1. **PURPOSE:** To describe the standardized laboratory procedure for whole genome sequencing of bacterial organisms on the Ion GeneStudio S5 benchtop sequencer, thus ensuring inter-laboratory comparability of the generated sequencing results.

2. **SCOPE:** To provide the PulseNet participants with a single protocol for KAPA library prep, Ion Chef template prep and Ion GeneStudio S5 run setup.

3. **DEFINITIONS:**
   - 3.1. **BSC:** Biosafety cabinet
   - 3.2. **DNA:** Deoxyribonucleic acid
   - 3.3. **dsDNA:** double-stranded DNA
   - 3.4. **EDTA:** Ethylenediaminetetraacetic Acid
   - 3.5. **GHS:** Globally Harmonized System
   - 3.6. **HS:** High Sensitivity
   - 3.7. **ISP**: Ion Sphere Particles
   - 3.8. **PCR:** Polymerase Chain Reaction
   - 3.9. **PPE:** Personal Protective Equipment
   - 3.10. **QC:** Quality Control
   - 3.11. **RNase:** Ribonuclease
   - 3.12. **SDS:** Safety Data Sheet
   - 3.13. **SOP:** Standard Operating Procedure
   - 3.14. **Tris-HCl:** Tris hydrochloride
   - 3.15. **WGS**: Whole Genome Sequencing

4. **RESPONSIBILITIES/PROCEDURE:**
   - 4.1. **PulseNet Public Health Laboratory:**
     - 4.1.1. Sequence isolate DNA and perform quality check of sequencing run.
     - 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 sequencing quality analysis, and provide support and troubleshooting with PHLs, as necessary.
     - 4.2.2. Communicate any suspected reagent issues to PHLs, as necessary.

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:** Personal injury can occur through inhalation, ingestions, skin and eye contact. See SDS for additional information and take proper precautions when handling the cartridges and waste. Ensure spent containers and unused contents are disposed of in accordance with governmental safety standards.
     - 5.2.1. KAPA Library Preparation Kit: See KAPA SDSs for additional information. Take proper precautions and wear appropriate PPE when handling reagents.
       - KAPA HiFi HotStart ReadyMix (2X) contains Tetramethylammonium chloride (GHS Category 1, 2 and 3 for specific target organ toxicity single exposure, aquatic environmental hazard, and oral acute toxicity).

5.2.2. The Ion 510™ & Ion 520™ & Ion 530™ Kit contains hydrogenated 1-Decene (GHS classification Category 1 and 4 for aspiration hazard and acute inhalation toxicity) and calcium hydroxide (GHS classification Category 2 and 3 for skin and eye irritation and specific target organ systemic toxicity). See ThermoFisher SDSs for additional information.

6. **REAGENTS:** Store reagents at room temperature (20-25°C) unless stated otherwise
   6.1. Agencourt AMPure XP for PCR Purification (5ml, Beckman Cat# A63880 or 60 ml, Beckman Cat#A63881). Store at 2-8°C.
   6.2. E-Gel™ SizeSelect™ II Agarose Gels, 2% (10 gels, ThermoFisher Cat# G661012)
   6.3. Ethanol, molecular-grade, 95-100% (Fisher Cat# BP2818-500 or equivalent)
   6.4. Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef (8 reactions, ThermoFisher Cat# A34461)
      6.4.1. Ion S5 Chef supplies
      6.4.2. Ion S5 Chef solutions
      6.4.3. Ion 510 & Ion 520 & Ion 530 Chef reagents. Store at -15°C to -25°C
      6.4.4. Ion S5 Sequencing solutions
      6.4.5. Ion S5 Sequencing reagents. Store at -15°C to -25°C
   6.5. Ion Sphere™ Quality Control Kit (1 Kit, ThermoFisher Cat# 4468656). Store at -15°C to -25°C.
      6.5.1. Ion Probes (Blue Cap)
      6.5.2. Alexa Fluor™ 488 Calibration Standard (Green Cap)
      6.5.3. Alexa Fluor™ 647 Calibration Standard (Red Cap)
      6.5.4. Annealing Buffer (White Cap)
      6.5.5. Quality Control Wash Buffer
   6.6. KAPA Fragmentation Kit (96 reactions, KAPA Cat# KK8602 or 24 reactions, KAPA Cat# KK8601). Store at -15°C to -25°C.
      6.6.1. KAPA Frag Enzyme
      6.6.2. KAPA Frag Buffer (10X)
      6.6.3. Conditioning Solution
      6.6.4. Stop Solution
   6.7. KAPA Library Preparation Kit Ion Torrent Platforms (48 reactions, KAPA Cat# KK8301). Store at -15°C to -25°C.
      6.7.1. End Repair Buffer (10X)
      6.7.2. End Repair Enzyme Mix
      6.7.3. Ligation and Nick Repair Buffer (5X)
      6.7.4. Ligation and Nick Repair Enzyme
      6.7.5. KAPA HiFi HotStart ReadyMix (2X)
      6.7.6. Library Amplification Primer Mix (10X)
   6.8. Molecular-grade water (Fisher Cat# BP24701 or equivalent)
   6.9. NEXTflex® DNA Barcodes - Ion Torrent Compatible (8 barcodes, Bioo Scientific Cat# NOVA-401001 or 32 barcodes Bioo Scientific Cat# NOVA-401003). Store at -15°C to -25°C.
   6.10. Qubit® dsDNA High Sensitivity (HS) Assay kit (100 samples, Invitrogen Cat# Q32851 or 500 samples, Invitrogen Cat# Q32854)
      6.10.1. dsDNA HS Reagent (Component A). Protect from light.
      6.10.2. dsDNA HS Buffer (Component B)
      6.10.3. dsDNA HS Standard #1 (Component C). Store at 4°C
      6.10.4. dsDNA HS Standard #2 (Component D). Store at 4°C
   6.11. TrackIt™ 100 bp DNA Ladder (100 applications, Invitrogen Cat# 10488058). Store at -15°C to -25°C
   6.12. Tris-HCl, 1M, pH 8.0 (Sigma-Aldrich, Cat# T3038-1L or equivalent). Store at 2-8°C.

7. **SUPPLIES:**
   7.1. Conical tubes, 10 ml and/or 15 ml (Fisher Scientific Cat# 14-959A and/or Fisher Cat# 14-959-53A or equivalent)
   7.2. Ice
   7.3. Microcentrifuge tubes, 1.5 ml, sterile (ThermoFisher Cat# AM12400 or equivalent)
   7.4. Microseal A film (BioRad Cat# MSA-5001)
   7.5. Microseal B film (BioRad Cat# MSB-1001)
   7.6. PCR plates, skirted, hard shell low profile, thin-wall, 96 well (BioRad Cat# HSP-9601 or equivalent)
   7.7. PCR tubes with or without caps, 0.2 ml, sterile (Fisher Cat# 14-230-220 or equivalent)
   7.8. Pipette tips, sterile, filtered: 20 µl, 200 µl and 1000 µl volumes (Rainin Cat# 17001865, 17001863 & 17001864 or equivalent)
   7.9. Qubit Assay Tubes (Invitrogen Cat# Q32856 or equivalent (clear, thin-wall 0.5-ml PCR tubes)).
   7.10. Serological pipets, 1ml to 10ml volumes (various catalog numbers)
   7.11. Solution basins, sterile (Fisher Scientific Cat# 13-681-504 or equivalent)
   7.12. **OPTIONAL**: Deepwell storage "MIDI" plates, 96 well (Fisher Cat# AB-0859 or equivalent)
   7.13. **OPTIONAL**: Eppendorf™ PCR Cooler (Fisher Cat# 05-403-00 or equivalent)

8. **EQUIPMENT:**
   8.1. E-Gel™ Power Snap Electrophoresis Device (Invitrogen, G8100)
   8.2. Ice buckets/containers
   8.3. Ion Chef™ Instrument (ThermoFisher Cat# 4484177)
   8.4. Ion GeneStudio S5 System (ThermoFisher Cat # A38194)
   8.5. Magnetic Stand-96 (ThermoFisher Cat# AM10027 or equivalent)
   8.6. Microcentrifuge for quick spins
   8.7. 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.*
   8.8. Microplate centrifuge
   8.9. Pipet-Aid
   8.10. Qubit 2.0, or 3.0 Flourometer, or equivalent for quantifying double-stranded DNA
   8.11. Thermal cycler, capable of accepting a skirted 96-well plate, with heated lid
   8.12. Vortex
   8.13. **OPTIONAL**: Bioanalyzer Instrument (Agilent or equivalent)

