United States Department of Agriculture

Food Safety and Inspection Service

MLG 42.01

Whole Genome Sequencing of Bacterial Isolates

This method describes the laboratory procedure for performing Whole Genome Sequencing (WGS) of bacterial strains using the Illumina MiSeq

Notice of Change

This version incorporates the Illumina DNA Prep Library Kit as the primary method for WGS library preparation as the Nextera XT Library Prep Kit PulseNet is being discontinued. The Illumina DNA Prep kit meets or exceeds performance metrics, can analyze more isolates on a single run, and improves turn-around times for Shiga toxin-producing *E. coli* (STEC).

This revision also includes a biosafety chart to further explain safety precautions regarding MLG 42.01.

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Introduction

Whole Genome Sequencing (WGS) is a laboratory procedure that determines the order of DNA bases in the genome of an organism in one process. The detailed DNA fingerprint generated can be used for regulatory purposes, surveillance, research, and other genomic applications. The following method describes the laboratory procedure for performing WGS of bacterial strains using the Illumina MiSeq® short-read technology by FSIS. Bacterial strains used for WGS are isolated and identified according to FSIS MLG chapters 4 (*Salmonella*), 8 (*Listeria monocytogenes*), 5C (*Escherichia coli*), and 41 (*Campylobacter*). The procedure is modeled after the Illumina technical documentation and the Centers for Disease Control and Prevention (CDC) PulseNet protocol for WGS. Additionally, quality control acceptance criteria for WGS data are described, including nucleotide balance, average quality and FASTA size.

Illumina's MiSeq® instrument uses reversible-terminator sequencing-by-synthesis technology to provide end-to-end sequencing to generate short reads, requiring a series of laboratory steps described in Figure 1. First, genomic DNA is extracted and isolated from bacterial cells. Then, DNA is enzymatically sheared into small fragments and tagged with Illumina-specific DNA tags (Tagmentation and Indexing). These indexes provide a unique identifier to allow DNA from multiple bacterial samples to be sequenced at once. Next, the short-tagged fragments of DNA (i.e. Tagmented and Indexed DNA) are purified for homogeneity. Samples are normalized to specific concentrations, mixed together as a pool, and loaded into the sequencer. The sequencer identifies the DNA bases that make up each bacterial sequence. Finally, the data is computationally analyzed to obtain a sequenced genome.

The methods described in this guidebook are for use by the FSIS laboratories. FSIS does not specifically endorse any of the mentioned test products and acknowledges that equivalent products may be available for laboratory use.

KEY DEFINITIONS

Base (or nucleobase): a functional unit of nucleotides in nucleic acids like DNA e.g. Adenine (A), Guanine (G), Cytosine (C), Thymine (T).

Read: set of nucleotide sequences produced by the sequencer, with corresponding identifier and quality scores

FASTA: a file format for contigs/genomes.

Contig: the product of assembling reads into longer contiguous sequences

Tagmentation: The transposome enzyme simultaneously cuts and adds adapter sequence tags to the bacterial genomic DNA in preparation for indexing

Indexing: The process of enzymatically adding unique index sequences to the tagged DNA pieces from the tagmentation step

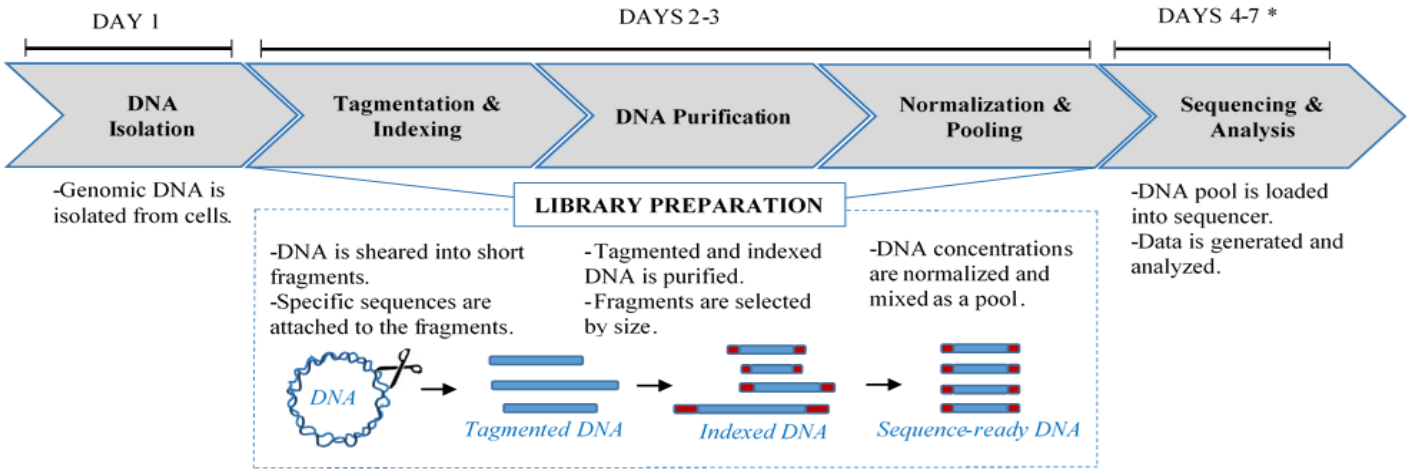


FIGURE 1: Overview and timeframe of WGS. Bacteria are isolated according to relevant FSIS MLG chapters, and fresh cultures are inoculated for WGS. Genomic DNA is isolated from cells and DNA libraries are prepared. Library preparation consists of Tagmentation, Indexing, size-selection of processed DNA, and pooling. The library is sequenced on Illumina MiSeq®, and the generated data is computationally analyzed. * *An additional 14 days may be necessary if sequences do not meet FSIS quality standards and need to be repeated.* Illustration Credit: Christian Rivera-Gonzalez

Safety Precautions

CDC guidelines for the handling of BioSafety Level 2 organisms should be followed whenever live cultures are used. The Safety Data Sheet (SDS) may be obtained from the manufacturer for the media, chemicals, reagents, and microorganisms used in the analysis. The personnel who will handle the material are to read the SDS prior to startup.

QUALITY CONTROL

Lab Quality Control Procedures

DNA extraction is to be performed from pure cultures, derived from a single colony.

DNA quantity is to be determined using a DNA-specific, intercalating dye-based fluorescence quantification method prior to further dilution (Qubit Broad Range). Minimum quantity required prior to dilution is 2 ng/µL.

Care is to be taken to avoid cross-contamination during DNA extraction and library preparation. Use aerosol resistant filter pipette tips and change gloves frequently.

To minimize carryover-contamination between sequencing runs, perform post-run instrument washes with bleach or establish an index-rotation scheme to ensure that the same index pair is not used for consecutive runs on the same instrument.

