reagent cartridges contain formamide (GHS classification Category 1B for reproductive toxicity), an aliphatic amide that is a potential reproductive toxin. Personal injury can occur through inhalation, ingestion, skin and eye contact.
5.2.2. PR2 buffer should not be discarded into drains (GHS Category 1 for skin sensitization).
5.2.3. Ethanol is flammable (GHS Flammability Category 2).

5.2.4. Sodium hydroxide is corrosive (GHS Category 1A and 1, GHS Category 3 for acute hazards to aquatic environment).

6. REAGENTS

6.1. MiSeq Reagent Kit Options:

- v2 Nano, 300 Cycles – Illumina, cat# MS-103-1001
- v2 Nano, 500 Cycles – Illumina, cat# MS-103-1003
- v2 Micro, 300 Cycles – Illumina, cat# MS-103-1002
- v2 300 Cycles – Illumina, cat# MS-102-2002 (single), MS-102-2022 (20-pack)
- v2 500 Cycles – Illumina, cat# MS-102-2003 (single), MS-102-2023 (20-pack)
- v3 600 Cycles – Illumina, cat# MS-102-3003

NOTE: ***PulseNet does not accept in any circumstances sequencing chemistries below 300 cycles.***

6.1.1. Box 1 of 2. Store at -15°C to -25°C, away from light.

- MiSeq Cartridge (see Section 5.2.1 for chemical safety information)
- Hybridization Buffer (HT1)

6.1.2. Box 2 of 2. Store at 2-8°C.

- Incorporation Buffer (PR2, see 5.2.2 for chemical safety information)
- Flow Cell

6.2. Ethanol, molecular-grade, 95-100% (Fisher Scientific, cat# BP2818-500 or equivalent)

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

6.4. Water, molecular grade (Fisher Scientific, cat# BP24701 or equivalent)

6.5. Sodium Hypochlorite, lab-grade, 4 – 8.25% (Fisher Scientific cat# SS290-1 or equivalent).
NOTE: *It is recommended to use laboratory grade sodium hypochlorite, due to possible additives in over-the-counter grade sodium hypochlorite, which may interfere with the instrument's fluidics.*

6.6. Sodium Hydroxide, suitable for cell culture, 1N (Millipore Sigma, cat# S2770-100ML or equivalent), pH should be ≥ 12.5
NOTE: *Recommended to use liquid over powdered. Also advisable to aliquot and freeze in 100 µl, single-use aliquots.*

6.7. Tween 20, molecular grade, (Millipore Sigma, cat# P9416-50ML or equivalent)

6.8. **OPTIONAL:** 10nM PhiX Control Kit v3 (Illumina, cat# FC-110-3001). Store at -25°C to -15°C.

6.9. **If sequencing Illumina DNA PCR-Free Prep libraries:** Illumina DNA PCR-Free Prep Sequencing and Indexing Primer (Illumina, cat. No. 20041797, 2 x 7.5 ml of V10 Read 1 Primer, 2 x 7.5 ml of V14 Read 2 Primer). **NOTE:** *the V14 Read 2 primer is not used for this procedure.*

7. SUPPLIES

7.1. Ice

7.2. Illumina MiSeq Wash Tray (n=2, one for bleach washes, another for water washes only)

7.3. Lint-free wipes (Fisher Scientific, cat# 06-666 or equivalent)

7.4. Lens paper (Fisher Scientific, cat# 11-996 or equivalent)

7.5. MiSeq Disposable Wash Tube (Illumina, at# MS-102-9999)
7.6. Wash Buffer Bottle for MiSeq, Nalgene container, 500 ml (n=2, for 0.5% Tween 20 and molecular grade water, Fisher Scientific, cat# 15-350-205 or equivalent). **ALTERNATIVELY:** Can use spent and rinsed PR2 bottles.
7.7. Microseal B film (BioRad, cat# MSB-1001)
7.8. 1.5 ml microcentrifuge tubes (Fisher Scientific, cat# 05-408-129 or equivalent)
7.9. Sterile pipette tips, filtered: 20 µl, 200 µl,1000 µl and 1000 µl extended length volumes (Rainin, cat# 30389225, 30389239, 30389212 and 30389223 or equivalent)
7.10. Wash bottle with nozzle (x3, one each for 0.5% Tween 20, 100% ethanol, and molecular grade water (Fisher Scientific, cat# FB0340922D or equivalent))

8. EQUIPMENT

8.1. MiSeq Benchtop Sequencer
8.2. Micropipettes, capable of volumes from 1 µl to 1000 µl
8.3. Heat block (Nextera XT, KAPA HyperPlus, QiaSeq FX and NEB Ultra II FS library preps only)
8.4. Ice buckets
8.5. Microcentrifuge
8.6. Microplate centrifuge or equivalent
8.7. **OPTIONAL**: UPS back-up for the MiSeq (Recommended: Staples, Cyberpower AVR Series Line Interactive 1.5 kVA UPS, cat# CP1500AVRLCD)
8.8. **OPTIONAL:** External encrypted hard drive or server for data transfer and storage if BaseSpace or networking of the instrument is not available (Suggested: CDW, DataLocker H350 Basic Hard Drive 1 TB USB 3.0, cat# 4075102)

9. PROCEDURE

NOTE: *It has been documented and observed that cluster density can be negatively affected by the use of ammonium-based cleaning products near sequencing equipment, including lab benches and pipets used for library preparation. Do not use quaternary ammonium compounds or wipes near or on sequencing equipment!*

9.1. Prepare the Reagent Cartridge for the MiSeq

9.1.1. Plan sufficient time for thawing the sequencing reagents (sequencing cartridge, HT1 buffer) and the V10 primer (if sequencing Illumina DNA PCR-Free Prep libraries). The HT1 and the VP10 primer buffer may be thawed on ice or at 2-8°C. See Table 1 for guidance regarding thaw time, method and storage for the sequencing cartridge:

Method of Thawing	Time Required to Thaw	Thawed Cartridge Storage Life (2 – 8ºC or on ice)	A cartridge should not be re-frozen after thawing
Room temperature water bath (opened)	~ 1 hour	24 hours[1]	
2 – 8ºC (unopened)	8-12 hours	7 days	

Table 1. Guidance for reagent kit thawing and storage after thawing.
[1]Illumina recommendation 6 h

9.1.2. Once thawed, invert the reagent cartridge 10 times to mix, and then visually inspect to verify that all positions are completely thawed and are free of precipitates.

9.1.3. Gently tap the cartridge on the bench to reduce air bubbles in the bottom of the reagent wells and ensure that the foil seals covering the wells are unobstructed.

9.1.4. Place the thawed reagent cartridge and HT1 on ice or set aside at 2 – 8°C until the sample is ready to be loaded.

9.2. **Chemical Denaturation of the Pooled Library DNA**

NOTE: *Refer to the available PulseNet Library Preparation SOPs for various kit options in prepping and pooling the libraries. If using the DNA Prep Checklist (PNL35.W3), pooling, denaturation and dilution and instrument loading are listed as steps #80-94.*

9.2.1. **Nextera XT, KAPA HyperPlus, QiaSeq FX and NEB Ultra II FS libraries only:** Pre-heat a heat block to 96°C ± 1.

9.2.2. Retrieve the pooled library plate/tubes, and if frozen, thaw on ice. Centrifuge at 800 – 1200 rpm (or 280 x g) for 1 minute.

9.2.3. Remove a 100 µl aliquot of 1 N NaOH from the freezer, and thaw on ice,

9.2.4. Dilute to 0.2 N by adding 400 µl of molecular grade water to the 100 µl aliquot of 1 N NaOH. Invert the tube several times to mix.

NOTE: *Freshly diluted NaOH should be used within 6 hours of dilution.*

9.2.5. Combine 5 µl of the pooled sample library and 5 µl of 0.2 N NaOH in a new 1.5 ml microcentrifuge tube (now at 1 or 2 nM).

9.2.6. Incubate for 5 minutes at room temperature to denature the dsDNA.

9.2.7. **IMMEDIATELY** add 990 µl of pre-chilled HT1 to the tube containing 10 µl of denatured pooled library after the incubation. Refer to Table 2 for initial and denatured library concentrations.

Library Prep Kit	Initial Library Pool Concentration	Denatured Library Concentration After Addition of 990 µl of HT1
Nextera XT, NEB Ultra II FS, QIAseq FX, & KAPA HyperPlus	2 nM	10 pM
Illumina DNA Prep, PulseNet Rapid Prep, Nextera XT, NEB Ultra II FS, QIAseq FX, & KAPA HyperPlus	4 nM	20 pM
Illumina DNA PCR-Free	2 nM	20 pM

Table 2. Concentrations for the initial library pool and the denaturated pool for PulseNet validated library preps

9.3. **Dilution to Desired Final Loading Concentration and Heat Denaturation of the Pooled Library DNA**

9.3.1. Determine desired final loading concentration, based on library preparation type and sequencing kit to be used. See Table 3 below for guidance.