9. **PROCEDURE:**
   NOTE1: *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.*
   NOTE2: *It is recommended to prep 16 or less samples at a time to ensure consistent quality throughout the libraries.*
   9.1. **Preparation of 10mM Tris-HCl pH 8.0**
      9.1.1. Add 10 ml of 1M Tris-HCl, pH 8.0 to 990 ml of molecular-grade water.
      9.1.2. Invert several times to mix.
      9.1.3. Store at 2-8°C for up to one year (indicate the initial, date of preparation, and date of expiry on the bottle).
   9.2. **Preparation of Sequencing Workbook**
      9.2.1. Prepare the "Initial Dilution" tab of the Ion Torrent S5 Workbook as described below:
      NOTE: *The workbook is designed with the following color scheme, in general:*

- *Tan/yellow fields should be filled in*
- *Blue fields contain formulas, which will auto-populate, and should not be altered*

9.2.2. Enter the Run Name in C2; this will be the Run ID (LabID-MachineID-YYMMDD).
9.2.3. Enter the chip type (or select from the dropdown) in C3.
9.2.4. Enter the Library Prep date (C4), Technician (C5).
9.2.5. Enter the Sample ID in appropriate location (Column B). This ID needs to be the same identifier entered in the PulseNet Key field in BioNumerics.
9.2.6. Determine which set of barcodes will be used and enter (or select from the dropdown) the barcode number into column E.
NOTE: *It is recommended to not use the same A barcode adapter within 2 consecutive runs on the same sequencer to reduce the amount of carryover.*
NOTE2: *The workbook will highlight same barcodes to avoid duplicate preps in a run.*
9.2.7. Enter the Genome Size Estimate (based on Table 1 below) into Column G.

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

9.2.8. Confirm that the number of isolates on the run is appropriate for the capacity of the chip type to be used. The sum of the genome sizes (in Mb) for the samples on the run (G37 in the workbook) will give the estimated DNA load of the run. This cannot exceed the DNA load allowance of the chip to be used. The DNA load for chip types can be found in Table 2 below.

Chip Type	Capacity (Mb)
510 chip	20MB
530 chip	125MB

Table 2. Estimated DNA Load (in Mb) Capacity for Ion Chips.

9.2.9. Enter the concentration of extracted DNA into column H.
NOTE1: *PulseNet has standardized the starting mass of DNA to 115.5ng.*

9.3. Normalization and fragmentation of DNA

9.3.1. Thaw KAPA Frag Buffer (10X) and Stop Solution at room temperature. Store reagents on ice until ready to use.
9.3.2. Ensure the pre-programmed thermal cycler is turned on prior to beginning the fragmentation, as some steps are time sensitive.
9.3.3. Label a 96-well PCR plate, or equivalent, with Run ID.
9.3.4. Add molecular-grade water (Column J in Initial Dilution tab) to each sample well.
9.3.5. Add DNA to the molecular-grade water (Column I in Initial Dilution tab) and mix well.
9.3.6. Add 5 µl of KAPA Frag Buffer (10X) to each sample well.
9.3.7. Add 10 µl of KAPA Frag Enzyme to each well and mix well by gently pipetting 5-10 times using a multichannel pipette.

NOTE1: *Fragmentation is time sensitive. It is important to perform step 9.3.7. quickly.*
NOTE2: *It is recommended to use PCR cooler or equivalent when assembling fragmentation reaction.*

9.3.8. Seal the plate with Microseal A (or equivalent) and incubate the plate at 37°C for 7 minutes, followed by a 10°C hold (volume is 50 µl) on a thermal cycler with the lid heated at ≤50°C.
NOTE: *It is recommended to pre-program a thermal cycler for this purpose.*

9.3.9. When the samples have reached 10°C, quickly add 5 µl of Stop Solution to each well and mix well by gently pipetting 5-10 times using a multichannel pipette.
NOTE: *It is recommended to aliquot the Stop Solution into a PCR strip tube during the programmed thermal cycle and use a multichannel pipette to add the solution.*

9.3.10. Incubate at room temperature for 5 minutes and proceed to post-fragmentation clean-up.

9.4. **Post-fragmentation Clean-up**

9.4.1. Before starting, prepare reagents:

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

Reagent	Volume per sample	Example: 16 samples
100% ethanol	0.4 ml	6.4 ml
Molecular-grade water	0.1 ml	1.6 ml

Table 3. Reagent volumes per sample for 80% ethanol.

9.4.1.2. Bring AMPure XP beads and 10mM Tris-HCl to room temperature.

9.4.2. Centrifuge the PCR plate at 800-1200 rpm (or 280 x g) for approximately 30 seconds to collect condensation, if needed.

9.4.3. Vortex AMPure XP beads until well suspended (15 – 30 seconds).

9.4.4. To each 55 µl of product, add 110 µl (2x volume) of AMPure XP beads.
NOTE1: *Deepwell MIDI plate may be used for the procedure.*
NOTE2: *Avoid bead carryover by confirming no droplets are on the pipette tip. This will affect the ratio of beads to PCR reaction, which affects fragment size selection.*

9.4.5. Gently pipette up and down 10-15 times to mix.

9.4.6. Incubate at room temperature for at least 5 minutes.

9.4.7. Place the plate on the magnetic stand for a minimum of 5 minutes or until the supernatant has cleared.

9.4.8. With the plate still on the magnetic stand, carefully remove and discard the supernatant.
NOTE: *If any beads are inadvertently aspirated into the tips, dispense the beads back into the wells and let the plate rest on the magnetic stand for another 2 minutes or until the supernatant has cleared.*

9.4.9. With the plate still on the magnetic stand, wash the beads by adding 200 µl of freshly prepared 80% ethanol to each well.
NOTE: *Do not resuspend the beads at any point during the wash steps or remove the plate from the magnetic stand.*

9.4.10. Incubate the plate on the magnetic stand for 30 seconds, then carefully remove and discard the supernatant.

9.4.11. With the plate still on the magnetic stand, perform a second 80% ethanol wash.

9.4.12. Incubate the plate on the magnetic stand for 30 seconds, then carefully remove and discard the supernatant.
9.4.13. With the plate on the magnetic stand, use a low volume pipette tip to remove any remaining ethanol droplets and allow the beads to air-dry for up to 3 minutes.
NOTE: *Exceeding the maximum drying period (3 minutes) could make resuspension of DNA fragments difficult. Over-drying is indicated by cracks in the bead pellets.*
9.4.14. Remove the plate from the magnetic stand.
9.4.15. Add 55 µl of 10mM Tris-HCl to each well.
9.4.16. Gently pipette up and down at least 10 times to mix, ensuring the beads are completely resuspended, and changing tips after each well.
9.4.17. Incubate at room temperature for 5 minutes.
9.4.18. Place the plate on the magnetic stand for at least 2 minutes or until the supernatant has cleared.
9.4.19. Carefully transfer 50 µl of the supernatant from the plate to a new set of wells for DNA end-repair.

9.5. **DNA End-Repair**
9.5.1. Thaw End Repair Buffer (10X) and End Repair Enzyme on ice.
9.5.2. Prepare end-repair master mix:

Reagent	Volume per Sample
Molecular grade Water	8 µl
End Repair Buffer (10X)	7 µl
End Repair Enzyme	5 µl

Table 4. Reagent volumes per sample for end-repair master mix.

