

Version 2 ▼

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© Illumina DNA Prep (M) Tagmentation Library Preparation for use on an Illumina MiSeq Sequencer V.2

Julie Haendiges¹, Narjol Gonzalez-Escalona¹, Ruth E Timme¹, Maria Balkey¹

¹US Food and Drug Administration

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GenomeTrakr
Tech. support email: genomeTrakr@fda.hhs.gov

Julie Haendiges
US Food and Drug Administration

ABSTRACT

This procedure outlines the protocol for whole genome sequencing of bacterial organisms using the Illumina DNA Prep library preparation kit for sequencing on an Illumina MiSeq sequencer.

This document applies to all laboratory personnel in the Division of Microbiology (DM) as well as laboratories in the GenomeTrakr Network.

Complete in order:

- 1. DNA Extraction (Manual DNA Extraction or Automated DNA Extraction using the Qiacube)
- Step-by-step procedures to obtain high quality DNA from isolates in TSB for whole genome sequencing

2. DNA Quantitation

- Quantitation of extracted DNA using the Qubit Flourometer
- 3. Library Preparation for WGS (Included SOP or Library Preparation using Illumina Nextera XT)
- Library preparation using NexteraXT or Illumina DNA Prep (previously Nextera DNA Flex)
- 4. Sequencing using Illumina MiSeq
- 5. Data Quality Checks and NCBI Submission

DOI

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Version created by Julie Haendiges

WHAT'S NEW

Updated genome loading recommendations for sequencing cartridges and pooling volumes to harmonize with CDC PulseNet

KEYWORDS

Illumina DNA Prep, WGS Library Preparation, GenomeTrakr, Whole Genome Sequencing

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GUIDELINES

Illumina DNA Prep (M) Tagmentation Kit contain 3 components:

Box 1 of 3:

- SPB (Store at 2-8°C)
- TSB (Store at room temperature)
- TWB (Store at room temperature)

Box 2 of 3: (Store at -25 to -15°C)

- RSB
- TB1
- EPM

Box 3 of 3: (Store at 2-8°C)

BLT

Abbreviations:

BLT: Bead-Linked Transposome dsDNA: Double-Stranded DNA
EPM: Enhanced PCR Mix
HT1: Hybridization Buffer
PCR: Polymerase Chain Reaction
PR2: Incorporation Buffer
RSB: Resuspension Buffer
SPB: Sample Purification Beads
TB1: Tagmentation Buffer 1
TSB: Tagment Stop Buffer
TWB: Tagment Wash Buffer

MATERIALS TEXT

MATERIALS

⊠ Ethanol (100%, Molecular Biology Grade) Fisher

Scientific Catalog #BP2818500

Technologies Catalog #Q32851

Inc. Catalog #20018708

Sodium Hydroxide 1N Sigma

Aldrich Catalog #S2770-100ml

Molecular grade water nuclease-free Contributed by users

⊠ Illumina DNA Prep (M) Tagmentation (96 Samples) Illumina,

Inc. Catalog #20018705

⊠ Illumina DNA Prep (M) Tagmentation (24 Samples) Illumina,

Inc. Catalog #20018704

⊠IDT for Illumina - DNA/RNA UD Indexes Set A (96 Samples) Illumina,

Inc. Catalog #20027213

Inc. Catalog #20027214

⊠IDT for Illumina - DNA/RNA UD Indexes Set C (96 Samples) Illumina,

Inc. Catalog #20027215

⊠IDT for Illumina - DNA/RNA UD Indexes Set D (96 Samples) Illumina,

Inc. Catalog #20027216

Supplies:

- Qubit Assay Tubes (Thermofisher cat# Q32856)
- Pipette Tips, sterile, filtered (assorted volumes)
- Conical Tubes, 10ml and/or 15ml (FisherSci cat# 14-959-53A or equivalent)
- Solution basins, sterile (FisherSci cat# 13-681-504 or equivalent)
- 96-well PCR Plates, semi-skirted, flat deck (FisherSci cat# AB-1400L or equivalent)
- Microcentrifuge tubes, 1.5 ml, sterile (Thermofisher cat# AM12400 or equivalent)
- Plate Seals (FisherSci cat# AB-0558 or equivalent)