NOTE: *Final loading concentration may also be affected by a particular instrument and/or user and may be adjusted above or below the recommended concentrations in Table 3 in order to achieve optimal cluster density (CD). However, loading concentration should **only** be adjusted if the accuracy of the molarity calculation has been verified first by determining the average fragment size of the library pool using fragment analysis. An average fragment size that significantly differs from the default 1000 bp used in the molarity calculation will result in CD that is outside of the targeted range. In that case adjusting the molarity calculation based on the observed fragment size and/or correcting the errors in the library preparation resulting in aberrant fragment size should be done BEFORE adjusting loading concentration.*

Library Prep Kit	Recommended Final Loading Concentration	
	v2 (target CD 800-1200 K/mm^2)	v3 (target CD 1200-1400 K/mm^2)
Nextera XT	10 pM	15 pM
Illumina DNA Prep	12 pM	15 pM
NEB Ultra II FS & KAPA HyperPlus	10 pM	ND[1]
QIAseq FX	8 pM	ND[1]
Illumina DNA PCR-Free, PulseNet Rapid Prep	12 pM	ND[1]

Table 3. Recommended loading concentrations for different library prep kits. [1]Not determined.

9.3.2. Refer to Table 4 below to obtain the final loading concentration of the pooled library and dilute accordingly:

Final Loading Concentration	Denatured Pool Concentration = 10 pM		Denatured Pool Concentration = 20 pM	
	Required Volume of HT1	Required Volume of Denatured Pool	Required Volume of HT1	Required Volume of Denatured Pool
8 pM	200 µl	800 µl	600 µl	400 µl
10 pM	NA	NA	500 µl	500 µl
12 pM	NA	NA	400 µl	600 µl
15 pM	NA	NA	250 µl	750 µl

Table 4. Required pool and HT1 volumes for dilution to desired final concentration.

9.3.3. Thoroughly mix the diluted libraries.
OPTIONAL: *PhiX control may be spiked in at this point. See Appendix PNL38-1 for instructions and more information.*

9.3.4. **Nextera XT, KAPA HyperPlus, QiaSeq FX and NEB Ultra II FS libraries only:** Heat the denatured, diluted library pool to 96 ± 1°C for 2 minutes in a heat block to ensure complete denaturation.

9.3.5. Place the library pool on ice. The heat-treated library pools need to be cooled on ice immediately after the heat treatment for at least 5 minutes prior to loading.
NOTE: *The DNA library may sit on ice or at 2 – 8°C until ready for loading (< 30 minutes). If greater than 30 minutes passes before starting the run, it may be necessary to re-denature and dilute the pool.*

9.4. **Prepare the MiSeq Instrument for the Run**

9.4.1. Prior to setting up the run, ensure that there is enough free disk space (100 GB) on the instrument. If there is less than 100 GB available, see Section 9.11.3. for instructions on deleting files prior to loading the instrument and starting a run.

9.4.2. Ensure that the Sample Sheet (and Sample Plate, if applicable) setup has been completed in the Local Run Manager (LRM) on the instrument. See Appendix PNL38-2 for instructions.
NOTE: *The workbooks contain separate tabs for different Illumina instruments: MiSeq LRM3&4, iSeq LRM, MiniSeq LRM, and the Sample Import Template for NextSeq.*

9.4.3. Open the MiSeq Control Software window, select “Sequence”.

9.4.4. Select "Local Run Manager", log in with your username and password, select appropriate BaseSpace streaming option, and confirm run parameters before selecting "Next". The instrument will provide a prompt to load the flow cell.

9.5. **Prepare and Load the Flow Cell**

9.5.1. Retrieve the flow cell and the Incorporation Buffer (PR2) from storage (2 – 8°C).
NOTE: *Do not keep or store the PR2 buffer on ice as it may form particulates.*

9.5.2. While wearing clean, powder-free gloves, carefully remove the flow cell from the container, without touching the glass of the flow cell.

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

9.5.4. Pat the flow cell dry with a lint-free wipe, using care around the black port gasket. Make sure to remove all excess fluid.

9.5.5. Wet a clean piece of lens paper with ethanol and clean the flow cell glass, making sure that the glass imaging area (B in Fig. 1 below) is free of streaks, lint, and tissue fibers.
NOTE: *Do not add ethanol directly to flow cell. Avoid getting ethanol on the flow cell gaskets (A & C in Fig. 1).*

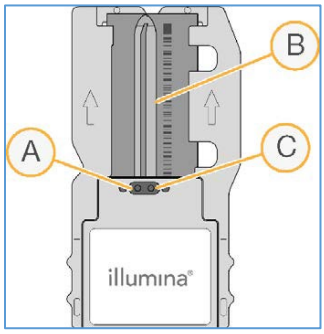


Figure 1. Flow cell. Port gaskets (A & C), imaging channels (B)

9.5.6. Dry any excess alcohol with the lens paper, and visually inspect to make sure that the flow cell ports (A & C) are free of obstructions, that the gasket is well-seated around the flow cell ports, and that there are no smudges or debris on the glass.

9.5.7. Raise the flow cell compartment door on the MiSeq and press the release button to open the clamp and remove the previously used flow cell.

9.5.8. Ensure that the flow cell stage is free of lint. If necessary, wipe with an alcohol wipe or similar and allow to dry.

9.5.9. Place the flow cell on the stage, close the flow cell clamp (it will click when closed fully), close the compartment door and select "Next" in the MiSeq Control Software. Loading of the PR2 bottle will be prompted.

9.6. **Load the Incorporation Buffer**

9.6.1. Gently invert the Incorporation Buffer (PR2) to mix, and then remove the lid.

9.6.2. Open the reagent compartment door and raise the sipper handle until it locks into place.

9.6.3. Remove the Wash Buffer bottle, re-cap, and set aside for future use.

9.6.4. Place the PR2 bottle where the MiSeq Wash Buffer bottle was previously located.

9.6.5. Remove the waste bottle and empty into an appropriate container for chemical waste, if necessary.
NOTE: *All liquid that accumulates in the MiSeq waste bottle should be disposed of as formamide waste, including liquid from wash cycles. Ensure that chemical waste is labeled and disposed of appropriately.*

9.6.6. Slowly lower the sipper handle. Make sure that the sippers lower completely into the PR2 and waste bottles.

9.6.7. 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.*

9.6.8. Select "Next" on MCS and screen will prompt for loading cartridge.

9.7. Prepare and Load the Cartridge

9.7.1. Remove the thawed reagent cartridge from storage, dry it thoroughly, and mix the reagents by inverting gently 10 times.
NOTE: *Mixing of reagents is very important! If the library pool is put into the cartridge prior to mixing the cartridge reagents, use an extended pipet tip to remove it, tape the sample reservoir, mix the cartridge and then re-load the pool.*

9.7.2. Tap the reagent cartridge on a hard surface to collect contents at the bottom of the reservoirs.

9.7.3. Use a clean 1000 µl pipette tip to pierce the foil seal over the reservoir labeled "Load Sample".

9.7.4. Load **600 µl** of the denatured DNA library into the **"Load Sample"** reservoir (Fig. 2).
NOTE1: *If sequencing Illumina DNA PCR-Free Prep libraries, add 600 µl of the thawed VP10 primer into the custom reservoir, position 18 (A in Fig. 2).*

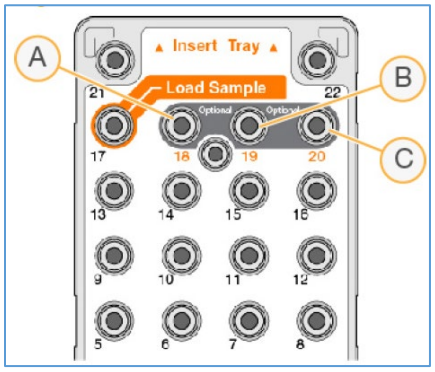


Figure 2. Reserved reservoirs in the sequencing cartridge: No. 17 for the library pool to be sequenced, no. 18-20 for custom primers.

NOTE2: *It is recommended to load the sample and primer toward the bottom of reservoir using an extended 1000 µl pipette tip to reduce the risk of droplets forming on the sides.* ***Take care to avoid droplets on the foil seal as the sample is dispensed.***

9.7.5. Tap gently on a hard surface to bring all contents to the bottom.

9.7.6. Open the reagent chiller door and remove the wash tray. If necessary, dry the bottom of the chiller compartment using absorbent wipes.
NOTE1: *Do NOT forcefully pull the wash tray; if there is resistance, the sippers may still be lowered. Wait until the MiSeq gives the prompt to load the cartridge and try again.*
NOTE2: *Do not leave the reagent chiller door open for extended periods of time.*

9.7.7. Slide the reagent cartridge into the chiller compartment until it stops.

9.7.8. Close the chiller door and check the screen to confirm that the RFID of the reagent cartridge has been read successfully.