NOTE: *It is recommended to increase number of samples during master mix calculation by 1-2 to ensure sufficient master mix volume. See workbook (S46-S48 in Initial Dilution tab) for reagent volumes.*

9.5.3. Gently vortex and quick spin the end-repair master mix.
9.5.4. Add 20 µl of End-Repair master mix to each sample well containing 50 µl of fragmented DNA and mix thoroughly by gently pipetting 10-15 times using a multichannel pipette.
9.5.5. Seal the plate with Microseal A (or equivalent) and incubate the plate at 20°C for 30 minutes, followed by a 10°C hold (volume is 70 µl) on a thermal cycler with the lid heated at 85°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-end-repair clean up should be commenced once the samples have reached 10°C.*

9.6. **Post-End-Repair Clean-up**
9.6.1. Before starting, prepare reagents:

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

Reagent	Volume per sample	Example: 16 samples
100% ethanol	0.4 ml	6.4 ml
Molecular-grade water	0.1 ml	1.6 ml

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

9.6.1.2. Bring AMPure XP beads and 10mM Tris-HCl to room temperature.

9.6.2. Centrifuge the PCR plate at 800-1200 rpm (or 280 x g) for approximately 30 seconds to collect condensation, if needed.
9.6.3. Vortex AMPure XP beads until well suspended (15 – 30 seconds).
9.6.4. To each 70 µl of product, add 120 µl (1.71x volume) of AMPure XP beads.
NOTE1: *Deepwell MIDI plate may be used for the procedure.*
NOTE2: *Avoid bead carryover by confirming no droplets are on the pipette tip. This will affect the ratio of beads to PCR reaction, which affects fragment size selection.*
9.6.5. Gently pipette up and down 10-15 times to mix.
9.6.6. Incubate at room temperature for at least 5 minutes.
9.6.7. Place the plate on the magnetic stand for a minimum of 5 minutes or until the supernatant has cleared.
9.6.8. With the plate still on the magnetic stand, carefully remove and discard the supernatant.
NOTE: *If any beads are inadvertently aspirated into the tips, dispense the beads back into the wells and let the plate rest on the magnetic stand for another 2 minutes or until the supernatant has cleared.*
9.6.9. With the plate still on the magnetic stand, wash the beads by adding 200 µl of freshly prepared 80% ethanol to each well.
NOTE: *Do not resuspend the beads at any point during the wash steps or remove the plate from the magnetic stand.*
9.6.10. Incubate the plate on the magnetic stand for 30 seconds, then carefully remove and discard the supernatant.
9.6.11. With the plate still on the magnetic stand, perform a second 80% ethanol wash.
9.6.12. Incubate the plate on the magnetic stand for 30 seconds, then carefully remove and discard the supernatant.
9.6.13. With the plate on the magnetic stand, use a low volume pipette tip to remove any remaining ethanol droplets and allow the beads to air-dry for up to 3 minutes.
NOTE: *Exceeding the maximum drying period (3 minutes) could make resuspension of DNA fragments difficult. Over-drying is indicated by cracks in the bead pellets.*
9.6.14. Remove the plate from the magnetic stand.
9.6.15. Add 35 µl of 10mM Tris-HCl to each well.
9.6.16. Gently pipette up and down at least 10 times to mix, ensuring the beads are completely resuspended, and changing tips after each well.
9.6.17. Incubate at room temperature for 5 minutes.
9.6.18. Place the plate on the magnetic stand for at least 2 minutes or until the supernatant has cleared.
9.6.19. Carefully transfer 30 µl of the supernatant from the plate to a new set of wells for adapter ligation and nick repair.

9.7. **Adapter Ligation and Nick Repair**
9.7.1. Thaw Adapter Ligation & Nick Repair Buffer (5X) and Adapter Ligation & Nick Repair Enzyme on ice.
9.7.2. Prepare adapter ligation and nick repair (AL&NR) master mix:

Reagent	Volume per Sample
Molecular grade Water	16 µl
Adapter Ligation & Nick Repair Buffer (5X)	14 µl
Adapter Ligation & Nick Repair Enzyme	6 µl

Table 6. Reagent volumes per sample for adapter ligation and nick repair master mix.

NOTE: *It is recommended to increase number of samples during master mix calculation by 1-2 to ensure sufficient master mix volume. See workbook (S52-S54 in Initial Dilution tab) for reagent volume.*

9.7.3. Gently vortex and quick spin the AL&NR master mix.

9.7.4. Add 36 µl of the AL&NR master mix to each sample well containing 30 µl of end-repaired DNA.

9.7.5. Add 2 µl of universal P1 adapter from NEXTflex® DNA Barcodes Kit to each sample well.

9.7.6. Add 2 µl of unique A barcode adapters (refer to Column E in Workbook) from NEXTflex® DNA Barcodes Kit to each sample well and mix thoroughly by gently pipetting 10-15 times using a multichannel pipette.

9.7.7. Seal the plate with Microseal A (or equivalent) and incubate the plate at the following conditions:

20°C for 15 minutes
65°C for 5 minutes
Hold at 10°C
(70 µl total volume)

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-ligation clean up should be commenced once the samples have reached 10°C.*

9.8. Post-Ligation Clean-up

9.8.1. Before starting, prepare reagents:

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

Reagent	Volume per sample	Example: 16 samples
100% ethanol	0.4 ml	6.4 ml
Molecular-grade water	0.1 ml	1.6 ml

Table 7. Reagent volumes per sample for 80% ethanol.

9.8.1.2. Bring AMPure XP beads and 10mM Tris-HCl to room temperature.

9.8.2. Centrifuge the PCR plate at 800-1200 rpm (or 280 x g) for approximately 30 seconds to collect condensation, if needed.

9.8.3. Vortex AMPure XP beads until well suspended (15 – 30 seconds).

9.8.4. To each 70 µl of product, add 70 µl (1x volume) of AMPure XP beads.
NOTE1: *Deepwell MIDI plate may be used for the procedure.*
NOTE2: *Avoid bead carryover by confirming no droplets are on the pipette tip. This will affect the ratio of beads to PCR reaction, which affects fragment size selection.*

9.8.5. Gently pipette up and down 10-15 times to mix.
9.8.6. Incubate at room temperature for at least 5 minutes.
9.8.7. Place the plate on the magnetic stand for a minimum of 5 minutes or until the supernatant has cleared.
9.8.8. With the plate still on the magnetic stand, carefully remove and discard the supernatant.
NOTE: *If any beads are inadvertently aspirated into the tips, dispense the beads back into the wells and let the plate rest on the magnetic stand for another 2 minutes or until the supernatant has cleared.*
9.8.9. With the plate still on the magnetic stand, wash the beads by adding 200 µl of freshly prepared 80% ethanol to each well.
NOTE: *Do not resuspend the beads at any point during the wash steps or remove the plate from the magnetic stand.*
9.8.10. Incubate the plate on the magnetic stand for 30 seconds, then carefully remove and discard the supernatant.
9.8.11. With the plate still on the magnetic stand, perform a second 80% ethanol wash.
9.8.12. Incubate the plate on the magnetic stand for 30 seconds, then carefully remove and discard the supernatant.
9.8.13. With the plate on the magnetic stand, use a low volume pipette tip to remove any remaining ethanol droplets and allow the beads to air-dry for up to 3 minutes.
NOTE: *Exceeding the maximum drying period (3 minutes) could make resuspension of DNA fragments difficult. Over-drying is indicated by cracks in the bead pellets.*
9.8.14. Remove the plate from the magnetic stand.
9.8.15. Add 105 µl of 10mM Tris-HCl to each well.
9.8.16. Gently pipette up and down at least 10 times to mix, ensuring the beads are completely resuspended, and changing tips after each well.
9.8.17. Incubate at room temperature for 5 minutes.
9.8.18. Place the plate on the magnetic stand for at least 2 minutes or until the supernatant has cleared.
9.8.19. Carefully transfer 100 µl of the supernatant from the plate to a new set of wells and proceed to double-sided size selection.

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.

9.9. **Double-Sided Size Selection**
NOTE: *The steps listed below are critical for efficient size selection, product recovery and read-length. Always check pipette tips for correct volumes and to 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.*

9.9.1. Before starting, prepare reagents:

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

Reagent	Volume per sample	Example: 16 samples
100% ethanol	0.4 ml	6.4 ml
Molecular-grade water	0.1 ml	1.6 ml

Table 8. Reagent volumes per sample for 80% ethanol.