Equipment, Reagents, Media, and Cultures

Table 1: Equipment for WGS

Equipment	Supplier	Purpose
Illumina MiSeq	Illumina	Sequencer
Thermocycler	General lab supplier	Perform Tagmentation and Indexing
200 and 1000 µL pipette tips	General lab supplier	Add and mix reagents
1.5 mL microcentrifuge tubes	General lab supplier	Elute, dilute, and store DNA
Qubit	Invitrogen, Catalog # Q33216	Quantify DNA concentration
Eppendorf LoBind 1.5 mL tubes	General lab supplier	Dilute DNA and denature sample libraries
Optically-Clear-wall 0.5 mL PCR tubes	General lab supplier	Quantify DNA concentration
2-20 µL, 20-200 µL, 200 µL, 1000 µL pipette tips	General lab supplier	Add and mix reagents
Centrifuge capable of at least 14,000 x g	General lab supplier	Spin 96-well PCR plate to mix reagents and for DNA extraction
Vortexer	General lab supplier	Mix reagents in tubes
MiSeq tube	Illumina, Catalog # MS-102-9999	Wash the template line
Ambion Magnetic Stand-96	Life Technologies, Catalog # AM10027	Purify and size-select DNA after Tagmentation and Indexing
Microseal A Film	Bio-Rad, Catalog # MSA5001	Seal 96-well PCR plate
Microseal B Film	Bio-Rad, Catalog # MSB1001	Seal 96-well PCR plate
Microseal F Seal	Bio-Rad, Catalog # MSF1001	Seal 96-well PCR plate
Thin-walled PCR Tube strips	General lab supplier	Dilute DNA and aliquot reagents
96 well PCR plates	General lab supplier	Perform Tagmentation and Indexing
PCR tube strip caps	General lab supplier	Dilute DNA and aliquot reagents
Rainin 200 µL Wide-orifice tips (RT-L200WFLR)	General lab supplier	Add beads and mix reagents
Vacuum Pump	Qiagen, Catalog # 84010	Extract and isolate genomic DNA from cells
Vacuum Manifold	Qiagen, Catalog # 19413	Extract and isolate genomic DNA from cells
Vacuum Connecting System	Qiagen, Catalog # 19419	Extract and isolate genomic DNA from cells
Vacuum Connectors	Qiagen, Catalog # 19407	Extract and isolate genomic DNA from cells

Table 2: Reagents for WGS

Reagent	Supplier	Purpose
200 proof Ethanol for molecular biology	General lab supplier	Wash DNA
10 mM Tris-HCl, pH 8.5	General lab supplier	Elute and dilute DNA
10 mM Tris-HCl with 0.1% Tween 20, pH 8.5	General lab supplier	Dilute DNA
1 M Tris-HCl, pH 8.0	General lab supplier	Elute and dilute DNA
0.5 M EDTA	General lab supplier	Enzymatic Cell Lysis Buffer
1 N NaOH	General lab supplier	Denature sample libraries
Laboratory-grade water	House system	Wash the instrument
100X TE Buffer Solution, pH 8.0	General lab supplier	Enzymatic Cell Lysis Buffer
Lysozyme	General lab supplier	Lyse Gram + cells
Triton X-100	General lab supplier	Enzymatic Cell Lysis Buffer
Tween 20	General lab supplier	Wash the instrument
Agencourt AMPure XP Beads	Beckman Coulter, Catalog # A63880	Purify and size-select DNA after Tagmentation and Indexing
MiSeq v2 cartridge 300 cycle	Illumina, Catalog # MS-102-2002	Sequence sample libraries
MiSeq v2 cartridge 500 cycle	Illumina, Catalog # MS-102-2003	Sequence sample libraries
Nano v2, 300 cycle	Illumina, Catalog # MS-103-1001	Sequence sample libraries
Micro v2, 300 cycle	Illumina, Catalog # MS-103-1002	Sequence sample libraries
PhiX V3 control	Illumina, Catalog # FC-110-3001	Control DNA
Qiagen DNeasy Blood and Tissue kit	Qiagen, Catalog # 69504	Extract and isolate genomic DNA from cells
Qubit dsDNA Broad Range (BR) Assay Kit	Invitrogen, Catalog # Q32850	Quantify DNA concentration
Qubit dsDNA High Sensitivity (HS) Assay Kit	Invitrogen, Catalog # Q32856	Quantify DNA concentration
Illumina DNA Prep, (M) Tagmentation (96 Samples, IPB)	Illumina, Catalog # 20060059	Prepare sample libraries
Illumina DNA/RNA UD Indexes Set A, Tagmentation (96 Indexes, 96 Samples)	Illumina, Catalog # 20091654	Prepare sample libraries
Illumina DNA/RNA UD Indexes Set B, Tagmentation (96 Indexes, 96 Samples)	Illumina, Catalog # 20091656	Prepare sample libraries

Illumina DNA/RNA UD Indexes Set C, Tagmentation (96 Indexes, 96 Samples)	Illumina, Catalog # 20091658	Prepare sample libraries
Illumina DNA/RNA UD Indexes Set D, Tagmentation (96 Indexes, 96 Samples)	Illumina, Catalog # 20091660	Prepare sample libraries
Nextera DNA CD Indexes (96 Indexes, 96 Samples)	Illumina, Catalog # 20018708	Prepare sample libraries
Nextera XT Library Prep Kit PulseNet (96 samples)	Illumina, Catalog # FC-133-1001	Prepare sample libraries
Nextera XT Index Kit (96 indexes, 384 sample)	Illumina, Catalog # FC-131-1002	Prepare sample libraries
Nextera XT Index Kit SetA (96 indexes)	Illumina, Catalog # FC-131-2001	Prepare sample libraries
Nextera XT Index Kit SetB (96 indexes)	Illumina, Catalog # FC-131-2002	Prepare sample libraries
Nextera XT Index Kit SetC (96 indexes)	Illumina, Catalog # FC-131-2003	Prepare sample libraries
Nextera XT Index Kit SetD (96 indexes)	Illumina, Catalog # FC-131-2004	Prepare sample libraries

Media
Media formulations are available in MLG Appendix 1, Media and Reagents.

a. Tryptic soy agar with 5% sheep blood (SBA)
b. Campy-Cefex plating medium
c. Brain Heart Infusion Agar

Cultures

Fresh (18 – 24 h) bacterial cultures on/in media yielding healthy growth following MLG protocols.

DNA Isolation

Prior to starting:

1. Aliquot 5 mL of 200 proof ethanol into a sterile tube each time prior to use.
2. When using a new extraction kit, add appropriate volumes of 200 proof ethanol to each wash buffer according to manufacturer's guidelines. Label and date each bottle after adding 200 proof ethanol with expiration date of one year. Store at room temperature.
3. Enzymatic Lysis Buffer (for Gram-positive bacteria) is made as follows: 5 mL 1 M Tris-HCl (pH 8.0), 1 mL 0.5M EDTA, 3 mL Triton X-100, and MilliQ water to a total volume of 250 mL. Mix gently (this may be done with a stir bar and stir plate). Prepared Enzymatic Lysis Buffer (ELB) is to be stored at room temperature and assigned an expiration date of one year.
4. Prepare 1X TE Buffer as follows: 10 mL 100X TE Buffer and 990 mL sterile water.
5. Prepare Lysozyme (20 mg/mL lysozyme stock solution) as follows: 100 mg Lysozyme, 5 mL 1X TE buffer, swirl to mix then aliquot approximately 200 µL in tubes. Store aliquots at ≤ -10°C for up to 1 year.

   Do not use kit-provided AE buffer for elution due to the presence of EDTA. Use 10 mM Tris-HCl (pH 8.5) as elution buffer on final step.