Equipment:

- Qubit 2.0 or 3.0 Fluorometer
- Thermocycler
- Microplate centrifuge
- Vortex
- Magnetic Stand-96 (Thermofisher cat# AM10027) (If possible, have two; one for pre-PCR and one for post-PCR)
- Micropipettes (Single and Multichannel)
- Ice bucket
- Microcentrifuge

SAFETY WARNINGS

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

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

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

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

EPM: GHS Category 4 for acute oral toxicity and Category 1 for specific organ toxicity. Contains tetramethylammonium chloride

BEFORE STARTING

Preparation of the Sequencing Workbook: The worksheet is an excel file that can be found in Appendix 1

Prepare the *Initial Dilution* tab of the DNA Flex Library Prep Workbook as described below:

Note: The workbook is designed with the following color scheme:

- · White fields should be filled in
- · Blue fields contain formulas, which will auto-populate, and should not be altered
- 1. Enter sample IDs for all isolates in column B.
- 2. Enter the organism in column C.
- 3. Enter the genome size in column D based on the table below:

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

4. Confirm that the number of isolates on the run is appropriate for the capacity of the reagent kit to be used. The sum of the genome sizes (Mb) for all samples on the run will be displayed in the workbook. This should not exceed the recommended DNA load values for the reagent kit.

MiSeq - V2 500 cycle: 100 Mb

V3 600 cycle: 160 Mb

Micro 300 cycle: 30 Mb

- 5. Enter the Qubit concentration of the gDNA in column E.
- 6. Determine which index kit will be used and select from the drop-down menu in column I. Select the index well positions in column J.
- 7. Enter the volume of extracted DNA to be used for library preparation in column F. GenomeTrakr has standardized the starting volume of DNA to 5 μ l (gDNA concentration of 20 50 ng/ μ l), however, the recommended quantity of input DNA is 100-500 ng. Our recommendation is to use at least 100 ng of input DNA. Individual laboratories may adjust input DNA volumes to ensure quantities fall within this range. The recommended minimum volume of input DNA is 2 μ l, if DNA is too concentrated, perform a dilution to bring input DNA volume above 2 μ l and proceed.

Dilute and Tagment Input DNA

1 Bring BLT (stored in refrigerator) and TB1 (stored in freezer) to room temperature.

Ensure that BLT is stored upright at all times, so that the beads remain submerged in the buffer

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- 2 Label a 96-well PCR plate with the Run ID.
- 3 Add molecular-grade water to the each sample well (from Column K of the workbook)
- 4 Add gDNA to the molecular-grade water (per volume in Column J) and mix well by gently pipetting 5 10 times.
- 5 Vortex BLT vigorously for 10 seconds, visually check the beads for complete resuspension and repeat vortexing if necessary.

Do not spin down the BLT tube, the beads must be resuspended

- 6 Vortex the TB1 and spin down the tube.
- 7 ***Scale up this step according to the number of reactions plus 3-4 for dead space volume/error***

Prepare the Tagmentation Master Mix:

Combine **□10** µl of TB1 with **□10** µl of BLT

Reagent	Volume per Sample			
TB1	10 μ1			
BLT	10 μ1			

Reagent volumes per sample for tagmentation master mix

Note: The 96 sample kit comes with 4 tubes of each reagent, each tube contains enough reagents for 24 samples

- 8 Vortex the tagmentation master thoroughly to make sure the BLT beads are evenly resuspended in the buffer.
- 9 Using fresh tips, transfer $\mathbf{20} \, \mu \mathbf{l}$ of tagmentation master mix to each sample well.