9.9.1.2. Bring AMPure XP beads and 10mM Tris-HCl to room temperature.

9.9.2. Centrifuge the PCR plate at 800-1200 rpm (or 280 x g) for approximately 30 seconds to collect condensation, if needed.
9.9.3. Vortex AMPure XP beads until well suspended (15 – 30 seconds).
9.9.4. To each 100 µl of product, add 45 µl (0.45x volume) of AMPure XP beads.
NOTE1: *Deepwell MIDI plate may be used for the procedure.*
NOTE2: *Avoid bead carryover by confirming no droplets are on the pipette tip. This will affect the ratio of beads to PCR reaction, which affects fragment size selection.*
9.9.5. Gently pipette up and down 10-15 times to mix.
9.9.6. Incubate at room temperature for at least 5 minutes.
9.9.7. Place the plate on the magnetic stand for a minimum of 3 minutes or until the supernatant has cleared.
9.9.8. With the plate still on the magnetic stand, carefully remove 140 µl of the supernatant and place in a new set of wells. Remove the plate from the magnetic stand for the second bead addition.
NOTE: *If any beads are inadvertently aspirated into the tips, dispense the beads back into the CAA and let the plate rest on the magnetic stand for another 2 minutes or until the supernatant has cleared.*
9.9.9. Add 20 µl (0.14x) of AMPure XP beads to the wells containing the 140 µl of supernatant.
9.9.10. Gently pipette up and down 10-15 times to mix.
9.9.11. Incubate at room temperature for at least 5 minutes.
9.9.12. Place the plate on the magnetic stand for a minimum of 2 minutes or until the supernatant has cleared.
9.9.13. With the plate still on the magnetic stand, carefully remove and discard the supernatant.
NOTE: *If any beads are inadvertently aspirated into the tips, dispense the beads back into the wells and let the plate rest on the magnetic stand for another 2 minutes or until the supernatant has cleared.*
9.9.14. With the plate still on the magnetic stand, wash the beads by adding 200 µl of freshly prepared 80% ethanol to each well.
NOTE: *Do not resuspend the beads at any point during the wash steps or remove the plate from the magnetic stand.*
9.9.15. Incubate the plate on the magnetic stand for 30 seconds, then carefully remove and discard the supernatant.
9.9.16. With the plate still on the magnetic stand, perform a second 80% ethanol wash.
9.9.17. Incubate the plate on the magnetic stand for 30 seconds, then carefully remove and discard the supernatant.
9.9.18. With the plate on the magnetic stand, use a low volume pipette tip to remove any remaining ethanol droplets and **IMMEDIATELY** remove the plate from the magnetic stand.
NOTE: *The bead pellets are small and do not need additional drying time.*
9.9.19. Add 25 µl of 10mM Tris-HCl to each well.
9.9.20. Gently pipette up and down at least 10 times to mix, ensuring the beads are completely resuspended, and changing tips after each well.

9.9.21. Incubate at room temperature for 5 minutes.
9.9.22. Place the plate on the magnetic stand for at least 2 minutes or until the supernatant has cleared.
9.9.23. Transfer 20 µl of the supernatant to a new set of wells in the PCR plate for library amplification.

9.10. **Library Amplification**
9.10.1. Thaw KAPA HiFi HotStart ReadyMix (2X) and Library Amplification Primer Mix (10X) on ice.
9.10.2. Add 25 µl of KAPA HiFi HotStart ReadyMix (2X) to each well containing 20 µl of clean adapter-ligated DNA.
9.10.3. Add 5 µl of Library Amplification Primer Mix (10X) to each well and mix well by gently pipetting 10-15 times using a multichannel pipette.
9.10.4. 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: 98°C for 45 seconds
Step 2: 6 cycles
98°C for 15 seconds
65°C for 30 seconds
72°C for 30 seconds
Step 4: 72°C for 1 minute
Step 5: Hold at 4°C
Total volume: 50 µl

9.10.5. Centrifuge plate for at 280 x g for 30 seconds and proceed to post-amplification clean-up.

9.11. **Post-Amplification Clean-up**
9.11.1. Before starting, prepare reagents:

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

Reagent	Volume per sample	Example: 16 samples
100% ethanol	0.4 ml	6.4 ml
Molecular-grade water	0.1 ml	1.6 ml

Table 9. Reagent volumes per sample for 80% ethanol.

9.11.1.2. Bring AMPure XP beads and 10mM Tris-HCl to room temperature.

9.11.2. Centrifuge the PCR plate at 800-1200 rpm (or 280 x g) for approximately 30 seconds to collect condensation, if needed.
9.11.3. Vortex AMPure XP beads until well suspended (15 – 30 seconds).
9.11.4. To each 50 µl of product, add 70 µl (1.4x volume) of AMPure XP beads.
NOTE1: *Deepwell MIDI plate may be used for the procedure.*
NOTE2: *Avoid bead carryover by confirming no droplets are on the pipette tip. This will affect the ratio of beads to PCR reaction, which affects fragment size selection.*
9.11.5. Gently pipette up and down 10-15 times to mix.
9.11.6. Incubate at room temperature for at least 5 minutes.
9.11.7. Place the plate on the magnetic stand for a minimum of 5 minutes or until the supernatant has cleared.

9.11.8. With the plate still on the magnetic stand, carefully remove and discard the supernatant.
NOTE: *If any beads are inadvertently aspirated into the tips, dispense the beads back into the wells and let the plate rest on the magnetic stand for another 2 minutes or until the supernatant has cleared.*

9.11.9. With the plate still on the magnetic stand, wash the beads by adding 200 µl of freshly prepared 80% ethanol to each well.
NOTE: *Do not resuspend the beads at any point during the wash steps or remove the plate from the magnetic stand.*

9.11.10. Incubate the plate on the magnetic stand for 30 seconds, then carefully remove and discard the supernatant.

9.11.11. With the plate still on the magnetic stand, perform a second 80% ethanol wash.

9.11.12. Incubate the plate on the magnetic stand for 30 seconds, then carefully remove and discard the supernatant.

9.11.13. With the plate on the magnetic stand, use a low volume pipette tip to remove any remaining ethanol droplets and allow the beads to air-dry for up to 3 minutes.
NOTE: *Exceeding the maximum drying period (3 minutes) could make resuspension of DNA fragments difficult. Over-drying is indicated by cracks in the bead pellets.*

9.11.14. Remove the plate from the magnetic stand.

9.11.15. Add 32 µl of 10mM Tris-HCl to each well.

9.11.16. Gently pipette up and down at least 10 times to mix, ensuring the beads are completely resuspended, and changing tips after each well.

9.11.17. Incubate at room temperature for 5 minutes.

9.11.18. Place the plate on the magnetic stand for at least 2 minutes or until the supernatant has cleared.

9.11.19. Carefully transfer 27 µl of the supernatant from the plate to a new PCR plate – this is the final product.

9.11.20. Measure the library concentrations using Qubit HS kit and record results in Column Q of the Initial Dilution tab in the Workbook.

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.***

9.12. **E-Gel Size-selection**

NOTE: *Each E-Gel cassette is able to accommodate 6 samples per run. Fill all empty wells with water if run consists of sample number less than 6.*

9.12.1. Centrifuge the library plate at 800-1200 rpm (or 280 x g) for approximately 30 seconds to collect condensation, if needed.

9.12.2. Ensure E-Gel™ Power Snap Electrophoresis Device is turned on and the gel type is set to E-Gel SizeSelect 2% (Run time: 20 minutes).

9.12.2.1. Press **Set up run** to start E-Gel protocol selection.

9.12.2.2. Press **Gel Type**

9.12.2.3. Select **E-gel SizeSelect 2%**

9.12.3. For each run, prepare 1:4 dilution of ladder by adding 5 µl of TrackIt™ 100 bp DNA Ladder to 20 µl of molecular grade water and keep on ice until ready to use.

9.12.4. Carefully remove the gel cassette from the package and gently remove the combs.
NOTE: *Take caution to avoid bending or creating suction in the wells during the removal, as samples may leak out through punctures.*

9.12.5. Insert gel cassette into the device, starting at the right edge. Press down on the left side of the cassette until it clicks into place.

9.12.6. Carefully load 25 µl of ladder into the top center well (Well L in Figure 1).

9.12.7. Load 25 µl of each sample into the top wells, or the sample wells (Wells 1-3 and 5-7 in Figure 1).

9.12.8. Fill the bottom wells, or the recovery wells with 25 µl of water (Wells 8-14 in Figure 1) and close the amber filter.

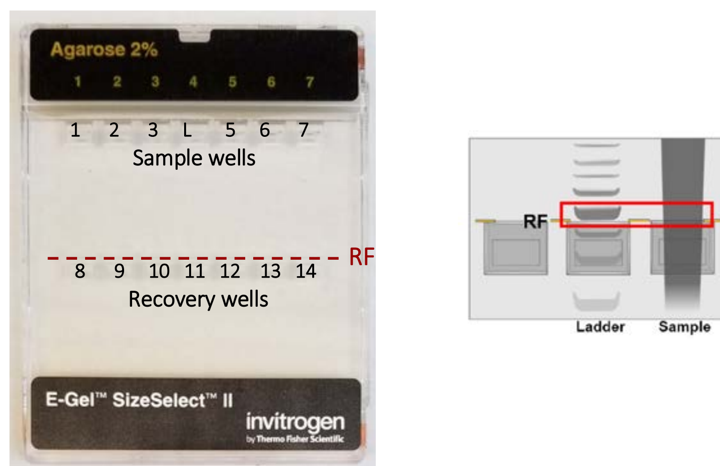

Figure 1. E-Gel SizeSelect II Gel Cassette

9.12.9. Ensure the correct gel type is selected on the device.

NOTE: *Adjust the duration of the gel run to 20 minutes using the + / - buttons or press in the duration field to open a number keyboard, if needed.*

9.12.10. Start the run.

NOTE1: *The reference run time for target library size of 480bp is 14-20 minutes. Monitor periodically for the movement of the bands by activating the Back light button for the initial 11-12 minutes.*

NOTE2: *For optimal viewing, dim the ambient lighting in the room for visualization.*

9.12.11. When 400-bp ladder band reaches the reference line (RF in Figure 1), pause the run.

9.12.12. Open the amber filter and carefully remove and discard all liquid from the recovery wells.

9.12.13. Load 25 µl of water to all recovery wells.

9.12.14. Resume the run for 30-60 additional seconds until 500-bp reference band reaches the reference line (around 14 minutes).