9.7.9. Close the reagent compartment door.

9.7.10. Review Run Parameters and ensure that they are correct (Experiment Name, Analysis Workflow, Read Length, etc.) then select "Next" to proceed to the Pre-Run Check.
NOTE: *The system performs a check of all run components, disk space, and network connections prior to run start. If any part of the pre-run check fails, a message will appear on the screen with general instructions describing the error or detailing how to correct it. For more information, see "Resolving Run Setup Errors" in the MiSeq System User Guide. If all items successfully pass the pre-run check, the system is ready to start the run.*

9.7.11. Select "Start Run".
NOTE1: *Instrument can be set to automatically start run after system checks. This can be designated under "Run Settings" in the MiSeq Control Software by checking the box "Start run after Pre-Run check. Do not prompt for confirmation."*
NOTE2: ***Once the run has been started, do not open the flow cell compartment or the reagent compartment doors, neither should the instrument monitor be engaged unless the run is to be stopped or paused.***
NOTE3: *Image capture on the MiSeq is sensitive to vibration. Performing tasks that cause vibration near/on the instrument during a run could cause the run to halt or adversely impact sequencing results.*

9.8. **Review Run Metrics**

9.8.1. Upon run completion, confirm that the sequencing run meets basic quality metrics, which will be shown on MCS when the run is complete (Fig. 3)

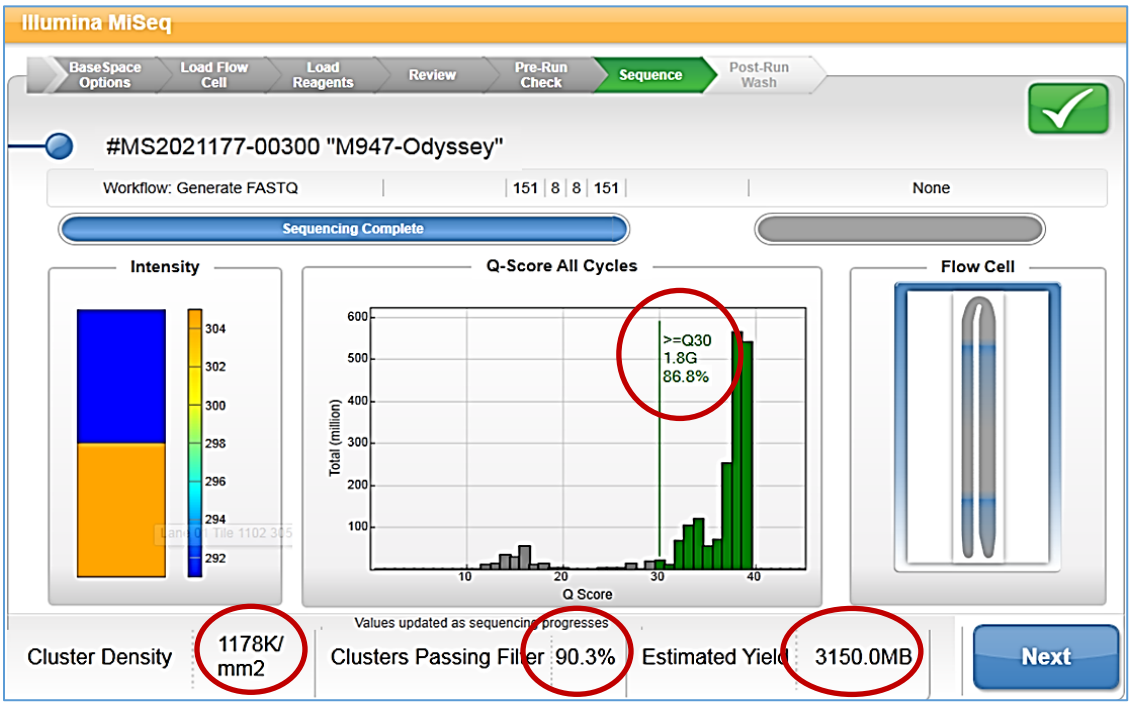


Figure 3. MiSeq Control Software Run Completion Screen. Primary metrics are circled in red.

NOTE: *These run metrics may also be found on the Summary Tab of SAV, see Fig. 4 below.*

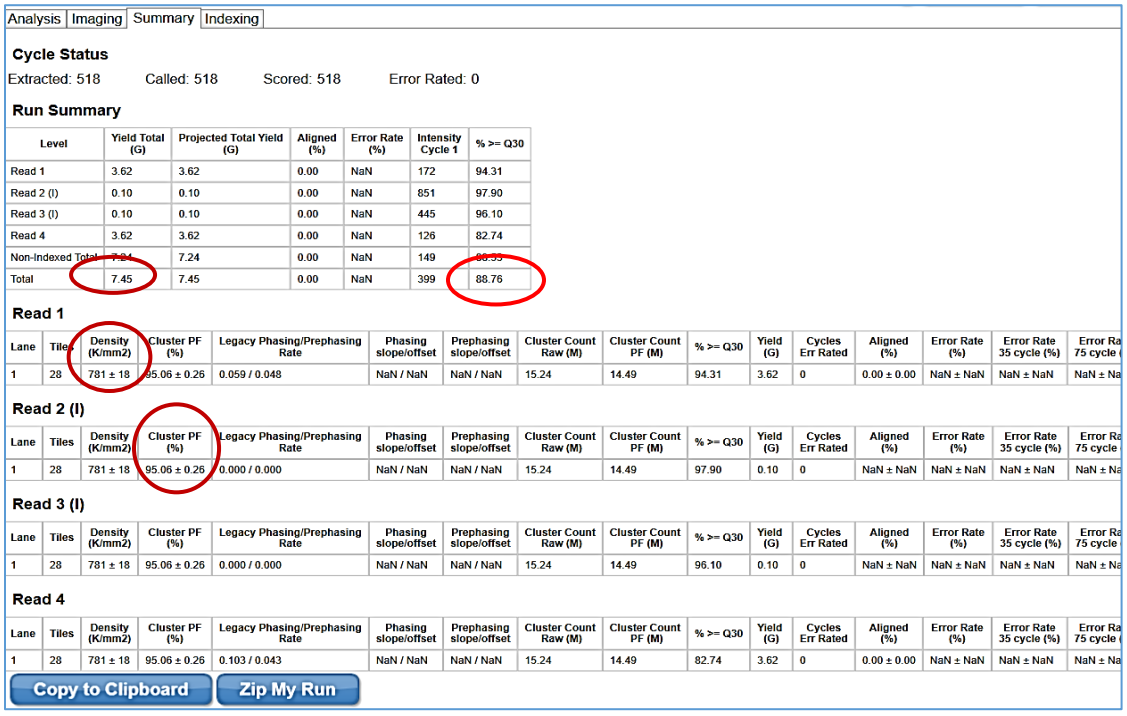


Figure 4. Screenshot of the SAV Summary Tab highlighting the key run metrics to be reviewed.

9.8.2. Record the primary run metrics on the workbook. Compare Q30, cluster density and clusters passing filter to PulseNet's recommended run metrics thresholds in Table 5.
NOTE: *If the run metrics do not meet the thresholds listed in Table 5, it is possible that some of the sequences may still pass the critical quality metrics outlined in PNQ07. Further analysis of the sequences is required to determine whether individual sequences are acceptable or need to be repeated.*

Kit Chemistry	Q30 (%)	Cluster Density[2] (K/mm^2)	Clusters Passing Filter (%)
v3, 500 cycle[1]	≥ 70	1200-1400	~ 80 or higher
v2, 500 cycle	≥ 75	800-1200	
v2, 300 cycle	≥ 80	800-1200	
Nano, v2 300 cycle	≥ 80	800-1200	
Nano, v2 500 cycle	≥ 75	800-1200	
Micro, v2 300 cycle	≥ 80	800-1200	

Table 5. Run metric thresholds for sequencing chemistries recommended by PulseNet.
[1]Kit standard is 600 cycles but PulseNet recommends sequencing at 500 cycles for better quality.
[2]Illumina recommendation 600-1200 K/mm^2. For a full run with optimized loading concentration, PulseNet recommendation is 800-1200 K/mm^2.

9.9. Export Data from the MiSeq

NOTE1: *This section is only necessary for users unable to stream to BaseSpace or if a secondary data repository (e.g., local server) is desired for local data analysis or storage.*
NOTE2: *There will be 2 fastq files per isolate for paired reads (R1 and R2). This data may be copied onto an external hard drive and moved to a computer workstation. Alternatively, the MiSeq instrument can also be connected to a local network share drive to directly transfer files from the instrument.*

9.9.1. Data is found on the D:\ drive → Run folder (choose the most recent run folder) → Alignment_1 → Run folder → Fastq.gz).
NOTE1: *It may take up to 1 hour for the MiSeq to generate fastq files post-run as the Real-Time Analysis Software lags behind the sequencing run. If using BaseSpace, the data can be accessed through the run or in a designated project.*
NOTE2: *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 LRM or corrective options in BaseSpace (see Appendix PNL38-3).*

9.10. Post-Run Washes and Reagent Disposal

9.10.1. Once the run is complete, select the "Start Wash" option at the bottom right-hand side of the screen.

9.10.2. Ensure the "Perform optional template line wash" checkbox is checked to proceed with a Post-Run bleach wash. **NOTE:** *Sodium hypochlorite (NaOCl) is used to reduce carry-over of nucleic acid from previous runs.*

9.10.3. Prepare a fresh dilution of molecular-grade sodium hypochlorite (NaOCl) using molecular-grade water. Refer to Table 6 below for serial dilution steps. Choose the first dilution according to the concentration of stock NaOCl.
NOTE1: *The second dilution (0.01%) may be prepared directly in a MiSeq wash tube/reservoir (A in Fig. 5).*
NOTE2: *Use of regular household bleach has not been validated and is discouraged.*

Dilution	Starting NaOCl Concentration	NaOCl Volume	Water Volume	Final NaOCl Concentration
First Dilution:	4%	30 µl	570 µl	**0.20%**
	5%	24 µl	576 µl	
	6%	20 µl	580 µl	
	8.25%	15 µl	585 µl	
Second Dilution:	0.20%	50 µl	950 µl	**0.01%**

Table 6. The calculations needed to prepare 0.2% and 0.01% dilutions from stock NaOCl.