Note: The master mix can be added to a reagent basin and distributed using a multichannel pipet

- 10 Pipette up and down 10 times mix the 50 μ l reaction to resuspend the beads.
- 11 Apply an adhesive PCR plate seal to the plate.

12 Place the plate into the thermocyler and run the tagmentation program. Program thermocyler to incubate at & 55 °C for © 00:15:00 followed by a & 10 °C hold with the lid heated at 8 100 °C 13 Check TSB for precipitate (if present, warm at 37 oC for up to 10 minutes and vortex) and ensure it is at room temperature prior to use. Upon completion of the incubation, remove the plate from the thermocycler. Proceed to the Post Tagmentation Cleanup step. Post Tagmentation Clean Up 15 Remove the plate seal. Add 10 µl of TSB to each sample (A multi-channel pipette can be used) Gently pipette up and down 10 times to mix and fully resuspend the beads in the 50 μ l reaction. Apply an adhesive PCR plate seal to the plate. 17 18 Place the plate into the thermocyler and incubate at § 37 °C for © 00:15:00 followed by a § 10 °C hold with the lid heated at § 100 °C While samples are incubating, thaw EPM (stored in freezer) on ice and thaw indices at room temperature. 20 Remove the plate from the thermocycler, quick spin the plate and remove the seal. 21 Place the plate on a magnet for \bigcirc **00:03:00** or until solution is clear. Note: The DNA is tagged with adapters and bound to the beads. 22 Using a multichannel pipette, remove the supernatant and discard. 23 Remove the plate from the magnet and add 100 µl of TWB directly to the pellet. Gently pipette to mix until beads are fully resuspended, try to avoid creation of foam from TWB.

- 24 Place the plate on the magnet for \bigcirc **00:03:00** or until solution is clear.
- 25 Remove the supernatant and discard.
- 26 Remove the plate from the magnet and add **100 μl** of TWB directly to the pellet. Gently pipette to mix until beads are fully resuspended.
- 27 Place the plate on the magnet for **© 00:03:00** or until solution is clear.
- 28 Remove the supernatant and discard.
- 29 Remove the plate from the magnet and add **100 μl** of TWB directly to the pellet. Gently pipette to mix until beads are fully resuspended.
- Place the plate with TWB on the magnet and allow to incubate until ready to proceed with adding the PCR master mix in the Amplify Tagmented DNA step. The plate should incubate for at least 3 minutes. It is important to keep the pellet in TWB to prevent overdrying of the beads.

Amplification and Index Addition of Tagmented DNA

- 31 Invert the EPM to mix, then briefly centrifuge.
- 32 Briefly centrifuge the Index plate.
- 33 ***Scale up this step according to the number of reactions plus 3-4 for dead space volume/error***

Prepare the PCR master mix:

Combine 20μ of EPM with 20μ of Molecular grade water

Reagent	Volume per Sample			
EPM	20 μ1			
Molecular grade water	20 μ1			

Reagent volumes per sample for PCR master mix

Note: The 96 sample kit comes with 4 tubes of each reagent, each tube contains enough reagents for 24 samples

- 34 Vortex and spin down the PCR master mix.
- Remove the third TWB wash from the samples while on the magnet. Remove any excess liquid from the plate using a small volume pipette.

Note: Removal of TWB is crucial, as it can impede PCR. Any foam remaining on the wells will not negatively impact the library.

- 36 Remove the plate from the magnet and immediately proceed to adding the master mix.
- 37 Add 340 µl of PCR master mix to each sample well. Gently pipette to mix to ensure beads are resuspended.