DNA Extraction from Gram-positive Bacterial Cultures

1. Label a 1.5 mL tube with a unique identifier for each sample.
2. Prepare a mastermix of 180 ± 1 µL of enzymatic lysis buffer and 20 ± 1 µL lysozyme (stock concentration 20 mg/mL) for each sample. Add 180 ± 1 µL of mastermix to the tube.
3. Resuspend approximately 1 µL loopful of colonies from a fresh (18 – 24 h) non-selective agar (sheep blood agar or brain heart infusion agar) in the mixture, vortex, and incubate on heatblock at 56 ± 1°C for 15 – 30 min. The tube should have a white precipitate.
4. Separately add 25 ± 1 µL of proteinase K (kit provided) and 200 ± 1 µL of AL Buffer to the tube. Do not combine proteinase K and AL Buffer prior to this step. If AL Buffer forms precipitate, heat to 56°C to dissolve before use.
5. Vortex the tube briefly.
6. Incubate on heatblock at 56 ± 1°C for 1 – 2 hours.
7. Add 200 ± 1 µL of 200 proof ethanol to the tube and vortex briefly.
8. Using a micropipette, transfer entire contents of tube to labeled Qiagen spin column. ****If using Vacuum manifold, proceed directly to "Optional Qiagen Vacuum Manifold Processing for Gram-positive Bacteria."**** Otherwise, proceed directly to step 9.
9. Centrifuge column at approximately 6,000 x g for 1 min.
10. Remove column from collection tube. Place column in new collection tube.
11. Add 500 ± 1 µL of buffer AW1 (kit provided) to the column and centrifuge at approximately 6,000 x g for 1 minute.
12. Remove column from collection tube. Place column in new collection tube.
13. Add 500 ± 1 µL of buffer AW2 (kit provided) to the column and centrifuge at max speed (14,000 – 20,000 x g) for 3 ± 1 minutes.
14. Carefully remove tubes from centrifuge. Empty the contents of the flow-through tube then centrifuge the tube again for 1 min at max speed (14,000 – 20,000 x g).
15. Transfer the column to a 1.5 mL tube and add 50 – 100 µL of 10 mM Tris-HCl (pH 8.5) to the column. Let column stand at room temperature for 1 minute. Reducing the elution volume can be used to increase the DNA concentration yield.
16. Centrifuge at approximately 6,000 x g for 1 minute. Discard the column and store the DNA appropriately (2 – 8°C for use within 24 h, ≤ -10°C for storage of more than 24 h).

Optional Qiagen Vacuum Manifold Processing for Gram-positive Bacteria
Using a vacuum manifold, if available, may be more efficient for high volume laboratories as it reduces the number of collection tube changes.

1. Add new VacConnectors on Vacuum Manifold for each sample, then place column on VacConnectors. Allow vacuum to drain the contents from the column.
2. Add 500 ± 1 µL of buffer AW1 to the column and allow to drain.
3. Add 500 ± 1 µL of buffer AW2 to the column and allow to drain.
4. Carefully remove column and place in collection tube. Centrifuge for 2 min at max speed (14,000 – 20,000 x g).
5. Transfer the column to a 1.5 mL tube and add 50 – 100 µL of 10 mM Tris-HCl (pH 8.5) to the column. Let column stand at room temperature for 1 minute. Reducing the elution volume can be used to increase the DNA concentration yield.
6. Centrifuge at approximately 6,000 x g for 1 minute. Discard the column and store the DNA appropriately (2 – 8°C for use within 24 h, ≤ -10°C for storage of more than 24 h).

DNA Extraction from Gram-negative Bacterial Cultures

1. Label a 1.5 mL tube for each sample.
2. Prepare a mastermix of 180 ± 1 µL ATL buffer (kit provided) and 20 ± 1 µL proteinase K (kit provided) per sample. Add 200 ± 1 µL of mastermix to the tube. ATL Buffer may form precipitate, warm up to 56°C to dissolve before use.
3. Resuspend approximately 1 µL loopful of colonies from a fresh (18 – 24 h) non-selective agar (sheep blood agar or brain heart infusion agar) in ATL buffer and proteinase K mix and vortex.
4. Incubate at 56 ± 1°C for 1 – 2 hours.
5. Create a mastermix of 200 ± 1 µL of AL Buffer and 200 ± 1 µL of 200 proof ethanol per sample. AL Buffer may form precipitate, warm up to 56°C to dissolve before use. Add 400 ± 1 µL of AL and ethanol mix to each sample tube. Vortex the tube briefly.
6. Using a micropipette, transfer entire contents of tube to labeled Qiagen spin column. ****If using Vacuum manifold, proceed directly to "Optional Qiagen Vacuum Manifold Processing for Gram-negative Bacteria."**** Otherwise, proceed directly to step 7.
7. Centrifuge column at approximately 6,000 x g for 1 min.
8. Remove column from collection tube. Place column in new collection tube.
9. Add 500 ± 1 µL of buffer AW1 to the column and centrifuge at approximately 6,000 x g for 1 minute.
10. Remove column from collection tube. Place column in new collection tube.
11. Add 500 ± 1 µL of buffer AW2 to the column and centrifuge at max speed (14,000 – 20,000 x g) for 3 minutes.
12. Carefully remove tubes from centrifuge, do not let flow-through contact column. Empty the contents of the flow-through tube then spin the tube again for 2 min at max speed (14,000 – 20,000 x g).
13. Transfer the column to a 1.5 mL tube and add 50 – 100 µL of 10 mM Tris-HCl (pH 8.5) to the column for *Escherichia coli* and *Salmonella*. Add 50 ± 1 µL of 10 mM Tris-HCl (pH 8.5) for *Campylobacter spp*. Incubate at room temperature for 1 minute. Reducing the elution volume can be used to increase the DNA concentration yield.
14. Centrifuge at approximately 6,000 x g for 1 minute. Discard the column and store the DNA appropriately (2 – 8°C for use within 24 h, ≤ -10°C for storage of more than 24 h).

Optional Qiagen Vacuum Manifold Processing for Gram-negative Bacteria
Using a vacuum manifold, if available, may be more efficient for high volume laboratories as it reduces the number of collection tube changes

1. Add new VacConnectors on Vacuum Manifold for each sample, then place column on VacConnectors. Allow vacuum to drain the contents from the column.
2. Add 500 ± 1 µL of buffer AW1 to the column and allow to drain.
3. 500 ± 1 µL of buffer AW2 to the column and allow to drain.
4. Carefully remove column and place in collection tube. Centrifuge for 2 min at max speed (14,000 – 20,000 x g).
5. Transfer the column to a 1.5 mL tube and add 50 – 100 µL of 10 mM Tris-HCl (pH 8.5) to the column for *Escherichia coli* and *Salmonella*. Add 50 ± 1 µL of 10 mM Tris-HCl (pH 8.5) for *Campylobacter spp*. Incubate at room temperature for 1 minute. Reducing the elution volume can be used to increase the DNA concentration yield.

6. Centrifuge at approximately 6,000 x g for 1 minute. Discard the column and store the DNA appropriately (2 – 8°C for use within 24 h, ≤ -10°C for storage of more than 24 h).