9.10.4. Insert the wash tube into position 17 in the designated bleach wash tray until the neck is flush with the tray (B in Fig. 5).
NOTE: *It is recommended to have 2 wash trays, one designated for bleach and Tween 20 and the other designated for water wash only.*

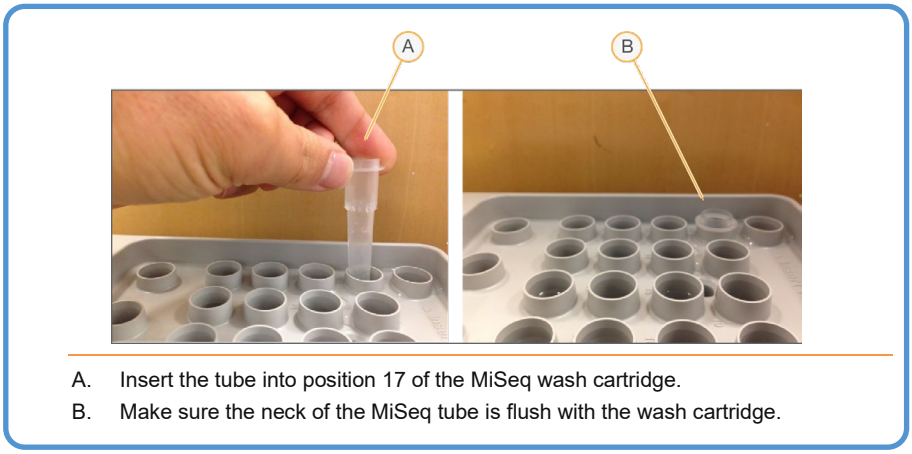


Figure 5. Inserting the 0.01% Sodium hypochlorite wash tube in wash tray.

9.10.5. Fill the remaining reservoirs with 0.5% Tween 20 wash solution.
NOTE: *0.5% Tween 20 can be prepared by adding 5 ml of Tween 20 into 995 ml of molecular-grade water. Only prepare 1 L of 0.5% Tween 20 at a time to ensure its used in a timely fashion. Over time, the detergent may degrade or separate out of solution and negatively affect sequencing quality. It is recommended to prepare fresh wash solution monthly or sooner if needed to reduce potential for contamination.*

9.10.6. Remove excess moisture from the surface of the wash cartridge with a lint-free wipe.

9.10.7. Open the reagent compartment door and chiller door and remove the used cartridge. Set the cartridge aside for disposal.
NOTE: *Reagent well 8 of the cartridge contains formamide and must be disposed of as hazardous chemical waste. See Section 5 and the SDS for safety details.*

9.10.8. Slide the wash tray into the chiller compartment until it stops, then close the chiller door.

9.10.9. Raise the sipper handle, remove the PR2 bottle and replace with a MiSeq Wash Buffer bottle containing 400-500 ml of 0.5% Tween 20 solution.
NOTE: *It is recommended to replace the Wash Buffer bottle with fresh 0.5% Tween prepared monthly or sooner to limit potential contamination.*

9.10.10. Remove the waste container and dispose of contents following the laboratory's hazardous chemical waste disposal methods (See Section 5.2.1. for safety information).
NOTE: *All liquid that accumulates in the MiSeq waste bottle should be disposed of as toxic formamide waste, including liquid from wash cycles. All formamide waste bottles must be clearly labeled as chemical waste and disposed of appropriately.*

9.10.11. Return the emptied waste container to the reagent compartment, lower the sipper arm, and close the reagent compartment door.

9.10.12. Select "Next" to begin the first Post-Run template line wash. This will take approximately 30 minutes.

9.10.13. Once the post-run template line wash is complete, select "Done" on the screen to return to the MiSeq Control Software home screen.

9.10.14. Immediately following the template line wash, a second Post-Run wash with molecular-grade water must be completed to ensure any trace amount of sodium hypochlorite is removed from the template line. From the home screen select "Perform Wash" > "Perform Post-Run Wash".

9.10.15. Select "Start Wash".
NOTE: *The "Template Line Wash" box should remain* ***un-checked****.*

9.10.16. Fill all reservoirs of the designated water wash tray with molecular-grade water.

9.10.17. Remove excess moisture from the surface of the wash cartridge with a lint-free wipe.

9.10.18. Remove the wash tray containing 0.5% Tween 20 and the bleach wash tube from the chiller compartment and rinse the wash tube thoroughly. Rinse the wash tray with de-ionized water (or equivalent) and let dry.

9.10.19. Place the water wash tray in the chiller compartment and close the door.

9.10.20. Raise the reagent sipper arm and replace the 0.5% Tween 20 wash bottle with a wash bottle containing 300-500 ml of molecular-grade water and lower the sipper arm.

9.10.21. Close the reagent compartment door and select "Next" to begin the wash.

9.10.22. Once the wash is complete, select "Done" to complete the wash cycle and return to the home screen. Keep the flow cell, wash bottle, wash cartridge and waste receptacle in place until the next sequencing run is to be performed.

9.11. **Instrument Maintenance**

	Maintenance Frequency		
	Post-Run	**Weekly**	**Monthly**
Type of Maintenance	Post-Run Template Line Wash, followed by Post-Run Water Wash	Post-Run Tween 20 Wash + Post-Run water wash	Maintenance Wash + Power cycle
Time Required	30 minutes + 20 minutes	20 minutes + 20 minutes	60 minutes+ 20 minutes
Reagents Needed	- 0.5% Tween 20 - 0.01% NaOCl - Molecular grade water	- 0.5% Tween 20 - Molecular grade water	- 0.5% Tween 20 - Molecular grade water

Table 7. Summary of the MiSeq maintenance.

9.11.1. Weekly Maintenance:

9.11.1.1. Wash: A Post-Run Wash with 0.5% Tween 20 should be performed to ensure proper instrument fluidics performance. If a sequencing run has been performed that week, the routine post-run wash followed by the water wash will suffice as the weekly wash (Step 9.10.). If the instrument has not been used, a manual post-run wash using 0.5% Tween 20 should be performed followed by a water wash.

9.11.2. Monthly Maintenance:

9.11.2.1. Maintenance Wash: To be performed at least once a month. This is a series of 3 washes (two 0.5 % Tween 20 washes and a water wash) that will require refilling of the wash tray after each wash cycle (20 minutes each). The total time for a maintenance wash is approximately 60 minutes.

9.11.2.1.1. Under Wash Options, select “Maintenance Wash”.

9.11.2.1.2. Fill the wash tray and wash buffer bottle with 0.5% Tween 20.

9.11.2.1.3. After the first two washes, top off the reservoirs of the wash tray and wash buffer bottle with molecular grade water.

9.11.2.2. Power cycle:

9.11.2.2.1. Select the “Manage Instrument” menu option on the home screen and then the “Shut Down” option.

9.11.2.2.2. Once the instrument has shut down (noted by a distinct click sound), reach behind the right-hand side of the instrument and flip off the power switch located near the power cord.

9.11.2.2.3. Let the instrument power down for at least 5 minutes and then flip the switch back on. The instrument will boot up the MCS in about 10 minutes.

9.11.3. Management of Disk Space on the Data (D:\) Drive:

9.11.3.1. The MiSeq requires 100 GB of free disk space on the D:\ drive before starting a sequencing run. **Data should be cleared only *after* any data transfer/back-up is**

complete, and if *no troubleshooting is necessary* for that run. The following instructions outline which files may be deleted and their locations:

9.11.3.1.1. Navigate to Data (D:\ Drive)/Illumina/MiSeqOutput/(most recent run folder)/. Within this folder select the "Images" and/or "Thumbnail_images" folders and delete (suggested for runs that are over one month old).

9.11.3.1.2. Navigate to Data (D:\ Drive)/Illumina/MiSeqAnalysis/. Delete any run folders excluding the most recent folder.