Note: The master mix can be added to a reagent basin and distributed using a multichannel pipet

- Add 10 μl of the index primer pair from the appropriate index wells in accordance with the sample sheet. The plate has a foil seal on it, P20 tips are sufficient to pierce the seal to pipette. The indexes are single-use only.
- 39 Use a pipette to gently mix a minimum of 10 times to ensure thorough mixing.
- 40 Apply an adhesive PCR plate seal to the plate.
- 41 Place the plate into the thermocycler and run the following pre-programmed settings with a heated lid at § 100 °C

Thermocycler protocol (for use with DNA inputs above 100ng)

```
Step 1: § 68 °C for © 00:03:00

Step 2: § 98 °C for © 00:03:00

Step 3: 5 cycles of:

§ 98 °C for © 00:00:45

§ 62 °C for © 00:00:30

§ 68 °C for © 00:02:00

Step 4: § 68 °C for © 00:01:00

Step 5: Hold at § 10 °C
```

42 Centrifuge plate at **280 x g** for **00:01:00**

43



This is a safe stopping point. The plate may be sealed and stored at $\$ 2 °C to $\$ 8 °C for up to 3 days.

Clean up Libraries

- **NOTE:** The steps listed below are critical for efficient size selection, product recovery and thus cluster generation and sequencing. Always check pipette tips for correct volumes and ensure that no beads have accidentally been aspirated. 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.
- 45 Before starting, prepare reagents:
 - 45.1 Prepare fresh 80% ethanol sufficient for all samples.
 - 45.2 Bring RSB to room temperature (from freezer) and vortex to mix.
 - 45.3 Bring SPB to room temperature (at least 30 minutes) from refrigerator. Vortex and invert SPB several times to full resuspend the beads.
- 46 Prepare the SPB Master mix:

Reagent	Volume/Reaction (μl)	Total Volume (μl) for 24-32 Samples			
SPB	45 μl	1350 μl			
Nuclease-Free Water	40 μl	1200 µl			

Note: The ratio of SPB:water has been validated by FDA-CFSAN for size-selection. CDC PulseNet uses a different ratio of SPB:water to select for large insert sizes.

47 If plate was retrieved from 4°C storage, centrifuge plate at 280 x g for 1 minute.

Remove the seal.

- 48 Place the sample plate on the magnet for \bigcirc 00:05:00
- Transfer 45 µI of supernatant (now containing the DNA) to a set of new wells on the sample plate.
- 50 Remove sample plate from the magnet.

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51 Note: The master mix can be added to a reagent basin and distributed using a multichannel pipet 52 Pipette to mix a minimum of 10 times or until thoroughly mixed. 53 Incubate at room temperature for © 00:05:00 54 Place the 96-well plate on the magnet for © 00:05:00 or until supernatant is clear. During incubation, vortex the stock SPB to resuspend the beads. 55 With the plate still on the magnet, transfer 125 µl of supernatant (containing the DNA) to a new set of wells. 56 Remove the plate from the magnet and add $\Box 15 \mu I$ of stock SPB to the supernatant. Gently pipet at least 10 times to mix. Incubate at room temperature for © 00:05:00 59 Place on the magnet for **© 00:05:00** or until clear. Remove and discard the supernatant (DNA is now bound to the beads) without disrupting the beads 60 Perform the steps below twice (for a total of two washes) 60.1 While the plate is on the magnet, add 180μ of the prepared 80% ethanol Note: Do not add directly to the bead and do not mix. 60.2 Incubate for © 00:00:30

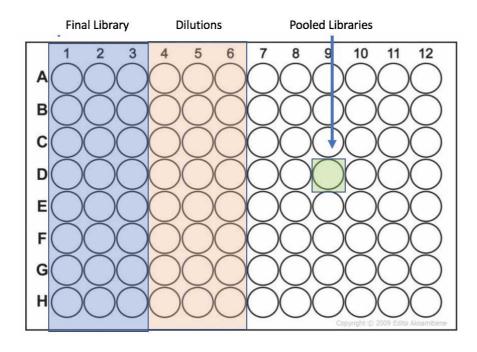
61 Use a pipette to remove any excess liquid from the plate.

Use a small volume pipette to get out any residual if necessary.