Quantification of dsDNA Using the Qubit Fluorometer

1. Sample prep**:** Label the lids of 0.5 mL PCR tubes needed for the number of samples and the two standards.
2. Prepare the Qubit working solution by diluting the dsDNA BR reagent 1:200 in dsDNA BR buffer.
3. For the standards, add 190 ± 1 µL of working solution and 10 ± 1 µL of each Qubit standard and vortex. For each sample, add 190 – 199 µL of working solution and 1 – 10 µL of extracted DNA to bring to 200 µL total volume and vortex. Check each tube for air bubbles and centrifuge if necessary.
4. While protecting from light, allow all samples and standards to incubate for at least two minutes at room temperature and read within 1 hour.
5. Calibrate Qubit: On the home screen, press **DNA** then **dsDNA Broad Range** as assay type. On the Standards Screen, select to run new calibration. To run new, insert Standard #1 in Qubit and close the lid and press Read. Do the same for Standard #2. Standards are used with each use of the fluorometer to provide a concentration of the samples relative to the two standards. Standard 2 must be at least 10x greater than Standard 1. The effectiveness of the kits should be evaluated on an ongoing basis. If a kit is deemed to provide out-of-spec results that affect library preparation, a new kit should be obtained.
6. At Sample Screen, insert your sample tube into the Qubit and press **Read**. Remove sample and insert next tube and press **Read Next Sample.** Repeat for all samples. The concentration for each sample is used for calculating dilution to 0.2 ng/µL in the next section.
7. For isolates using the Illumina DNA Prep, repeat the DNA extraction process for any isolates that do not achieve an initial DNA concentration of ≥ 4.0 ng/µL.
   For isolates using the Nextera XT Library Prep Kit PulseNet, repeat the DNA extraction process for any isolates that do not achieve an initial DNA concentration of ≥ 2.0 ng/µL.
8. Multiple isolates or organisms can be processed, and later combined into a pool for sequencing. Ensure that the number of isolates in the pool is appropriate based on the Illumina sequencing kit used. The sum of the genome sizes (Table 3) should not exceed the DNA load allowance listed in Table 4. Examples are listed in Table 5.

Table 3: Estimated Genome Size per Organism

Organism	***Estimated Genome Size***
Escherichia coli	6 Mb
Salmonella spp.	5 Mb
Listeria monocytogenes	3 Mb
Campylobacter spp.	1.8 Mb

Table 4: Recommended DNA Load Capacity for Illumina Sequencing Kits

Illumina Sequencing Kit	*Total DNA Load (Illumina DNA Prep)*	*Total DNA Load (Nextera XT)*
MiSeq v2, 300c	80 – 90 Mb	40 – 60 Mb
MiSeq v2 500c		60 – 80 Mb
Nano v2 300c	13 Mb	
Micro v2, 300c	25 – 35 Mb	

Table 5: Examples of Proper Pools

Pool	*Isolates*	*Number of Isolates Pool*	*Individual genome size*	*Total DNA in Pool*	*Illumina Sequencing Kit*	*Illumina Library Prep Kit*
1	*Salmonella spp.*	18	5 Mb	90 Mb	MiSeq v2, 300c	DNA Prep
2	*Campylobacter spp.*	50	1.8 Mb	90 Mb	MiSeq v2, 300c	DNA Prep
3	*E. coli*	16	6 Mb	90 Mb	MiSeq v2, 300c	DNA Prep
4	*Campylobacter spp.*	7	1.8 Mb	12.6 Mb	Nano v2, 300c	DNA Prep
5	*Salmonella spp.*	7	5 Mb	35 Mb	Micro v2, 300c	DNA Prep
6	*Campylobacter spp.*	19	1.8 Mb	34.2 Mb	Micro v2, 300c	DNA Prep
7	*Salmonella spp.*	12	5 Mb	60 Mb	MiSeq v2, 300c	Nextera XT
8	*E. coli*	12	6 Mb	80 Mb	MiSeq, v2 500c	Nextera XT
9	*Campylobacter spp.*	28	1.8 Mb	50.4 Mb	MiSeq v2, 300c	Nextera XT
10	*L. monocytogenes*	20	3 Mb	60 Mb	MiSeq v2, 300c	Nextera XT
11	*Mixed (Salmonella + L. monocytogenes)*	10 (*Salmonella*) 2 (*L.mono*)	5 Mb *(Salmonella)* 3 Mb (*L.mono*)	50 Mb 6 Mb 56Mb	MiSeq v2, 300c	Nextera XT

Library Preparation

DNA Library Preparation for MiSeq Sequencer

FSIS laboratories have the option to use either the Illumina DNA Prep or the alternative, Illumina Nextera XT library prep kit. FSIS laboratories can elect to use the alternative Nextera XT Library Prep Kit PulseNet as outlined below for reasons including: unavailability of Illumina DNA Prep kits, interruption in reagent supply chain, or results comparison.

The initial step in making a DNA library is tagmentation. In this step, isolated DNA is mixed with transposomes (engineered enzyme), and through their enzymatic activity the DNA is cut into short fragments. The tagmented DNA is then used for indexing. Specific sequences are added to the DNA fragments to prepare samples for indexing. These sequences provide specificity to each sample and are recognized in the sequencer. Once these reactions are completed the processed DNA fragments are cleaned to obtain homogenous size fragments and to remove unprocessed DNA, enzymes, and salts. Finally, processed DNA is normalized to a specific concentration and mixed together as a pool.

Illumina DNA Prep Library Kit

The Laboratory Standard Operating Procedure for PulseNet Illumina DNA Prep Library Preparation, PNL35 and the Laboratory Standard Operating Procedure for Whole Genome Sequencing on MiSeq, PNL38 are followed.

The Illumina DNA Prep chemistry uses On-Bead tagmentation: bead-linked transposomes (BLTs) simultaneously fragment DNA and tag sequencing primers.

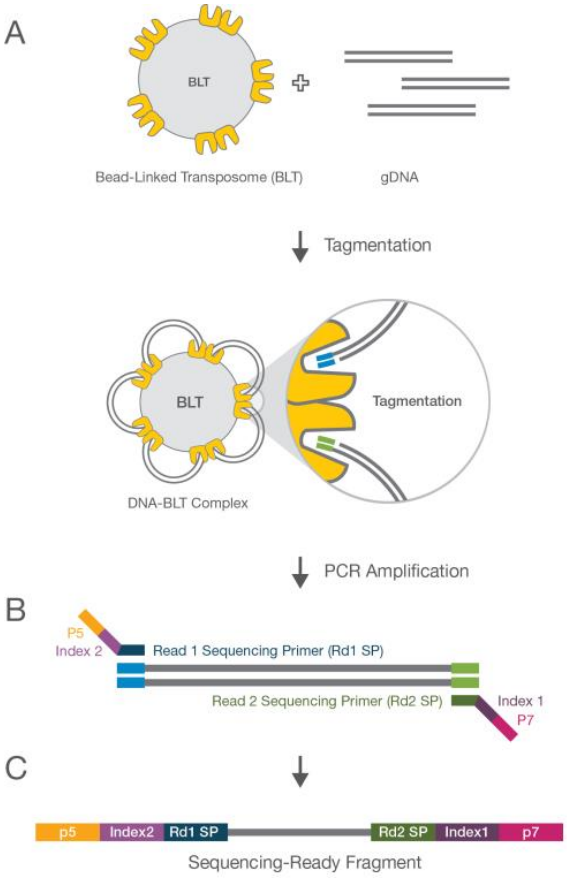


Illustration Credit: Illumina

Alternative Method: Nextera XT Library Prep Kit PulseNet

Selection of Isolates for Sequencing and Dilution of DNA for Tagmentation (0.2 ng/µL).