NOTE: *It is important to monitor the bands with the Back light during this time, as the duration of run time and current may vary slightly from each lot of E-Gel.*

9.12.15. Stop the run and collect 20 µl of samples from recovery wells and transfer to a new PCR plate, taking caution to avoid piercing the agarose during collection.

9.12.16. Measure the library concentrations using Qubit HS kit in **ng/ml** and record in Column R of the Initial Dilution tab of the Workbook.

NOTE: *Fragment analysis can be performed using Bioanalyzer or equivalent and results recorded in Column S of the Initial Dilution tab of the Workbook.*

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.

9.13. **Normalizing and Pooling the Libraries**

9.13.1. If libraries in the PCR plate are frozen, thaw on ice and centrifuge at 800 – 1200 rpm (or 280 x g) for 30 seconds.

9.13.2. For each library, dispense molecular-grade water into a clean well of the PCR plate according to the volumes listed in Column J of the Library Dilution tab of the workbook.

9.13.3. Ensure that DNA is well mixed, then transfer 1 µl of each library to the corresponding well to obtain the desired concentration.
NOTE: *The recommended concentration for GeneStudio S5 is 90pM. Adjust the loading concentration as needed (Cell L21).*

9.13.4. Mix the dilutions by pipetting up and down 5-7 times with a multichannel pipet.

9.13.5. For each sample library, transfer the volume listed in the "Pooling Volume" column (Column L in Library Dilution tab) into a single well on the PCR plate or in a new 0.2 ml tube for a total pooled volume of 30 µl.
NOTE: *The pooling factor is based on the genome size. When genomes of different sizes are run together, the amount of each single library is added proportionately to genome size to reduce the over-representation of small genomes in the pooled library.*

9.13.6. Mix the pooled library by pipetting up and down 10 times and place on ice until ready to use.

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.

9.14. **Library Templating with Ion Chef**

NOTE1: *The Ion Chef templates, enriches, and loads two library pools into two different chips per reaction. User must take this into account and plan two runs at once for the best use of reagents.*
NOTE2: *The chip loading centrifuge must be load balanced. If for some reason only one chip is being prepared for sequencing, be sure to use a dummy chip to balance the centrifuge.*
NOTE3: *Ensure all components are clean and dry before loading them onto the Ion Chef Instrument.*
NOTE4: *All components will be loaded with the "Ion" logo right side up and readable.*

9.14.1. Remove the Ion 510 & 520 & 530 Chef Reagents cartridge from the freezer **45 minutes** before use and allow to warm to room temperature. Record the barcode numbers found on the tabs of tube 1 and tube 2 (Figure 2) for entry into the S5 Torrent Server.

9.14.2. Pipet 25µl of each library pool into the bottom of each tubes (Figure 2). Record which pool went into which position for entry into S5 Torrent Server.

9.14.3. Remove and designate two new chips from the box and record the barcodes for entry into S5 Torrent Server.

9.14.4. Plan run on S5 Torrent Server (See appendix PNL41-1).

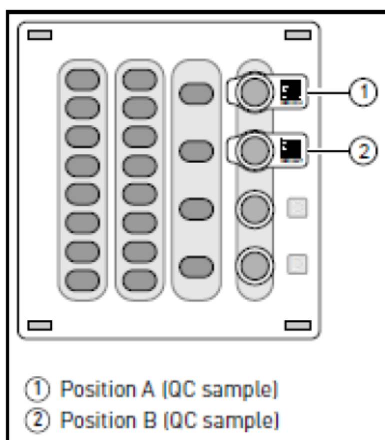

Figure 2. Diagram of Ion 510 & 520 & 530 Chef Reagents Cartridge

9.14.5. Load the pipette tip racks and PCR plate

9.14.5.1. Touch the Open Door symbol to unlock the Ion Chef door and wait for latch to open.

9.14.5.2. Lift the door until the latch mechanism engages and retrieve Ion S5 Chef Supplies from room temperature.

9.14.5.3. Unwrap a new tip cartridge and remove the cover to expose the tips and load it into the New Pipette Tip Position (#1 in Figure 3).

9.14.5.3.1. Pull the catch forward, and then pivot the locking bracket upwards. Load the Tip Cartridge into the New Pipette Tip Position, then pull the bracket downwards and push the catch backwards to lock into place.

9.14.5.4. Load an empty pipette tip rack into the Waste Pipette Tip position (#4 in Figure 3).

9.14.5.5. Unwrap the PCR plate and load into the thermal cycler sample block (#3 in Figure 3).

9.14.5.6. Unwrap and slide a new frame foil seal underneath the automated heated cover (#2 in Figure 3).

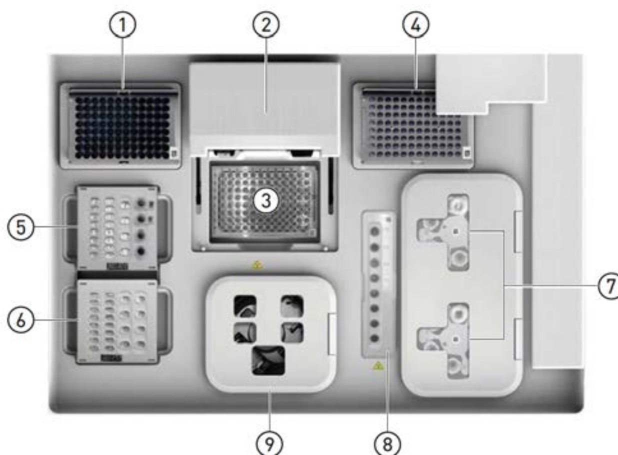

Figure 3. Illustration of the Interior of the Ion Chef Instrument

9.14.6. Load the Reagents and Solutions Cartridges.

9.14.6.1. Gently tap the Ion 510 & 520 & 530 Chef Reagents cartridge on the bench to ensure the reagents are on the bottom of the tubes.

9.14.6.2. Load the cartridge into the Reagents station (#5 in Figure 3) so that it snaps into place and is level on the deck.

9.14.6.3. Uncap all of the tubes in the Reagents cartridge.

9.14.6.4. Retrieve the Ion S5 Chef Solutions cartridge from room temperature and gently tap on the bench to ensure the reagents are on the bottom of the tubes.

9.14.6.5. Load the cartridge into the Solutions station (#6 in Figure 3) until it snaps into place and is level on the deck.

9.14.7. Load the Recovery Tubes and Enrichment Cartridge

9.14.7.1. Open the hinged lid of the Recovery Centrifuges (#7 in Figure 3) and load six Recovery Tubes into each centrifuge.

9.14.7.2. Place a Recovery Station Disposable Lid over each centrifuge with the port oriented toward the rear of the instrument and press down. Make certain the lids snap completely into place.

9.14.7.3. Close the hinged lid of the Recovery Centrifuges.

9.14.7.4. Load the Enrichment Cartridge (#8 in Figure 3), then press down to ensure it is level with the instrument deck.

9.14.8. Load the Chip-loading centrifuge

9.14.8.1. Load each sequencing chip into a centrifuge bucket (two detachable buckets inside centrifuge #9 in Figure 3), and then attach a Chip Adapter to the assembly.

9.14.8.1.1. Place the chip in the chip-loading bucket, and then align the wells of the Chip Adapter to the wells of the chip, orienting the adapter on the chip so the chip barcode is visible.

9.14.8.1.2. Place the adapter onto the chip, then insert the stationary tabs at the reservoir end of the adapter into the slots of the bucket.