9.11.3.1.3. Empty the Recycle Bin.

9.11.3.1.4. Reboot the system following large data deletions.

9.11.3.1.4.1. On the MCS home screen, select "Manage Instrument" then "Reboot". It will take approximately 10 minutes for the system to reboot and start the MiSeq Control Software.
NOTE: *When performing the reboot, having an external hard drive plugged in may cause an error at the start-up.*

9.11.3.2. **BaseSpace Users:** It is recommended to maintain a duplicate copy of the run folder locally on the instrument as a backup. Doing the following will ensure that a run folder will be generated in the Data drive file folders, in addition to being streamed to BaseSpace:

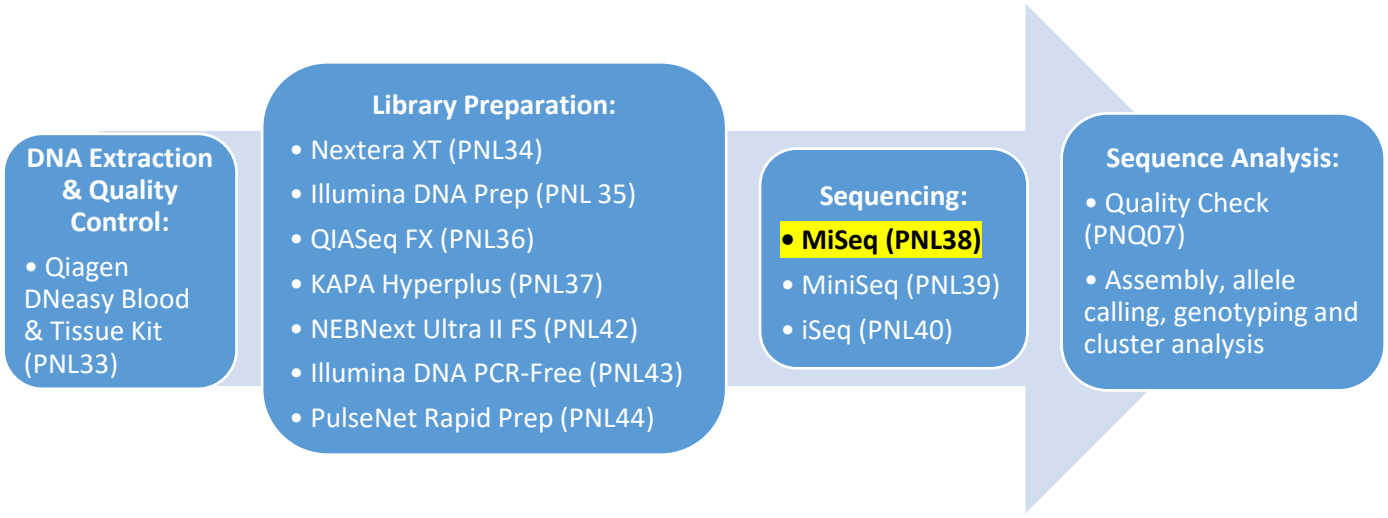
9.11.3.2.1. From the MCS home screen, select "Run Options".

9.11.3.2.2. Under the "Run Settings" tab, check the "When using BaseSpace, replicate analysis locally on MiSeq" checkbox.

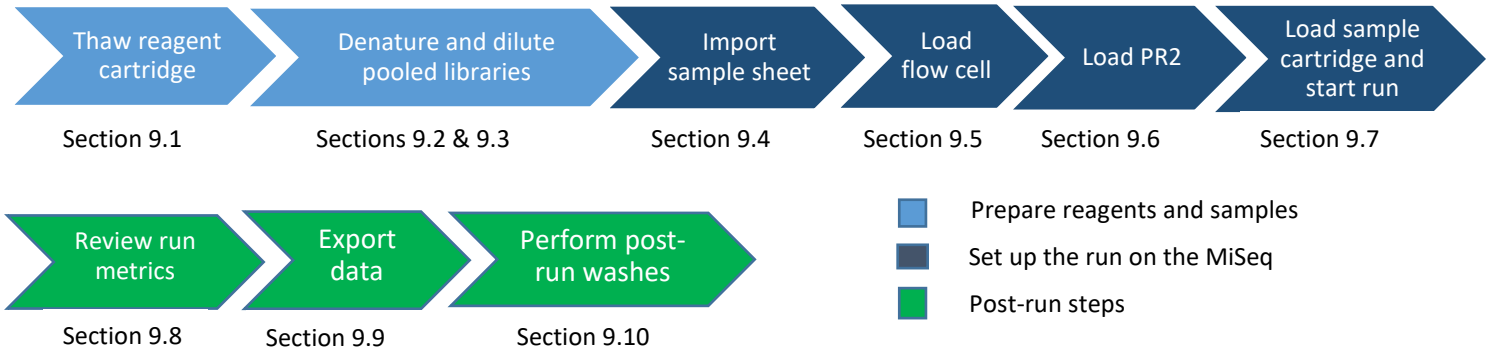
9.11.3.2.3. Select "Save and Return" to save these changes and return to the home screen.
NOTE: *This run folder may be deleted if run data was successfully streamed to BaseSpace and local data storage is not required.*

10. FLOW CHARTS:

10.1. PulseNet WGS Workflow



10.2. MiSeq Sequencing Workflow



11. RELATED DOCUMENTS:

Document Number	Title
PNL33	**DNA Extraction and QC SOP**
PNL34	**Nextera XT Library Prep SOP**
PNL34.W1	**Nextera XT Library Prep Workbook**
PNL35	**Illumina DNA Prep SOP**
PNL35.W1	**Illumina DNA Prep Workbook, 96 CD/UD Indexes, separate sample sheets for MiSeq LRM, MiniSeq LRM, and iSeq LRM, and Sample Import Template for NextSeq LRM**
PNL35.W3	**Illumina DNA Prep Checklist**
PNL36	**QIAseq FX Library Prep SOP**
PNL36.W1	**QIAseq FX Library Prep Workbook**
PNL37	**KAPA HyperPlus Library Prep SOP**
PNL37.W1	**KAPA HyperPlus Library Prep Workbook**
PNL42	**NEB Next Ultra II FS Library Prep SOP**
PNL42.W1	**NEB Next Ultra II FS Library Prep Workbook**
PNL43	**Illumina DNA PCR-Free Prep SOP**
PNL43.W1	**Illumina DNA PCR-Free Workbook**
PNL43.W2	**Illumina DNA PCR-Free Checklist**
PNL44	**PulseNet Rapid Prep SOP**
PNL44.W1	**PulseNet Rapid Prep Workbook**
PNL44.W2	**PulseNet Rapid Prep Checklist**
PNQ07	**Illumina Sequence Data QC SOP**

12. REFERENCES:

12.1. Illumina, Inc. MiSeq Product Documentation. Instructions for Operating and Maintaining the MiSeq Instrument. (Document # 200046664 v00). November 2023. https://support.illumina.com/sequencing/sequencing_instruments/miseq/documentation.html

12.2. Illumina, Inc. Local Run Manager v3 Software Guide (Document # 1000000111492 v01). March 2022. https://support.illumina.com/content/dam/illumina-support/documents/documentation/software_documentation/local-run-manager/local-run-manager-v3-software-guide-1000000111492_01.pdf

12.3. Illumina, Inc. MiSeq RUO Software System Customer Release Notes (Document Number: 200049209 Rev. 00). Effective Date: 17-NOV-2023. https://support.illumina.com/content/dam/illumina-support/documents/downloads/software/miseq/200049209_00_MiSeq%20RUO%20Software%20System%20v4.1.0%20Customer%20Release%20Notes.pdf

12.4. Illumina, Inc. MiSeq System Custom Primers Guide (15041638 v01). March 2016. https://emea.support.illumina.com/downloads/miseq-system-custom-primers-guide-15041638.html

12.5. Illumina, Inc. Impact of Ammonium Based Cleaning Products on Sequencing Run Performance. November 10, 2021. https://support.illumina.com/bulletins/2019/09/impact-of-ammonium-based-cleaning-products-on-sequencing-run-per.html

12.6. Illumina, Inc. How to edit a sample sheet and requeue an analysis in BaseSpace Sequence Hub (illumina.com). https://support.illumina.com/bulletins/2018/03/how-to-edit-a-sample-sheet-and-requeue-a-miseq--hiseq--or-novase.html

12.7. Illumina, Inc. MiSeq System. Denaturate and Dilute Libraries Guide. (Document # 15039740 v10). February 2019. https://support.illumina.com/content/dam/illumina-support/documents/documentation/system_documentation/miseq/miseq-denature-dilute-libraries-guide-15039740-10.pdf

13. CONTACTS:

13.1. PulseNet NGS Lab: pulsenetngslab@cdc.gov

13.2. Illumina Technical Support: techsupport@illumina.com

14. AMENDMENTS:

14.1. **01/31/2019:**

- Pulled procedures pertaining to setting up a run on the MiSeq out of PNL32 to create separate document (PNL38)

14.2. **02/02/2021**

- Added additional definitions
- Updated reagent and supply catalog numbers
- Added information for the NEBNext Ultra II FS and QIAseq FX library prep
- Added the “Review Run Metrics” section (9.8.)
- Added the workflow charts
- Added related documents
- Updated references
- Updated the PhiX instructions
- Updated the MiSeq sample sheet instructions by adding LRM

14.3. **02/01/2023**
- Added information for DNA PCR-Free Prep library prep kit
- Updated Table 1 (thawing and post-thaw storage of the cartridge)
- Additional figures added for clarity
- Added Table 7 summarizing the instrument maintenance
- Updated notes
- Updated references
- Former appendix PNL38-2 (MiSeq sample sheet set up) was separated into two appendixes by the software type: LRM (PNL38-2) and IEM (PNL38-3). Former appendix PNL38-3 (requeuing the analysis) is now appendix PNL38-4.
- Retired the MiSeq LRM job aid PNL38.JA1. This information is now included in the appendix PNL38-2.