Using the reading (ng/µL) from the Qubit fluorometer, dilute the extracted DNA to 2 ng/µL in 50 ± 1 µL molecular-grade water. To get the volume of extracted DNA to add to molecular grade water, use the formula C1V1 = C2V2 modified as follows:

$$V1 = \frac{50\ \mu L(\frac{2\ ng}{\mu L})}{Concentration(\frac{ng}{\mu L})}$$

The amount of water = 50 µL – (volume of extracted DNA)

For example: To dilute a 25 ng/µL template:

$V1 = \frac{50\ \mu L(\frac{2\ ng}{\mu L})}{25(\frac{ng}{\mu L})}$ resulting in V1 = 4 µL. The amount of water is 50 µL - 4 µL = 46 µL.

Add 4 µL of DNA template to 46 µL of water to achieve a 2.0 ng/µL concentration.

The resulting 2.0 ng/µL tube can be stored at ≤ -10°C for up to 3 months.

Tagmentation of DNA

1. Add 5 ± 1 µL of the diluted DNA (2 ng/µL) to 45 ± 1 µL of molecular-grade water to dilute the extracted DNA to 0.2 ng/µL. This will serve as the template for the tagmentation reaction.
2. Thaw ATM, NPM, and TD (Nextera XT Library Prep Kit PulseNet) on ice. Store NT at room temperature.
3. Aliquot the following reagents into PCR strip tubes for easier multi-channel pipetting purposes:
    a. ATM (7 ± 1 µL)
    b. NT Buffer (7 ± 1 µL)

NTA Plate (Nextera XT Tagment Amplicon Plate) Preparation (96 Well Plate Format)

4. To each well, add 10 ± 1 µL TD Buffer, 5 ± 1µL diluted DNA (0.2 ng/µL) and 5 ± 1 µL ATM
5. Pipette the mixture (20 ± 1 µL volume) up/down 5x to mix reagents.
6. Seal the plate using Microseal B and centrifuge for 1 ± 0.5 min (approximately 280 x g).
7. Place the plate on the thermocycler and heat at 55 ± 1°C for 5 ± 1 min followed by cooling to 10 ± 1°C. Immediately proceed to step 8.
8. Add 5 ± 1 µL of NT buffer and pipette up/down (quickly multi-channel pipette the NT with each aliquot previously prepared).
9. Seal the plate using Microseal B and centrifuge for 1 min (approximately 280 x g).
10. Hold the plate for 5 ± 1 min at room temperature.

Indexing of Tagmented DNA

Thaw index primers from **Nextera XT Index Kit at room temperature and use immediately or store on ice**.

11. Add 15 ± 1 µL of NPM into NTA plate wells (from tagmentation)
12. Add 5 ± 1 µL of each primer (Index 2 then Index 1) and pipette up/down 5x to mix reagents.
13. Seal the plate with Microseal A and centrifuge for 1 min at approximately 280 x g.
14. Perform PCR on the mixture (50 ± 1 µL total)

Use the following reaction parameters:

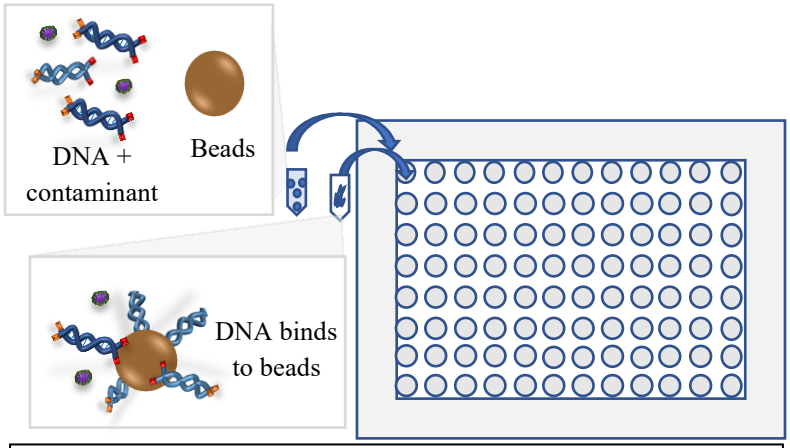
72°C for 3 minutes
95°C for 30 seconds
12 cycles:
95°C for 10 seconds
55°C for 30 seconds
72°C for 30 seconds
72°C for 5 minutes
Hold at 4°C
Optional Stopping point 2 days at 2 – 8°C

DNA Purification

PROCESS STEPS

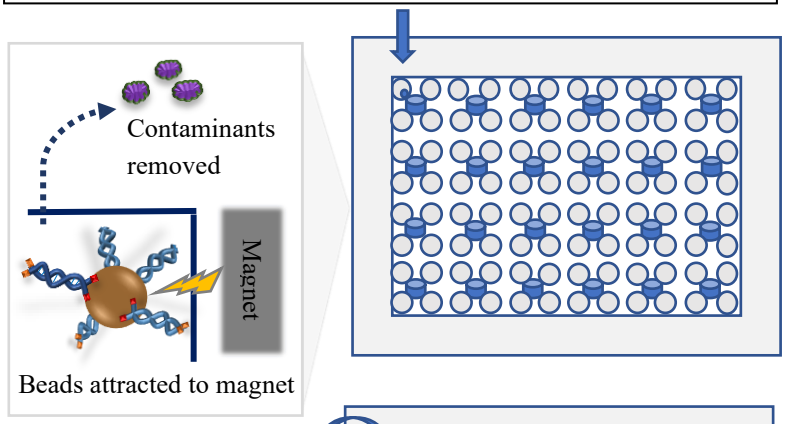
1. Add paramagnetic beads and DNA aliquot to a deep well plate.
2. Allow DNA indexed fragments to bind to beads.
3. Place the deep well plate onto the magnetic stand.
4. Remove the supernatant from each well to clean up DNA fragments.
5. Purify DNA indexed fragments using 80% Ethanol wash. Remove additional supernatant and repeat ethanol wash.
6. Allow washed DNA indexed fragments to air dry to remove any residual liquid.
7. Remove deep well plate from the magnet stand. Elute purified DNA indexed fragments into solution using resuspension buffer.
8. Place deep well plate onto magnetic stand again to attract paramagnetic beads.
9. Transfer supernatant containing purified DNA indexed fragments into to a tube or a new deep well plate. The samples are now ready to be quantified for WGS library pooling.

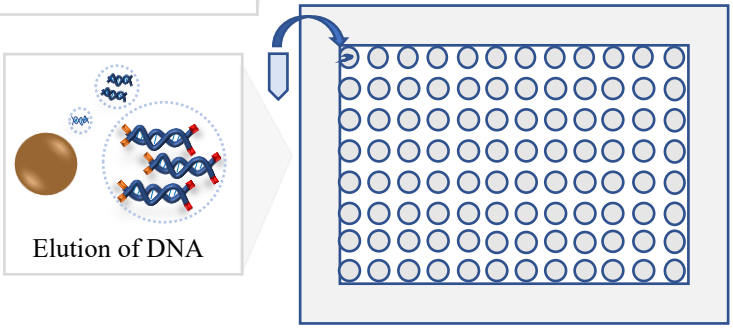
Steps 1-2



Magnets will attract paramagnetic beads bound to the DNA, leaving contaminants behind in the supernatant. Avoid disrupting beads during ethanol washes and allow beads to air dry while remaining on the magnet stand.