9.14.8.1.3. Gently squeeze the flexible tabs at the other end of the adapter into the bucket slots until the adapter locks into place.

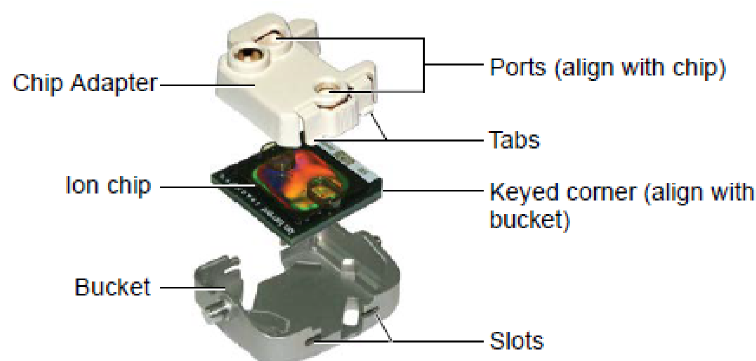

Figure 4. Instructions for Loading the Chip into the Centrifuge Bucket.

9.14.8.2. Confirm that the tabs at all four corners of the adapter are fitted into the slots in the centrifuge bucket. Loading can fail if the adapter is not attached securely.

9.14.8.3. Load the adapter/chip/bucket assemblies into the Chip-loading centrifuge.

9.14.8.4. Ensure the centrifuge is load-balanced, and the chip buckets are seated securely and oriented correctly in the centrifuge. Then close the lid of the Chip-loading centrifuge.
NOTE: *The chip that corresponds to the library pool in position B should be in position 90°clockwise from the double hole in the rotor bucket cover at rest. Confirm the chips and the library pools are in matching positions to ensure correct loading of the library.*

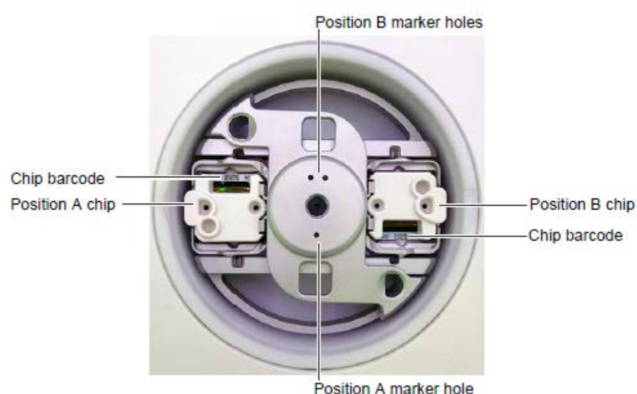

Figure 5. Illustration of Chip-loading Centrifuge and loading position.

9.14.9. Confirm that the consumables are correctly installed before continuing.

9.14.9.1. Confirm that each cartridge is at the correct location and in the correct orientation.

9.14.9.2. Press down on all cartridges to confirm that they are firmly pressed into place.

9.14.9.3. Confirm that all tubes on the Ion 510 & 520 & 530 Chef Reagents cartridge are uncapped and firmly pressed into place.

9.14.9.4. Confirm that the centrifuge lids are installed correctly so that the port is oriented toward the rear of the instrument.

9.14.9.5. Confirm that the tube and chip buckets are seated securely in the rotor arms of the Chip-loading and Recovery centrifuges, and that the consumables they contain are correctly installed.

9.14.10. Start the Ion Chef run

9.14.10.1. Touch **Set up run** on Ion Chef.

9.14.10.2. Touch **Quick Start**.
NOTE: *For initial setup,* ***Step by Step*** *may be selected for detailed step by step guide.*

9.14.10.3. When prompted, close the instrument door by first lifting it slightly to disengage the locking mechanism, and then push down on the door until the lock engages.

9.14.10.4. When prompted, touch **Start Check** to start the Deck Scan. Wait while the instrument scans the barcodes of all consumables and reagents to confirm their presence and compatibility.
NOTE: *If Ion Chef displays warnings, ensure all consumables are installed correctly and present. The chip centrifuge cover must be clicked into rest position that clearly displays chip barcodes (Figure 5).*

9.14.10.5. When the Deck Scan is complete, touch **Next** to display the Data Destination screen.

9.14.10.6. Confirm that the instrument displays the correct kit type, chip type, chip and tube barcodes, and Planned Run. If the correct planned runs do not display, touch the dropdown menu to select the Planned Run for each chip, and then touch **Next**.
NOTE: *If you have not entered the runs into the S5 Torrent Server, they will not show up. Enter the run and return to the Chef Instrument (See appendix PNL41-1).*

9.14.10.7. On the Run Options screen, choose **Pause** for the option to analyze templating efficiency and touch **Start Run** to begin the run.

9.14.10.8. The machine will run overnight and pause at the time indicated in step 9.14.10.7.

9.14.10.9. Transfer all liquids from tubes 1 and 2 into two labelled 0.5 ml PCR tubes to analyze the templating efficiency with Qubit for Ion QC (See appendix PNL41-2).

9.14.10.10. Close the door and touch **Continue** to resume the run.
NOTE: *It is not possible to re-pause the run or re-open the door after it has been closed. Ensure both samples have been retrieved before locking the instrument to continue.*

9.15. Initializing the GeneStudio S5

9.15.1. After resuming Ion Chef run, remove Ion S5 Sequencing Reagents Cartridge from the freezer and allow to thaw for 30-60 minutes.
NOTE1: *Do not remove the cartridge from its packaging until immediately before loading. Unused cartridge may be returned to storage if sequencing run is delayed.*
NOTE2: *It is recommended to begin initializing S5 about 50 minutes before the Chef finishes its run.*

9.15.2. In the S5 main menu, touch **Initialize**. The door, chip, and reagent cartridge clamps will unlock.

9.15.3. Remove the used Ion S5 Wash Solution bottle, then remove and empty the waste reservoir.

9.15.4. Reinstall the empty waste reservoir.

9.15.5. Remove the expended Ion S5 Sequencing Reagents cartridge and set aside for disposal.

9.15.5.1. Remove the CO_2 scrubber (red, top right corner on the cartridge, see Figure 6) with Ion S5 Cartridge Tool and dispose according to applicable hazardous waste regulations.

9.15.5.1.1. Invert the cartridge over an appropriate absorbent material to drain all residual liquid.

9.15.5.1.2. Wearing gloves, insert the Ion S5 Cartridge Tool firmly into the CO_2 scrubber until the flange stops on the top of the cartridge.

9.15.5.1.3. Pull straight up on the tool while holding the nucleotide reagent cartridge down.

9.15.5.1.4. The remaining nucleotide reagent cartridge may be disposed of as laboratory waste.

Figure 6. Removal of CO_2 scrubber from expended Ion S5 Sequencing Reagents cartridge

9.15.6. Replace with the new thawed Ion S5 Sequencing Reagents cartridge.

9.15.7. Invert a new Ion S5 Wash Solution bottle 5 times and swirl at an angle to mix thoroughly.

9.15.8. Remove the red cap and install.

9.15.9. Ensure that a used sequencing chip is properly seated in the chip clamp and the chip clamp is pushed in all the way.

9.15.10. If necessary, install a new Ion S5 Cleaning Solution bottle.

NOTE: *The Ion S5 Cleaning Solution bottle contains sufficient reagent to complete 4 - 5 cleanings.*

9.15.11. Close the door, and then touch **Next**.

9.15.12. When initialization is complete, touch **Home**. Initialization takes around 50 minutes.

9.16. Cleaning Ion Chef and Starting a Run on GeneStudio S5

NOTE: *When the Ion Chef run is complete, unload and sequence the chips immediately or store away from light in a sealed container at 4°C for up to 6-8 hours. Allow stored chips to come to room temperature in the dark before attempting to load into the sequencer.*

9.16.1. Unload Chip and start cleaning Chef

9.16.1.1. Press the **Open Door** button on Ion Chef to open the door.

9.16.1.2. Open the lid of the Chip-loading centrifuge, and unload both chip/bucket assemblies from the Ion Chef instrument.

9.16.1.2.1. Remove the chip/bucket assembly from the Chip-loading centrifuge.

9.16.1.2.2. Apply pressure to both ends of the Chip Adapter, then remove and discard it.

9.16.1.2.3. Grasp the chip by its edges, carefully lift the chip out of the bucket, then set it aside on a clean, static-free surface. Return the buckets to the Chip-loading centrifuge.