14.4. **07/03/2024**
- Removed IEM and LRM versions earlier than 3 from the SOP and PNL35.W1. Consequently, Appendix PNL38-3 (instructions for the sample sheet set up in IEM) and instructions for requeuing the analysis using MSR in Appendix PNL38-4 were removed. Appendix PNL38-4 (Requeuing the analysis) became PNL38-3.
- Added language on emphasizing that loading concentration should only be adjusted after library fragment size has been verified
- Added Related Documents PNL44, PNL44.W1 and PNL.W2 (Rapid Library Prep SOP and associated workbooks)
- Updated the catalog number for obsolesced product [DNA PCR-Free Prep Sequencing Primer (cat# 20041496) containing only VP10 primer mix only discontinued October 2023]
- Removed the heat denaturation step from the SOP for the Illumina DNA Prep libraries
- Removed all references to BioNumerics
- Updated recommendation to prepare fresh 0.5% Tween wash buffer monthly or sooner and to include water wash after tween washes.
- Removed the weekly reboot as a maintenance recommendation
- Adjusted the lower acceptable threshold for CD from the Illumina recommended 600 to more realistic 800
- Removed links to outdated references

15. APPROVAL SIGNATURES:

Approved By: PATRICIA C. LAFON -S (Affiliate) Digitally signed by PATRICIA C. LAFON -S (Affiliate) Date: 2024.07.08 08:32:17 -04'00' Date: 07/08/2024
PulseNet QA/QC Personnel

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

Approved By: Peyton A. Smith -S Digitally signed by Peyton A. Smith -S Date: 2024.07.06 15:22:36 -04'00' Date: 07/06/2024
PulseNet WGS Technical Lead

Approved By: Efrain M. Ribot -S Digitally signed by Efrain M. Ribot -S Date: 2024.07.08 08:06:17 -04'00' Date: 07/08/2024
PulseNet Reference Outbreak Surveillance Team Lead

16. APPENDICES:

Appendix PNL38-1

Addition of PhiX Control

1. **Purpose**

PhiX is a 500 bp balanced genome of a bacteriophage and is recommended by Illumina as an instrument control which may help indicate how well the sequencing instrument is performing. A PhiX run after instrument maintenance or repair is recommended.

2. **Procedure**

NOTE1: *According to Illumina, 12.5 pM and 20 pM PhiX dilutions produce optimal cluster density for v2 and v3 reagents, respectively.*

NOTE2: *If using a previously prepared PhiX denatured dilution, proceed to Step 2.7.*

2.1. Prepare 0.2 N NaOH by adding 400 µl of molecular-grade water to a 100 µl aliquot of 1 N NaOH and place on ice until ready to use.

2.2. Combine 1 µl of 10 nM PhiX library from the stock and 4 µl of RSB (or the diluent, e.g., EBT, Tris-HCl, your library prep kit uses to normalize libraries/library pool) to obtain 2 nM PhiX library in a new 1.5 ml microcentrifuge tube.

2.3. Combine 5 µl of 2 nM PhiX library and 5 µl of 0.2 N NaOH and mix well.

2.4. Incubate for 5 minutes at room temperature to denature the PhiX library into single strands.

2.5. **IMMEDIATELY** add 490 µl of pre-chilled HT1 to the tube containing 10 µl of denatured 1 nM PhiX library after the incubation to obtain 20 pM of denatured PhiX library.

2.6. Label, date and initial the microcentrifuge tube. Denatured 20 pM PhiX libraries may be stored up to three weeks at -15º to -25ºC.

2.7. If using v2 sequencing reagents, dilute the denatured 20 pM PhiX library to 12.5 pM by adding 62.5 µl of 20 pM denatured PhiX library to 37.5 µl of pre-chilled HT1.

2.8. Determine percentage of PhiX (N%) to add to run. See below for general guidance:

- If troubleshooting a library preparation: 1-10%.
- If troubleshooting a MiSeq instrument issue: 5% or above (depending on Illumina tech support guidance) is appropriate.
- If using PhiX routinely in every run: 1% is recommended.
- If loading libraries with low sample diversity, or spiking in amplicon: 5-10%.

2.9. Combine N x 10µl of 12.5 pM (or 20 pM) PhiX with 1000 – (N x 10) µl of sample library of desired concentration.

- For example: for 1% PhiX addition, add 10 µl of 12.5 pM PhiX to 990 µl of denatured 10-20 pM sample library.

2.10. Place on ice until ready to proceed with heat denaturation of sample library (see section 9.3.4.) and addition of PhiX.

3. **PhiX Expected Results**

3.1. The "Aligned (%)" in SAV/BaseSpace upon run completion should match the percentage of PhiX spiked into a run. If not, this may be an indication of an instrument or library issue.

3.1.1. Libraries that are smaller than the size of the PhiX library (500 bp) will outcompete the PhiX, resulting in lower aligned percentage.

NOTE: *Libraries with low GC content, such as Campylobacter are prone to over-tagmentation particularly with the Nextera XT prep resulting in shorter fragments.*

3.1.2. Libraries that are larger than the PhiX will result in greater percentage of PhiX aligned than what was spiked in.

NOTE: *Libraries with high GC content, such as Mycobacterium tuberculosis tend to under-tagment particularly with the Nextera XT prep resulting in larger fragments.*

3.2. Contact Illumina Tech Support and Cc pulsenetngslab@cdc.gov for troubleshooting assistance if ncessary

Appendix PNL38-2

MiSeq Sample Sheet Setup Using LRM

NOTE: ***MiSeq sample sheet can be set up either by importing the SampleSheet tab from the library prep workbook (section 1) or by entering the information manually (section 2).***

1. Generate and Import a Sample Sheet from the PulseNet Workbook

1.1. Ensure that the Initial Dilution tab (Nextera XT) or the Library Prep tab (DNA Prep) is filled out properly. These tabs will auto-populate the sample sheets with the necessary information. Check each field in the Sample Sheet tab to ensure that all of the data has been accurately populated based on the Initial Dilution (Nextera XT) or the Library Prep (Illumina DNA Prep) tab. The Description and Sample_Project fields may be blank.
NOTE: *The index sequences are auto-populated from the Indices tab. Do not modify or delete this tab.*

1.2. Convert the appropriate Sample Sheet tab to a .csv (either by saving as or exporting as a .csv comma delimited file).

1.2.1. To save as .csv:

1.2.1.1. Open the "File" menu option and select "Save As".

1.2.1.2. Change "Save as type:" to "CSV (Comma delimited)".

1.2.1.3. Navigate to the desired file folder location and save the file using the Plate ID as the filename (i.e., LabID-MXXXX-YYMMDD).

1.2.1.4. Click "OK" to save only the active sheet, and "Yes" in the following window to keep using the CSV format.

OR

1.2.2. To export as .csv:

1.2.2.1. Open the "File" menu option and select "Export".

1.2.2.2. Select "Change File Type".

1.2.2.3. Choose "CSV (Comma delimited)".

1.2.2.4. Save and select "OK" to save only the active sheet.

1.3. Open the CSV file in WordPad or NotePad and delete any commas listed after the final sample. See Fig. 6 below.
NOTE: *This must be done in order to avoid errors when importing the sample sheet.*

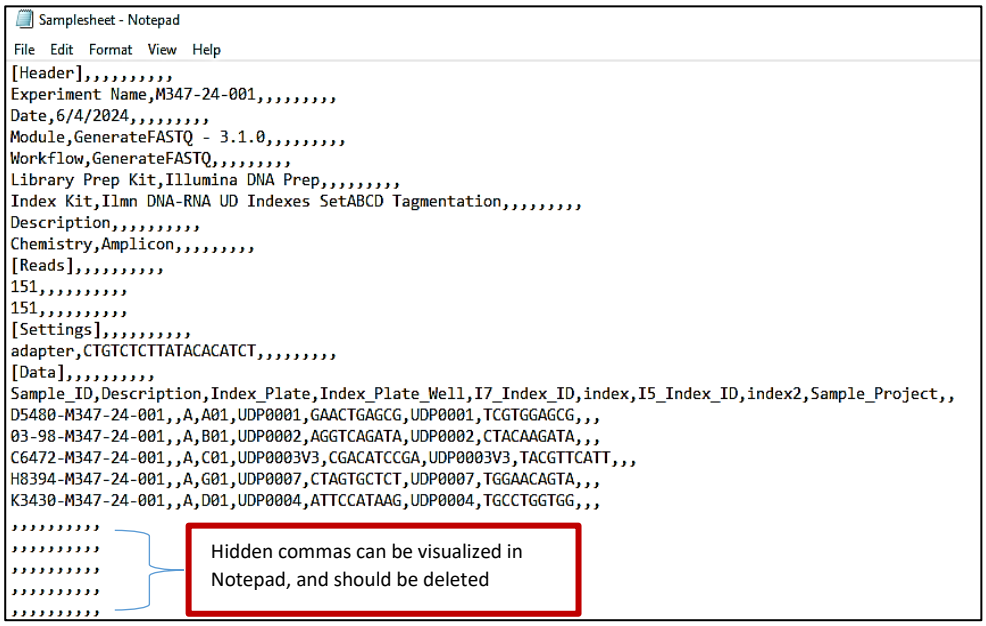


Figure 6. Sample sheet tab in Notepad, depicting extra commas to be deleted.