Steps 3-6





Magnets will pull beads out of suspension, leaving behind purified DNA in the supernatant.

Step 8-9

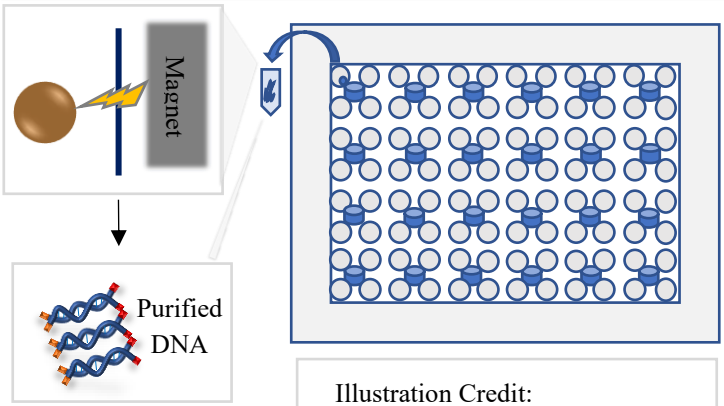


Illustration Credit:
Christian Rivera-Gonzalez

Cleanup of Indexed Fragments

Beads have to be vortexed periodically to remain in solution.

1. Thaw RSB from Nextera XT Library Prep Kit on ice (takes approximately 1 hour). Warm Ampure beads to room temperature (already aliquoted into 500 – 1000 µL aliquots) and make 80% ethanol wash solution at time of use according to chart.
2. Vortex Ampure beads for approximately 30 sec. Using wide-orifice tips, add 20 ± 1 µL of Ampure beads to 40 ± 1 µL of volume from the NTA plate into a deep well plate.
3. ***Using wide orifice pipette tips to avoid clogging and ensure mixing***, pipette up and down 10x to mix reagents and let stand at room temperature for at least 5 minutes. Increasing incubation time can increase quality and yield.
4. Place the plate on the Ambion Magnetic Stand-96 for 2 ± 1 minutes.
5. Remove the supernatants using a multi-channel pipette.
6. To each well, add 190 ± 10 µL 80% Ethanol with plate on the Ambion Magnetic Stand-96. Incubate for 30 ± 1 second, remove supernatant.
7. Repeat 80% ethanol wash.
8. Using a 20 µL pipette tip, extract any residual liquid and *dry approximately 10 – 11 minutes*. Exceeding the maximum air-dry period (11 minutes) can make resuspension difficult and is not recommended. Over-drying is indicated by cracks in the bead pellets.
9. Remove the plate from the magnet and add 45 ± 2 µL RSB. Mix with multi-channel pipette 10x using wide-orifice tips or shaker 2 ± 1 minutes. Incubate for 2 ± 1 minutes at room temperature.
10. Place the plate on the Ambion Magnetic Stand-96 for 2 ± 0.5 minutes.
11. Pipette 40 ± 1 µL of AMpure-cleaned supernatant into a new PCR plate labeled **CAN** (clean amplified NTA plate) and seal with Microseal B film or pipette into Eppendorf LoBind 1.5 mL tubes and label as CAN.

Optional Stopping Point *≤ -10℃ for up to 3 months*

Ethanol Wash Dilution Calculations (80%)

# samples	Total 80% needed (*µL*)	100% EtOH (*µL*)	H_2O (*µL*)
9	3600	2880	720
10	4000	3200	800
11	4400	3520	880
12	4800	3840	960
13	5200	4160	1040
14	5600	4480	1120
15	6000	4800	1200
16	6400	5120	1280
17	6800	5440	1360
18	7200	5760	1440
19	7600	6080	1520
20	8000	6400	1600
21	8400	6720	1680
22	8800	7040	1760
23	9200	7360	1840
24	9600	7680	1920
25	10000	8000	2000
26	10400	8320	2080
27	10800	8640	2160
28	11200	8960	2240
29	11600	9280	2320
30	12000	9600	2400
31	12400	9920	2480
32	12800	10240	2560
33	13200	10560	2640
34	13600	10880	2720
35	14000	11200	2800
36	14400	11520	2880
37	14800	11840	2960
38	15200	12160	3040
39	15600	12480	3120
40	16000	12800	3200
41	16400	13120	3280
42	16800	13440	3360
43	17200	13760	3440
44	17600	14080	3520
45	18000	14400	3600
46	18400	14720	3680
47	18800	15040	3760
48	19200	15360	3840
49	19600	15680	3920

QUALITY CONTROL

PhiX Quality Assurance Considerations

DNA from the bacteriophage PhiX, provided by Illumina, is well balanced in nucleotide composition and serves as an overall MiSeq® run performance control and helps provide important run information during troubleshooting. After a run, analysts may evaluate the effectiveness of PhiX addition by viewing the aligned reads percentage in Sequence Analysis Viewer (SAV). The percentage of aligned reads should correlate to the percent PhiX added during pooling (1%). The percentage of reads should be roughly 1 ± 0.75% in a typical MiSeq® run.

PhiX Control Stepwise Dilutions

Quantify post-cleaning DNA (CAN) using Qubit dsDNA High Sensitivity (HS) kit as follows:

1. Add 2 ± 1 µL 10 nM PhiX (stock concentration) to 3 ± 1 µL 10 mM Tris-HCl with 0.1% Tween 20 (pH 8.5) to bring concentration to 4 nM. Add 5 ± 1 µL 0.2 N NaOH (see Library Pooling and Denaturation for preparation), vortex to bring concentration to 2 nM.
2. Centrifuge briefly and incubate for 5 ± 1 min at Room Temperature
3. Add 990 ± 1 µL Pre-chilled HT1 to bring concentration to 20 pM.
4. Vortex and leave on ice.

The 20 pM dilution can be stored at ≤ -10°C and used for 3 weeks.

KEY DEFINITIONS

Sequence Analysis Viewer (SAV): Illumina provided application that allows users to view selected metrics after run completion

Library Pooling and Denaturation

1. Quantify post-cleaning DNA (CAN) using Qubit dsDNA High Sensitivity (HS) kit as follows:
   a) Sample Prep: Label the lids of the Qubit assay tubes needed for numbers of samples and the two standards.
   b) Prepare the Qubit working solution by diluting dsDNA HS reagent 1:200 in dsDNA HS buffer (1 µL of reagent into 199 µL buffer) or use the commercially available HS working solution.
   c) For the standards, add 190 ± 1 µL of working solution and 10 µL of each Qubit standard and vortex. For the samples, add 190 – 199 µL of working solution and 1 – 10 µL of DNA template to bring to 200 µL total volume and vortex. Incubate all samples and standards for a minimum of two minutes at room temperature (protected from light)
   d) Reading the samples: On the Qubit home screen, press **DNA** then **dsDNA High Sensitivity** as assay type. On the Standards Screen, select to run new calibration. To run new, insert Standard #1 in Qubit and close the lid and press Read. Do the same for Standard #2. Standards are run to provide a concentration of the samples relative to the two standards. Standard 2 must be at least 10x greater than Standard 1. The effectiveness of the kits should be evaluated on an ongoing basis. If a kit is deemed to provide out-of-spec results that affect library preparation and sequence quality and other metrics, a new kit should be obtained.
   e) At Sample Screen, insert your sample tube into the Qubit and press **Read**. Remove sample and insert next tube and press **Read Next Sample.** Repeat for all samples. Post-cleaning DNA concentration shall be ≥ 1.33 ng/µL. If the reading(s) does not meet this criteria, a new library must be performed for that sample.