62 Allow beads to air dry for up to **© 00:05:00** (minimum of 3 minutes)

Note: Do not allow beads to over-dry. If bead pellet appears to be cracking, immediately resuspend beads regardless of drying time.

- 64 Incubate at room temperature for **© 00:02:00**
- 65 Place the plate on the magnet for \bigcirc **00:02:00**
- Prepare a new 96-well plate as in below that is labelled with run date and intials. Transfer **25 μl** of the supernatant to a new 96-well plate. This is the final library.



Example 96-well plate set up

67 **(II**

If ready to proceed go to the Pooling Libraries step. Otherwise, this is a safe stopping point. The plate may be sealed and stored at & -20 °C for up to 30 days.

Quantification, Normalization and Pooling of Libraries

- Quantify each sample using the Qubit dsDNA High Sensitivity kit. See SOP titled "DNA Quantification using the Qubit Fluorometer" for more detailed information on performing DNA quantification.
- Enter the Qubit values into the "Prep Workbook" tab on the DNA Prep Library worksheet. The DNA Prep worksheet can be found in section 8 of this SOP. The dilution values will be calculated automatically.
- 70 For v2 sequencing chemistry, it is recommended to dilute to 2nm; and for v3 sequencing chemistry to dilute to 4nm.
- 71 Dilute each sample according to the values on the worksheet in the designated dilution wells on the 96-well plate.
- Pool 35 μl of each diluted library into the specified well and pipet to mix. **Note: if sequencing** *Campylobacter*, pool 32.5 μl of diluted sample.

Denaturing Pooled Library

73 Prepare a fresh aliquot of 0.2N NaOH. (This should be made fresh for each run)

Note: It is recommended to make aliquots of 1N NaOH and store in the freezer.

- 74 Transfer 5 µl of the diluted library to a new Eppendorf LoBind tube.
- 75 Add **5** µl of 0.2N NaOH and pipette to mix.
- 76 Incubate at room temperature for **© 00:05:00** to denature the dsDNA.
- 77 Immediately add **3990 μl** of HT1 and pipette to mix. The concentration for a 2 nm start is 10 pM and for 4 nm is 20 pM.
- 78 Dilute the denatured library to the final desired loading concentration.

		red Pool ion = 10 pM	Denatured Pool Concentration = 20 pM			
Final Loading Concentration	Required Volume of HT1	Required Volume of Denatured Pool	Required Volume of HT1	Required Volume of Denatured Pool		
8 pM	200 μ1	800 μ1	600 μ1	400 μl		
9 pM	100 μ1	900 μ1	550 μl	450 μl		
10 pM	NA	NA	500 μ1	500 μ1		
12 pM	NA	NA	400 μ1	600 µl		
14 pM	NA	NA	300 μ1	700 μl		
15 pM	NA	NA	250 μ1	750 µl		
18 pM	NA	NA	100 μ1	900 μ1		
20 pM	NA	NA	NA	NA		

Note: The final loading concentration for optimal Cluster Density may need to be adjusted based on data from previous runs.

79 Mix by repeated inversion of sample tube.

Optional: Denatured PhiX control can be spiked in at this point.

- Heat the denatured DNA library to § 96 °C for © 00:02:00 in a heat block to ensure complete denaturation of all dsDNA in the sample.
- 81 Immediately cool in an ice-water bath for at least **© 00:05:00** prior to loading.

The DNA library may sit on ice or at 8 2 °C - 8 8 °C until ready for loading (<30 minutes)

- 82 Proceed to loading $\Box 600 \, \mu I$ of the denatured ibrary to the thawed cartridge.
- 83 Illumina MiSeq Operation and Maintenance: See "Procedure for Operation and Maintenance of the Illumina MiSeq for Whole Genome Sequencing" SOP for further instructions.

Appendix

84 Appendix 1

DNA Prep Library Workbook

(i) GT_DNA Prep Library Worksheet.xlsx

85 Appendix 2

Nextera DNA CD Indexes (96 indexes, plated)

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