1.4. Transfer the .csv file to the instrument using a flash drive or similar:

1.4.1. On the MiSeq, open Chromium and log into LRM.

1.4.2. On the LRM home page, select "Create Run".

1.4.3. Select the "GenerateFASTQ" analysis module (Fig. 7).

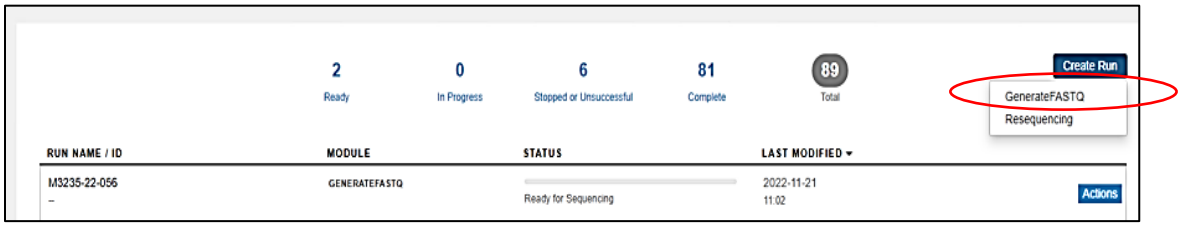


Figure 7. GenerateFASTQ module option in Local Run Manager.

1.4.4. Select "Import Sample Sheet" and navigate to the .csv file generated from the workbook.

1.4.5. Ensure that ALL run parameters, samples, and index information imported correctly (Fig. 8). This includes Run ID, Library Prep Kit, Index Kit, **Adapter Trimming,** Read Length, **Custom Primers (if sequencing Illumina DNA PCR-Free Prep libraries)**, etc.

NOTE: *If the Library Prep Kit defaults to "Custom" (as in the example in Fig. 9 below); do not proceed. Ensure that the commas in the exported sample sheet were deleted (Step 1.3).*

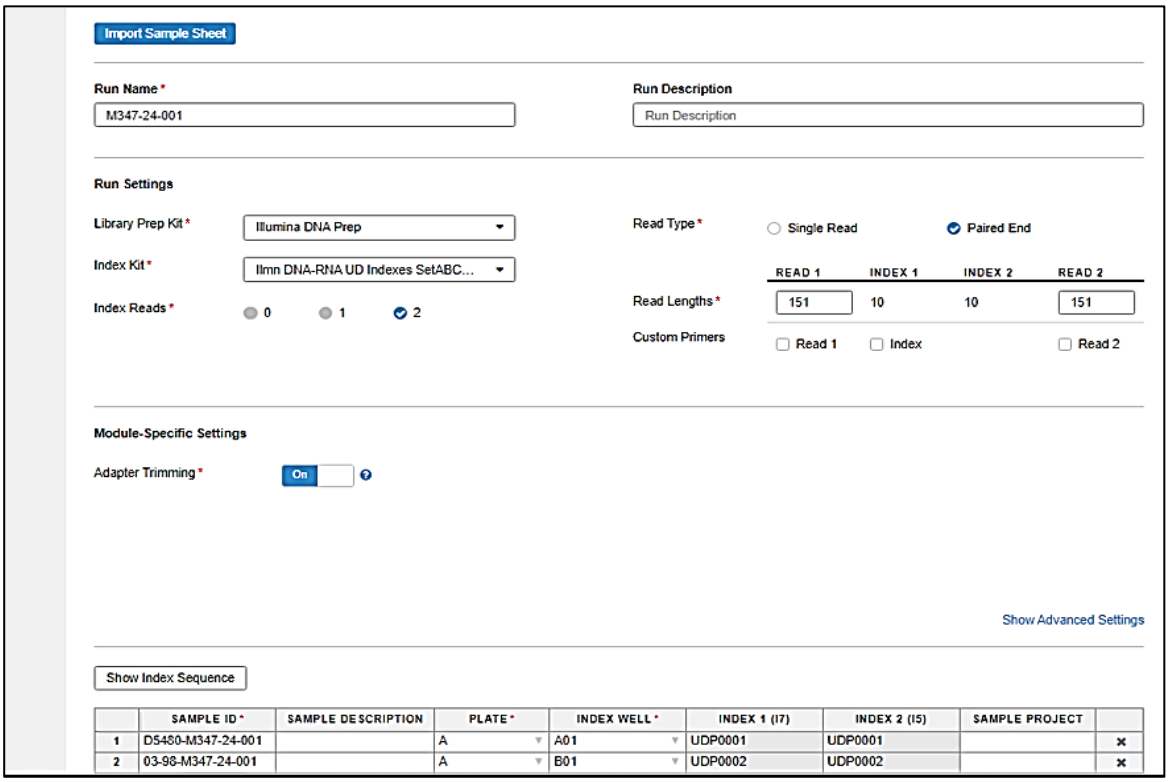


Figure 8. Screen shot of LRM with run parameters correctly populated for Illumina DNA Prep 300c run.

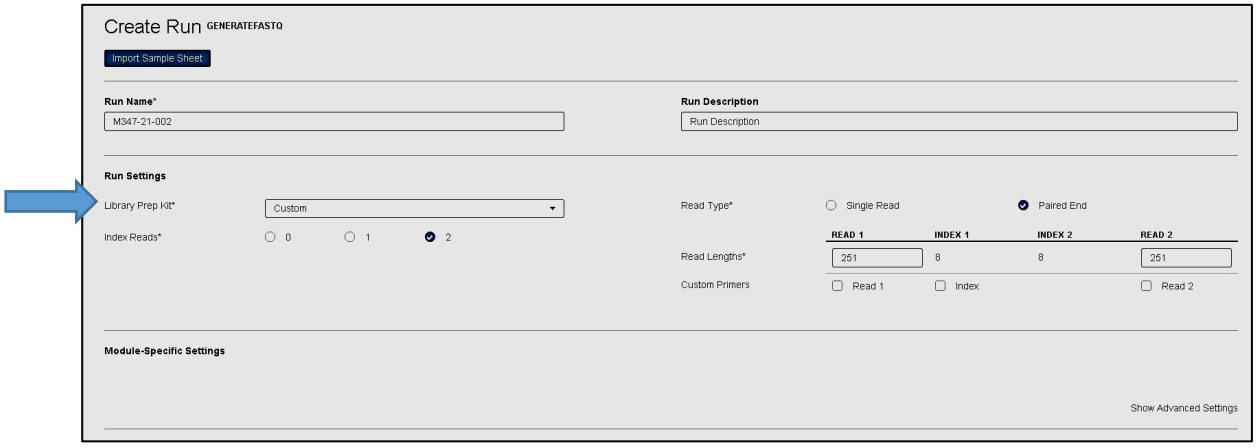


Figure 9. Screen shot of Local Run Manager with Library Prep kit type defaulting to "Custom".

1.4.6. Upon confirming all run parameters and sample information are correct, and **Adapter Trimming is toggled to the on position**, select "Save Run". The run will now be displayed on the LRM home page.

1.4.7. Close Chromium. The run will now be saved in LRM and will show up on the MiSeq Control Software homepage for sequencing.

2. Manually Enter Run Information in LRM:

2.1. On the MiSeq, open Chromium.
2.2. On the LRM home page, select "Create Run" to set up run parameters.
2.3. Select the "GenerateFASTQ" analysis module.
2.4. Manually enter run information:
  2.4.1. Run Name: enter the Plate ID (i.e., LabID-MXXXX-YYMMDD)
  2.4.2. Run Description
  2.4.3. Run Settings:
    2.4.3.1. Library Prep Kit: select appropriate kit in drop-down
    2.4.3.2. Read Type: select "Paired End"
    2.4.3.3. Index Reads: select "2"
    2.4.3.4. Read lengths: input desired number of cycles, depending on kit.
      2.4.3.4.1. For 500 cycles, type "251" for READ 1 and READ 2.
      2.4.3.4.2. For 300 cycles, type "151" for READ 1 and READ 2.
    2.4.3.5. **If sequencing Illumina DNA PCR-Free libraries:** Custom primers: "CustomRead1PrimerMix (C1)".
  2.4.4. **Adapter Trimming**: confirm box is in the **"On" setting.**
  2.4.5. Input sample information, including sample ID and indexes used.
2.5. Select "Save Run". The run will now be displayed on the LRM home page.
2.6. Close Chromium.