   If a sample will be excluded from a run, then the sample sheet needs to be edited before loading.

2. Pre-heat a heat block to 96 ± 1°C.
3. Thaw cartridge in room temperature water (to the maximum line) and then place on ice or in refrigerator (2 – 8°C). Alternatively, the cartridge may be thawed in the refrigerator for 8 – 24 hours.
4. Thaw HT1 buffer (found in cartridge container) and keep on ice. Calculate the sample volume for a 4 nM library (or 3 nM or 2 nM only if yield was too low for 4 nM). The conversion factor for ng/µL to nM shall be 1.5. Dilute template in 10 mM Tris-HCl with 0.1% Tween 20 (pH 8.5) to desired volume (e.g. 50 µL). *With lower post-cleaning concentrations, more sample volume from CAN step is used, limiting the need for dilution to get the optimal library concentration.* Pool the needed volume from each sample library into one tube for a minimum volume of 50 µL.
5. Prepare fresh 0.2 N NaOH from 1 N stock concentration each day of use (for example, add 200 µL of PCR water to 50 µL 1N NaOH) to denature the pooled library. 1 N NaOH should be kept frozen (≤ -10°C) in aliquots (e.g. 50 µL) in tubes for ease of use. Discard diluted NaOH after usage.
6. Combine 5 ± 1 µL of 0.2 N NaOH and 5 ± 1 µL of the pooled library.
7. Vortex gently and centrifuge. Incubate for 5 ± 1 min at Room Temperature
8. Add 990 ± 1 µL of pre-chilled HT1 vortex and store on ice.
9. Dilute library using Table 6.

Table 6: Library calculations

	2 nM (10 pM DNA)		**3 nM** (15 pM DNA)		**4 nM** (20 pM DNA)	
Final Loading concentration	***Denatured DNA***	***HT1***	***Denatured DNA***	***HT1***	***Denatured DNA***	***HT1***
7 pM	420 µL	180 µL	280 µL	320 µL	210 µL	390 µL
8 pM	480 µL	120 µL	320 µL	280 µL	240 µL	360 µL
9 pM	540 µL	60 µL	360 µL	240 µL	270 µL	330 µL
10 pM	600 µL	0 µL	400 µL	200 µL	300 µL	300 µL
11 pM			440 µL	160 µL	330 µL	270 µL
12 pM			480 µL	120 µL	360 µL	240 µL
13 pM			520 µL	80 µL	390 µL	210 µL
14 pM			560 µL	40 µL	420 µL	180 µL
15 pM			600 µL	0 µL	450 µL	150 µL
16 pM					480 µL	120 µL
17 pM					510 µL	90 µL
18 pM					540 µL	60 µL
19 pM					570 µL	30 µL
20 pM					600 µL	0 µL

10. Add 3.75 ± 1 µL of diluted PhiX control (20 pM) to 600 ± 1 µL (1%) of denatured and diluted sample library. Please see Quality Control: PhiX Control Stepwise Dilutions for diluted PhiX control preparation instructions.
11. Incubate PhiX/library mixture at 96 ± 1°C for 2 ± 1 minutes.
12. Keep tube on ice for at least 5 ± 1 minutes until ready to load into the cartridge. PhiX/library mixture may be stored up to three weeks at -15°C to -25°C.

    MiSeq instruments contain the MiSeq Control Software program for operation. The program provides step-by-step instructions to operate the instrument and load the cartridge. Please refer to the Illumina MiSeq® System Guide for additional details.

13. Shut down and restart the MiSeq instrument before loading as per manufacturer instructions defined in Illumina MiSeq System User Guide.
    a. From the Home screen, select **Manage Instrument**.
    b. Select **Shut Down**. This command shuts down the software
    c. Toggle the power switch to the OFF position.

    Any time that the instrument is switched off, wait a ***minimum*** of 60 seconds before turning the power switch back to the ON position to prevent instrument damage.

Loading the Pooled Library onto the MiSeq®

14. Mix the solutions in the cartridge by inverting 5 – 10 times after all the ice is gone before loading the library.
    a. Tap on bench to settle components (not on the main bench if a machine is running).
    b. Pre-pierce the foil in the sample hole with pipette tip.

c. Add your sample (approximately 600 µL) to the cartridge and tap gently to settle the liquid to bottom of the well.
d. Prepare flow cell by cleaning off buffer with Milli-Q water and dry with a KimWipe tissue. Wipe with lens cleaning tissue to ensure no lint is left on the flowcell. If there is residue on the glass, wipe down the flow cell with 80 % ethanol (avoid the gasket) and ensure no liquid is visible on the flow cell before loading into the machine.
e. Empty the waste container if needed.
f. Invert PR2 bottle a few times to mix. Discard PR2 cap to prevent confusion with the wash lids.
g. Follow the on-screen prompts to start the run. This includes loading the following: (a) Flow cell (b) PR2 bottle (c) Sample sheet selection (d) Cartridge with Pooled Library loaded
h. Load and make sure pre-run check is satisfactory, then proceed with sequencing.

MiSeq Post-Run Steps

Monitoring the Progress of the MiSeq Run

The onboard diagnostics of the MiSeq should provide several indications that the run has successfully begun. The cluster density should populate around Cycle 10 and should ideally be in the range of 600 – 1300 k/mm^2 for Version 2 chemistry. Pass filter and Q30 percentage should begin to populate on the screen during the run. Pass filter should ideally be ≥ 80% and Q30 should be ≥ 80%.

These metrics apply to the entire run and failure to meet these metrics does not necessarily indicate that individual samples on the run will fail to meet quality control metrics. One may choose to accept individual sequences instead of an entire run, based on the sample acceptance Quality Metrics Criteria.

Post-run Wash

1. To perform the Post-run wash, fill each of the wells of the wash cartridge with 6 ± 1 mL of 0.5% Tween 20. The remainder of the 0.5% Tween 20 (approximately 350 mL) will be used as the wash bottle for post-run wash.
2. After the run, discard the waste according to state and federal regulations.
3. Pour the remaining PR2 into the sink and triple rinse the bottle before disposing of it following state and federal regulations.
4. Place the wash cartridge with 0.5% Tween 20 solution and Tween 20 bottle into the MiSeq® and do post-run wash by following on-screen prompts. The flow cell stays in place and is not removed until the next MiSeq® run.
5. Discard used flow cells in sharps bins.

Post-Run Template Line Wash

1. Prepare fresh sodium hypochlorite solution (NaOCl) each day of use by adding approximately 36 µL of 5% NaOCl to approximately 864 µL of laboratory-grade water. This will result in a 1:25 NaOCl dilution.