NOTE: *Gloves may cause static electricity damage and should not be used when handling the chips.*

9.16.1.2.4. Place the chip to be sequenced second into a sealed container and store away from light at 4°C for up to 6-8 hours.

9.16.1.3. Move the empty tip rack from New Pipette Tip Position (#1 in Figure 3) and place in the Waste Pipette Tip position (#4 in Figure 3).

9.16.1.4. Discard all other consumables and wipe all condensation with lint-free wipe.

9.16.1.5. Close door and touch **Next** on the screen that appears after run completion.

9.16.1.6. Confirm all consumables have been removed from the Ion Chef and touch **Next**.

9.16.1.7. Touch **Start** to begin cleaning.

9.16.2. Start the sequencing run on GeneStudio S5

NOTE: *It is recommended to start the run soon after initialization is complete, however Fisher reports successful runs have been started as long as 24 hours after initialization.*

9.16.2.1. Touch **Run** on home screen

9.16.2.1.1. When the mechanism unlocks, slide the chip clamp out and remove the chip used to initialize.

9.16.2.1.2. Place the loaded chip in the chip clamp with the chip notch in the bottom corner towards the back of the instrument.

NOTE: *Do not force the chip into the clamp. If the chip does not fit easily in the clamp, confirm that the notch is oriented towards the back.*

9.16.2.1.3. Slide the chip clamp in fully, then close the instrument door and touch **Next.**

9.16.2.2. Confirm that the correct Planned Run has been auto-populated and ensure the Post-Run Clean is enabled and touch **Review**.

9.16.2.3. Confirm that the remaining pre-populated settings are correct, or touch **Edit** to make changes if needed.

9.16.2.4. Confirm that the instrument is closed, then touch **Start run**.

NOTE1: *Run process can be monitored on S5 Torrent Server in Monitor tab.*

NOTE2: *Post-run clean is automatically performed upon run completion. Leave chip, cartridge, wash solution, and cleaning solution in the instrument when idle.*

9.17. Locating and Downloading Run FASTQ files

9.17.1. Open internet browser on a computer connected to the same network as the S5 instrument and login to S5 Torrent Server (Refer to appendix PNL41-1).

9.17.2. In **Data** tab, find the appropriate run name and click the report name in light blue corresponding to the run.

9.17.3. Scroll down to **Plugins.**

9.17.4. Under FileExporter, click **<u>Compressed Files</u>** and save the zipped FASTQ files to a designated location.

10. FLOW CHART: N/A

11. RELATED DOCUMENTS:

Document Number	Title
PNL33	DNA Extraction and QC SOP
PNL41.W1	Ion Torrent S5 Workbook

12. **REFERENCES:**

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12.4. ThermoFisher, User Guide: Ion Sphere Quality Control Kit. (Pub No. MAN0017531). February 2018. https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2FMAN0017531_IonSphereQCKit_UG.pdf&title=VXNlciBHdWlkZTogSW9uIFNwaGVyZSBRdWFsaXR5IENvbnRyb2wgS2l0
12.5. ThermoFisher, User Guide: Ion 510 & Ion 520 & Ion 530 Kit - Chef - Instructions for automated template preparation, chip loading, and sequencing. (Pub No. MAN0016854). December 2018. https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2FMAN0016854_510_520_530_Kit_Chef_UG.pdf&title=VXNlciBHdWlkZTogSW9uIDUxMCAmYW1wOyBJb24gNTIwICZhbXA7IElvbiA1MzAgS2l0IC0gQ2hlZiAtIEluc3RydWN0aW9ucyBmb3IgYXV0b21hdGVkIHRlbXBsYXRlIHByZXBhcmF0aW9uLCBjaGlwIGxvYWRpbmcsIGFuZCBzZXF1ZW5jaW5n

13. **CONTACTS:**

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

14. **APPENDICES:**

14.1. PNL41-1: Planning a Run on S5 Torrent Server
14.2. PNL41-2: Qubit for Ion QC

15. **AMENDMENTS:**

15.1. **06/03/2019:** 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 PNL41-1

Planning a Run on S5 Torrent Server

1. Exporting a Sample Sheet from the Ion Torrent S5 Workbook

1.1. After completing the Initial Dilution tab in Ion Torrent S5 Workbook, select the SampleSheet tab.

1.2. Check the Barcode and Sample Name field in the Samplesheet to ensure that all of the data has been accurately populated.
NOTE: *The index numbers are populated from the List of Barcodes tab. Do not modify or delete this tab.*

1.3. To export/save the Sample Sheet, open the File menu option and select “Save As.”

1.4. Change the saved file type to CSV (Comma delimited) by selecting it from the drop-down menu.

1.5. Navigate to the desired file folder location and save the file using the Run ID as the filename (e.g. LabID-MachineID-YYMMDD).

1.6. Click “OK” to save only the active sheet, and “Yes” in the following window to keep using the CSV format.

1.7. Open CSV file in wordpad or notepad and delete unnecessary rows of “,,,,,,,,” and save.

2. Planning a run on S5 Torrent Server

2.1. On the S5 GeneStudio instrument home screen, touch **Settings.**

2.2. In Settings screen, touch **Instrument Settings.**

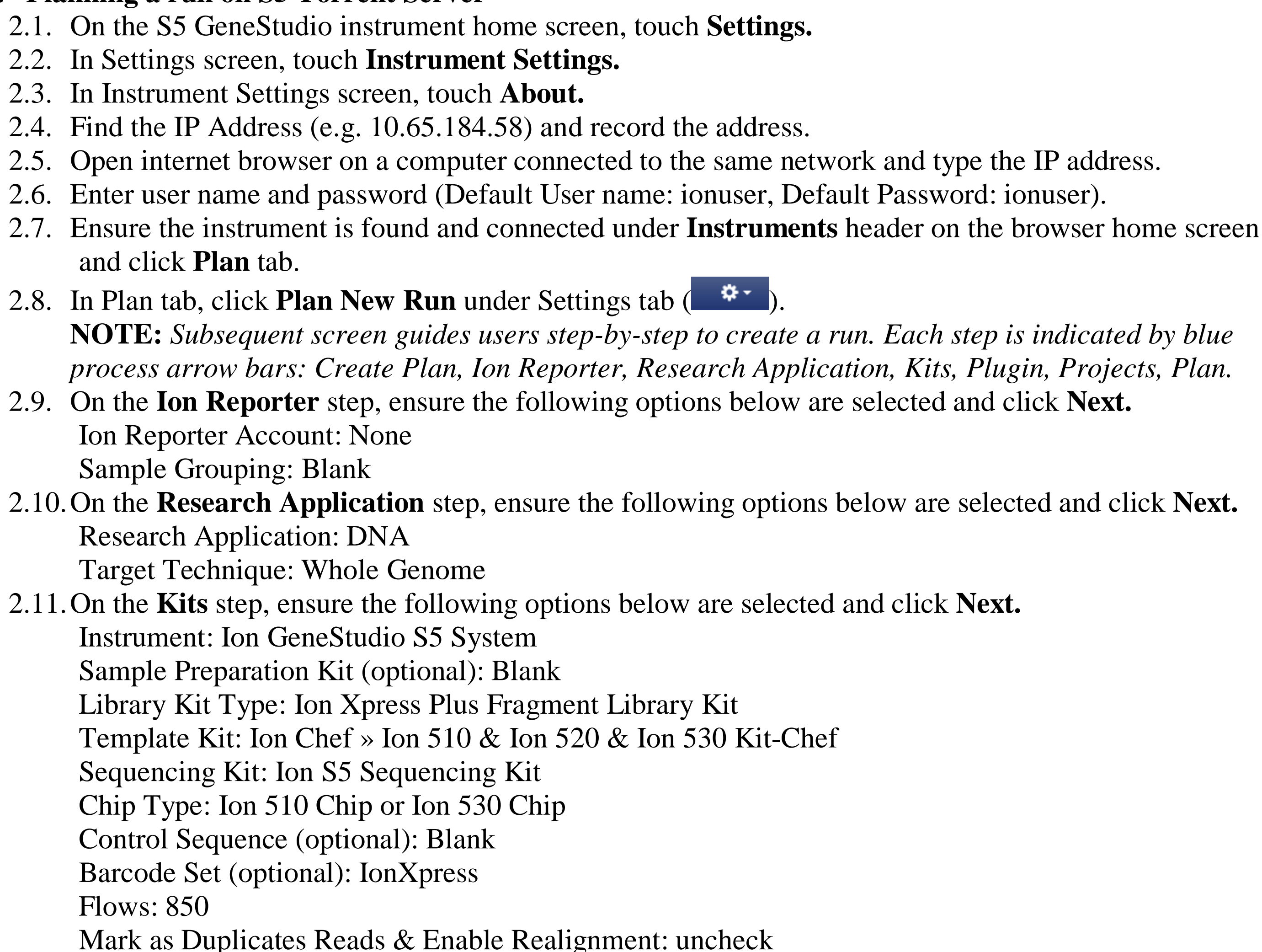
2.3. In Instrument Settings screen, touch **About.**

2.4. Find the IP Address (e.g. 10.65.184.58) and record the address.

2.5. Open internet browser on a computer connected to the same network and type the IP address.

2.6. Enter user name and password (Default User name: ionuser, Default Password: ionuser).

2.7. Ensure the instrument is found and connected under **Instruments** header on the browser home screen and click **Plan** tab.