Appendix PNL38-3

Requeuing a Run for Analysis

1. Purpose

Requeuing a run may be necessary if an indexing or sample naming error has been identified (i.e., the index assignments in the sample sheet are not accurate). Prior to requeuing analysis, prepare a corrected sample sheet. This will be used by the instrument for the new analysis, to re-parse the data and to rename the associated fastq files. A run can be requeued for analysis either using LRM (section 2.1.) or BaseSpace (section 2.2.).

2. Procedure

2.1. Requeue a Run Using LRM and MCS v3.0 or More Recent

2.1.1. Open Chromium to log into LRM.
2.1.2. Find the run to be requeued.
2.1.3. Click "Actions".
2.1.4. Click "Requeue".
2.1.5. Select "Edit Setup" when prompted by the software.
2.1.6. Import the new sample sheet and proceed.
2.1.7. The reanalyzed data (i.e., fastq files) will be stored in the "Alignment_2" folder of the run analysis and output folders.

NOTE*: If a run was accidentally completed with the Library Prep Kit listed as "Custom", indexes and adapter trimming cannot be edited using requeue for analysis as described above. Instead, create a new run in LRM (recommended to use the same Run ID as the run that needs requeuing, with a "v2" at the end) and import the corrected sample sheet (with the new "v2" Run ID). After setting up this run in LRM, right click and select "Import" on the LRM home screen (Fig.10). Copy the file directory for the run that needs to be re-analyzed into the dialog box (Fig. 11); ensure that the output folder is correct and select "Import Run". The resulting run will have properly demultiplexed and adapter trimmed fastq files.*

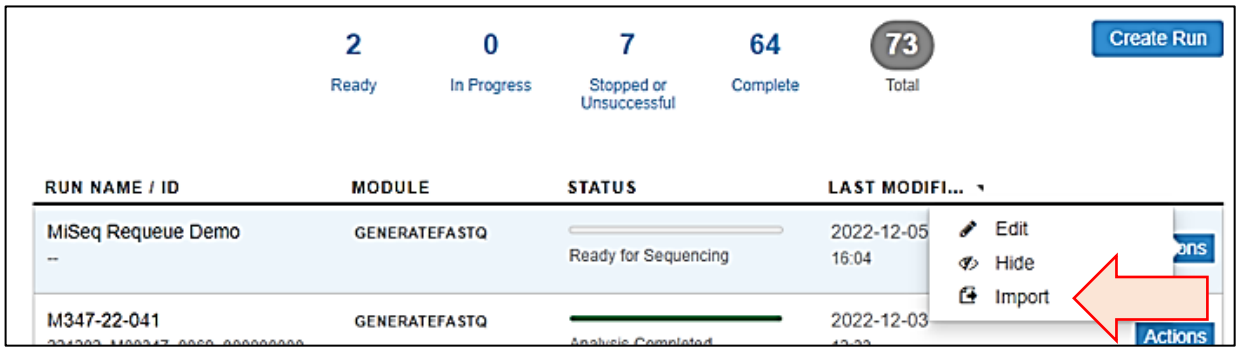


Figure 10. The drop-down menu to select "Import" for a manual re-analysis of run data.

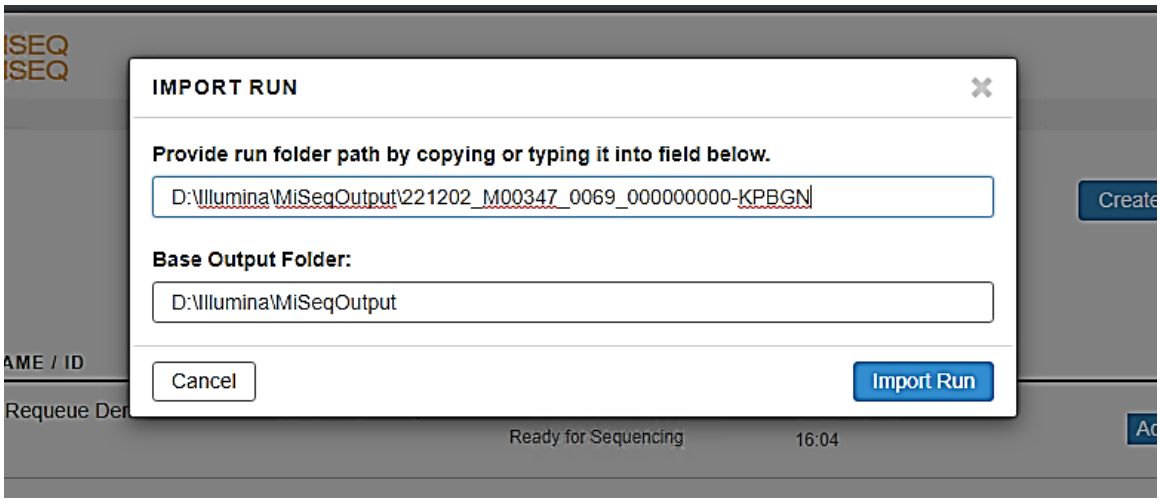


Figure 11. Selecting the run to be re-analyzed and the output folder in the "Import run" pop-up window.

2.2. Requeue a Run Using BaseSpace Sequence Hub

NOTE1: *In order to requeue the analysis within BaseSpace, you must be the owner of the run. A shared run does not allow permissions to requeue analysis. Additionally,* ***a requeued run can only be submitted up to five times in BaseSpace Sequence Hub****. If assistance with additional requeues is needed, email Illumina Technical Support, and be prepared to share your run ID and sample sheet.*

NOTE2: *LRM and BaseSpace will requeue a new sample sheet independent of each other. If a new sample sheet is requeued with LRM, it will not update the information on BaseSpace.*

NOTE3: *Follow the general instructions for requeuing in BaseSpace on the Illumina website: https://knowledge.illumina.com/software/cloud-software/software-cloud-software-reference_material-list/000001321*

- *In order to fix the* ***sample sheet****, refer to the following instructions at the Illumina webpage: https://help.basespace.illumina.com/runs/fix-sample-sheet*
- *In order to fix the* ***index assignment****, refer to the following instructions at the Illumina webpage: https://help.basespace.illumina.com/sequence/fix-indexes*

2.2.1. After logging into your BaseSpace account, select the "Runs" tab.
2.2.2. Select the blue hyperlink for the run you wish to requeue.
2.2.3. Click on the "Status" icon and from the drop-down menu, select "Requeue" and "Sample Sheet" (Fig. 12).

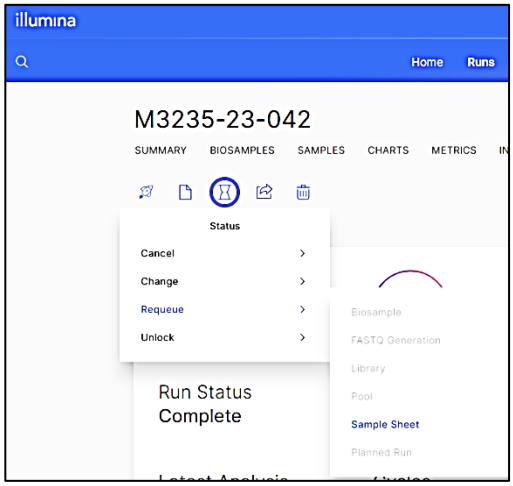


Figure 12. Finding the sample sheet for the run to be requeued.

2.2.4. Select “Load Original” to load the original sample sheet (Fig. 13).

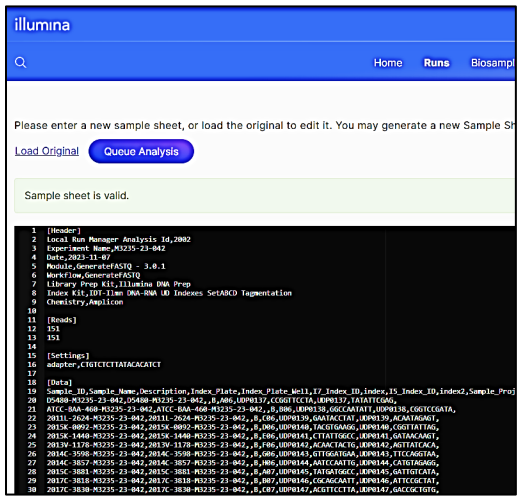


Figure 13. Copy and paste the corrected sample sheet for the run to be requeued.

2.2.5. Copy and paste from a plain text editor the corrected sample sheet and click on “Queue Analysis” (Fig. 13).

2.2.6. Status on the run “Summary” tab will display “Analyzing” during re-analysis process and “Complete” when finished.