2. Add approximately 50 µL of the 1:25 NaOCl dilution to approximately 950 µL of laboratory-grade water in a MiSeq tube and place the tube containing the 0.01% NaOCl into position 17 on the wash cartridge.
3. Fill the remaining wells on the wash cartridge with with 6 ± 1 mL of 0.5% Tween 20, and with the remainder of the 0.5% Tween 20 (approximately 350 mL) will be used as the wash bottle for template line wash.
4. When the run is complete, select **perform optional template line wash** on the post-run screen. This will prompt the MiSeq for the bleach wash.
5. After the run, remove the waste according to state and federal regulations.
6. Dispose remaining PR2 per state and federal regulations.
7. Place the wash cartridge with 0.5% Tween 20 and 0.01% NaOCl solution and Tween 20 bottle into the MiSeq® and do post-run wash by following on-screen prompts. The flow cell stays in place and is not removed until the next MiSeq® run.
8. Discard used flow cells in sharps bins.

Bioinformatics

Calculate nucleotide balance

For each FASTQ file determine the total number of each nucleotide (A, T, C, G). Next determine the ratio of A to T and C to G using the following equations: (total #of As)/(total # of Ts) and (total #of Cs)/(total # of Gs). For each sample there should be 4 nucleotide balance values an A:T and C:G for the read 1 FASTQ and the read 2 FASTQ.

Calculate average quality

For each FASTQ file, every 4th line is converted to its corresponding decimal value from the native ASCII value. Next 33 is subtracted from each decimal value to account for the proper offset. The resulting values are then summed and divided by the total number of base pairs to obtain the average quality score.

Calculate coverage

For each FASTQ file, the total number of reads is multiplied by read length and divided by genome size in base pairs. The following genome sizes will be used for calculation of coverage:

Salmonella	5 Mbp
E.coli	6 Mbp
Listeria monocytogenes	3 Mbp
Campylobacter	1.8 Mbp

KEY DEFINITIONS

FASTQ: raw data file format containing reads and quality metrics used for bioinformatics analysis

Coverage: the average number of times that each base in a genome is sequenced given a number of reads, read length, and the assumption that the reads are randomly distributed. The average number of reads used to generate a contig/genome

Perform Assembly

De novo assembly is performed using available assembly algorithms from the manufacturer.

Assess assembly size

Assembly size is estimated based on the FASTA file size in which 1 MB is approximately 1,000,000 base pairs.

KEY DEFINITIONS

De novo Assembly: A reconstruction of original genome sequence from reads using software based on aligning possible overlaps

NCBI: National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/)

Phylogenetic Tree: A diagram that shows the evolutionary relationships among a group of organisms based on their genetic characteristics.

SNP: Single Nucleotide Polymorphism: differences between the nucleotides at the same genomic position of two or more isolates

Quality Metrics Criteria

Quality metrics are subject to change based on unique analysis needs and/or federal partners' recommendations and minimum requirements. The current quality criteria used are based on "PulseNet Standard Operating Procedure for Illumina MiSeq Data Quality Control" (https://www.cdc.gov/pulsenet/pathogens/wgs.html)

Uploading Sequences to NCBI and Further Analysis

If sequences meet or exceed the recommended quality metrics, the sequences and corresponding metadata will be uploaded to NCBI.

NCBI BioSample
Limited isolate metadata (isolate identifier, organism, isolation source, collection year, collection state) is submitted to the NCBI BioSample Database which assigns a unique identifier (BioSample Accession/SAMN#). Once BioSample information is published it is publicly available (https://www.ncbi.nlm.nih.gov/biosample/).

NCBI Sequence Read Archive
FASTQ files are uploaded to the NCBI Sequence Read Archive (SRA) Database which assigns a unique identifier (SRA Accession/SRR#). The FASTQ files are converted to the SRA format and are linked to the unique BioSample identifier for the isolate and published to the SRA database. Once SRA information is published, raw reads can be publicly downloaded (https://www.ncbi.nlm.nih.gov/sra/).

Once sequences are uploaded to NCBI, NCBI performs phenotypic predictions (e.g. antimicrobial resistance, stress, and virulence) and clusters genetically similar isolates into phylogenetic trees based on SNPs.

Biosafety Chart

Safety Information and Precautions

1. Required protective equipment: Nitrile or latex gloves, lab coat, and safety glasses
2. Hazards

Procedure Step	*Hazard*	*Recommended Safety Procedures*
DNA Isolation- Extraction of *Salmonella*	If ingested, *Salmonella* causes gastrointestinal illness, but can also infect the urine, blood, bones, joints, or the nervous system.	Follow CDC guidelines for manipulating Biosafety Level 2 (BSL-2) pathogens.
DNA Isolation- Extraction of Shiga Toxin-Producing *Escherichia coli* (STEC)	If ingested, STEC infections can cause severe illness or death. A portion of STEC infections will develop hemolytic uremic syndrome (HUS).	Follow CDC guidelines for manipulating Biosafety Level 2 (BSL-2) pathogens.
DNA Isolation- Extraction of *Listeria Monocytogenes*- Risk to Pregnant and/or Immunocompromised Individuals	Pregnant women are more susceptible to get listeriosis than other healthy adults. Listeriosis may cause miscarriage, premature labor, birth defects, or infant death. Listeriosis can also lead to severe illness or death of the pregnant individual. Listeriosis can cause severe illness or death in immunocompromised individuals.	Follow CDC guidelines for manipulating Biosafety Level 2 (BSL-2) pathogens. Pregnant women and immunocompromised individuals should avoid working with *Listeria monocytogenes* at all. Laboratories should inform pregnant women and immunocompromised individuals of the risks of working with *Listeria monocytogenes*. Reasonable accommodations should be made to ensure the safety of individuals in these health groups.
DNA Isolation- Extraction of *Campylobacter*	If ingested, *Campylobacter* can cause gastrointestinal disease that may result in life-threatening infections.	Follow CDC guidelines for manipulating Biosafety Level 2 (BSL-2) pathogens.

DNA Isolation- Extraction of bacterial cultures with ethanol DNA Purification and Cleanup of Indexed Fragments- use of ethanol wash solution	Ethanol is acutely toxic by oral, dermal, or inhalation means. Highly flammable.	Wear gloves when using ethanol and avoid breathing the fumes directly. Avoid handling ethanol around heat or open flame.
DNA Isolation- Preparation of the enzymatic lysis buffer for Gram positive bacteria uses Ethylenediaminetetraacetic Acid (EDTA)	EDTA may cause damage to respiratory tract organs through prolonged and repeated exposure.	Avoid breathing the fumes directly. A mask may be worn to decrease exposure to EDTA.
DNA Isolation- Preparation of the enzymatic lysis buffer for Gram positive bacteria uses Triton X-100	Triton X-100 causes skin irritation, serious eye damage, and is harmful if swallowed.	Wear gloves and eye protection when handling. Wash skin thoroughly after use.
DNA Isolation- Preparation and use of lysozyme stock solution for Gram positive bacteria uses Lysozyme	Lysozyme may cause allergy or asthma symptoms or breathing difficulties if inhaled.	Avoid breathing the fumes directly. A mask may be worn to decrease exposure to Lysozyme.

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Contact Information and Inquiries

Inquiries about methods can be submitted through the USDA website via the "Ask USDA" portal at https://ask.usda.gov or please contact:

Microbiology Section
Laboratory Quality Assurance, Response, and Coordination Staff
USDA/FSIS/OPHS
950 College Station Road
Athens, GA 30605
OPHS.LQAD@usda.gov.

This method has been validated, reviewed, approved, and deemed suitable and fit for purpose for use in the USDA FSIS Field Service Laboratories.

William K. Shaw, Jr., PhD
Executive Associate for Laboratory Services