2.8. In Plan tab, click **Plan New Run** under Settings tab ().
NOTE: *Subsequent screen guides users step-by-step to create a run. Each step is indicated by blue process arrow bars: Create Plan, Ion Reporter, Research Application, Kits, Plugin, Projects, Plan.*

2.9. On the **Ion Reporter** step, ensure the following options below are selected and click **Next.**
Ion Reporter Account: None
Sample Grouping: Blank

2.10. On the **Research Application** step, ensure the following options below are selected and click **Next.**
Research Application: DNA
Target Technique: Whole Genome

2.11. On the **Kits** step, ensure the following options below are selected and click **Next.**
Instrument: Ion GeneStudio S5 System
Sample Preparation Kit (optional): Blank
Library Kit Type: Ion Xpress Plus Fragment Library Kit
Template Kit: Ion Chef » Ion 510 & Ion 520 & Ion 530 Kit-Chef
Sequencing Kit: Ion S5 Sequencing Kit
Chip Type: Ion 510 Chip or Ion 530 Chip
Control Sequence (optional): Blank
Barcode Set (optional): IonXpress
Flows: 850
Mark as Duplicates Reads & Enable Realignment: uncheck

Advanced Settings: Customize
Templating Protocol: Chef Protocol – 400 bp
Forward Library Key: Ion TCAG
Test Fragment Key: ATCG
Base Calibration Mode: Default Calibration
Forward 3’ Adapter: Ion P1B
Flow Order: Use Instrument Default

2.12. On the **Plugins** step, check the following options below and click **Next.**
FastQC
FileExporter
Optional: AssemblerSPAdes

2.13. On the **Projects** step, check the appropriate project name and click **Next.**

2.14. On the **Plan** step, enter the run information.
Run Plan Name: LabID-MachineID-YYMMDD
Analysis Parameters: Default (Recommended)
Sample Tube Label: Refer to 9.14.1
Chip Barcode: Refer to 9.14.3

2.15. Click **Load Samples Table** and navigate to the folder location where Sample Sheet was saved.

2.16. Select Sample Sheet and **Load.**

2.17. Ensure all information has been entered correctly and click **Plan Run.**

Appendix PNL41-2

Qubit for Ion QC

1. **Prepare the Calibration Standard**
   NOTE: *Calibration standards do not need to be read each time. Measure once when a new kit is opened.*
   1.1. From the Ion Sphere™ Quality Control Kit, thaw Alexa Fluor™ 488 and Alexa Fluor™ 647 Calibration Standard reagents.
   NOTE: *Both the Alexa Fluor™ 488 and Alexa Fluor™ 647 molecules are sensitive to light. Avoid exposure to light.*
   1.2. Transfer 200 µl of each standard into two separate Qubit Assay tubes and label the tubes.
   1.3. Press **Ion** on Qubit Fluorometer to access Alexa Fluor™ 488 and Alexa Fluor™ 647 measurement options.
   1.4. Press **AF 488**, insert the corresponding tube, close the lid and press **Read**.
   NOTE: *The lettering on the Read button changes from red to white when finished reading.*
   1.5. Record the RFU value in Cell B3 in the Qubit Calculator tab of the workbook and remove the tube.
   1.6. Press **Home** to return to home screen.
   1.7. Press **Ion** on Qubit Fluorometer to access Alexa Fluor™ 488 and Alexa Fluor™ 647 measurement options.
   1.8. Press **AF 647**, insert the corresponding tube, close the lid and press **Read**.
   NOTE: *The lettering on the Read button changes from red to white when finished reading.*
   1.9. Record the RFU value in Cell B4 in the Qubit Calculator tab of the workbook and remove the tube.

2. **Prepare and Measure the Samples**
   2.1. Thaw the Ion Probes, Annealing Buffer, and (if needed) Quality Control Wash Buffer.
   NOTE: *Aliquot 1.5ml of Quality Control Wash Buffer and store at -15°C to -25°C. Approximately 2-2.5ml of wash buffer is needed for two library pools.*
   2.2. Transfer the entire volume of liquid from tube 1 and 2 on the Chef Reagents Cartridge into two labelled 0.5 ml PCR tubes.
   2.3. Centrifuge the PCR tubes at 14,500 x*g* for 4 minutes.
   2.4. Remove supernatant to reduce the total volume per sample to approximately 10 µl.
   NOTE: *Probe pellet may not be visible. Carefully pipet from the opposite wall of tube, away from the pellet.*
   2.5. Add 10 µl Annealing Buffer and 1 µl Ion Probes directly to each 0.5 ml PCR tube containing the ISPs, then mix well by pipetting up and down.
   2.6. Transfer to two 0.2 ml PCR tubes and incubate on thermal cycler at the following conditions:

   Step1: 95°C for 2 minutes
   Step2: 37°C for 2 minutes
   Step 3: Hold at 4°C
   (21 µl total volume)

   NOTE1: *It is recommended to pre-program a thermal cycler for this purpose.*
   2.7. Transfer the contents back to two 0.5 ml PCR tubes, and perform three washes with 200 µl Quality Control Wash Buffer to remove the unbound probes.
   NOTE: *Probe pellet may not be visible. Carefully pipet from the opposite wall of tube, away from the pellet.*
      2.7.1. Add 200 µl Quality Control Wash Buffer to the PCR tube.
      2.7.2. Vortex to mix and centrifuge at 14,500 x*g* for 4 minutes.

2.7.3. Carefully reduce the volume to ~10 µl by removing the supernatant with a pipette.
2.7.4. Perform steps 2.7.1 through 2.7.3 two additional times for a total of three washes.

2.8. After the final wash, reduce volume to 10 µl and add 190 µl Quality Control Wash Buffer. Mix thoroughly by pipetting and transfer entire sample to a Qubit Assay tube.
2.9. Create a negative control by adding 200 µl of Quality Control Wash Buffer to a new Qubit Assay tube.
2.10. Press **Ion** on Qubit Fluorometer to access Alexa Fluor™ 488 and Alexa Fluor™ 647 measurement options.
2.11. Press **AF 488**, insert the sample tube, close the lid and press **Read**.
NOTE: *The lettering on the Read button changes from red to white when finished reading.*
2.12. Record the RFU value in Column B of Qubit Calculator tab of the workbook and remove the tube.
2.13. Insert negative control tube, close the lid and press **Read**.
2.14. Record the RFU value in Column D of Qubit Calculator tab of the workbook and remove the tube.
2.15. Press **Home** to return to home screen.
2.16. Press **Ion** on Qubit Fluorometer to access Alexa Fluor™ 488 and Alexa Fluor™ 647 measurement options.
2.17. Press **AF 647**, insert the sample tube, close the lid and press **Read**.
NOTE: *The lettering on the Read button changes from red to white when finished reading.*
2.18. Record the RFU value in Column C of Qubit Calculator tab of the workbook and remove the tube.
2.19. Insert negative control tube, close the lid and press **Read**.
2.20. Record the RFU value in Column E of Qubit Calculator tab of the workbook and remove the tube.
2.21. Enter the template kit- and lot-specific Conversion Factor in Column F in the Qubit Calculator tab of the Workbook. Go to https://www.thermofisher.com/order/catalog/product/4468656?SID=srch-srp-4468656 for updated Qubit Conversion Factors Guide under Documents.
2.22. The Percent Template ISPs will be auto-calculated and displayed in Column G for each pool.
NOTE: *The recommended optimal range for Ion S5 GeneStudio system is 10-25%. If the results are outside the desired percent template ISPs range, then increase or decrease the library input accordingly.*