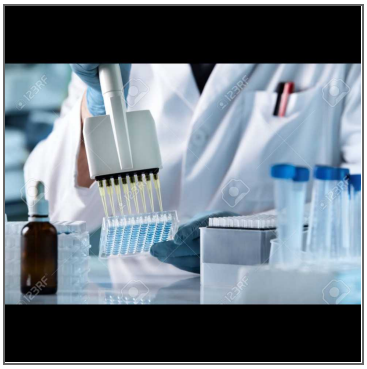

Illumina DNA Prep (M) Tagmentation Library Preparation for use on an Illumina MiSeq Sequencer V.3

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OPEN ACCESS

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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

GUIDELINES

Illumina DNA Prep (M) Tagmentation Kit: The components of the kit have different storage conditions, please follow these recommendations.

Room Temperature Storage:
IPB (Store upright)
TSB
TWB

-25 to -15ºC Storage:
RSB
TB1
EPM

2-8ºC Storage:
BLT (Store upright)

Abbreviations:
BLT: Bead-Linked Transposome
CD: Combinatorial Dual
dsDNA: Double-Stranded DNA
EPM: Enhanced PCR Mix
HT1: Hybridization Buffer
IPB: Illumina Purification Beads
PCR: Polymerase Chain Reaction
RSB: Resuspension Buffer
TB1: Tagmentation Buffer 1
TSB: Tagment Stop Buffer
TWB: Tagment Wash Buffer
UD: Unique Dual

MATERIALS

MATERIALS

Ethanol (100%, Molecular Biology Grade) **Fisher Scientific Catalog #BP2818500**

Qubit® dsDNA HS assay kit, 100 reactions **Life Technologies Catalog #Q32851**

or

Qubit 1X dsDNA HS **Thermo Fisher Scientific Catalog #Q33230 or Q33231**

Sodium Hydroxide 1N **Sigma Aldrich Catalog #S2770-100ml**

Molecular grade water nuclease-free

Illumina DNA Prep, (M) Tagmentation (96 Samples, IPB) **Illumina, Inc. Catalog #20060059**

Illumina DNA Prep, (M) Tagmentation (24 Samples, IPB) **Illumina, Inc. Catalog #20060060**

Nextera DNA CD Indexes (96 samples) **Illumina, Inc. Catalog #20018708**

IDT for Illumina - DNA/RNA UD Indexes Set A (96 Samples)**Illumina, Inc. Catalog #20027213**

IDT for Illumina - DNA/RNA UD Indexes Set B (96 Samples)**Illumina, 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**

Illumina DNA/RNA UD Indexes Set A, Tagmentation (96 Indexes, 96 Samples) **Illumina, Inc. Catalog #20091654**

Illumina DNA/RNA UD Indexes Set B, Tagmentation (96 Indexes, 96 Samples) **Illumina, Inc. Catalog #20091656**

Illumina DNA/RNA UD Indexes Set C, Tagmentation (96 Indexes, 96 Samples) **Illumina, Inc. Catalog #20091658**

Illumina DNA/RNA UD Indexes Set D, Tagmentation (96 Indexes, 96 Samples) **Illumina, Inc. Catalog #20091660**

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 4.0 Fluorometer (or older versions 2.0 and 3.0)
- Thermocycler with heated lid

- 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

Safety information

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 START INSTRUCTIONS

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 (Cell D43). This should not exceed the recommended DNA load values for the reagent kit.

Sequencing Kit (cycles)	DNA Load (Mb) Runs without *Vibrio*, *Escherichia* or *Shigella*	DNA Load (Mb) Runs containing *Vibrio*, *Escherichia* or *Shigella*
MiSeq		
v2 300	**90**	**90**
v2 500	**100**	**100**
v3 600	**200**	**175**
Micro (300)	**35**	**30**
Nano (500)	**13**	**13**
Nano (300)	**13**	**10**
MiniSeq		
Mid Output	**60**	**60**
High Output	**100**	**100**
iSeq		
iSeq v2	**35**	**25**

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 µl (gDNA

concentration of 20 - 50 ng/µl), 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 µl, if DNA is too concentrated, perform a dilution to bring input DNA volume above 2 µl 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

2 Label a 96-well PCR plate with the Run ID.

3 Add molecular-grade water to the each sample well (from Column G of the workbook)

4 Add gDNA to the molecular-grade water (per volume in Column F) 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 µl
BLT	10 µl

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 Mix thoroughly to make sure the BLT beads are evenly resuspended in the buffer.

9 Using fresh tips, transfer 20 µL 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 µl 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 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.

14 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.

16 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 µl reaction.

17 Apply an adhesive PCR plate seal to the plate.

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

19 While samples are incubating, thaw EPM 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 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 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.

30 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 of Tagmented DNA and Index Addition

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 20 µL of EPM with 20 µL of Molecular grade water

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

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.

35 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 40 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

38 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.

Dual Size Selection and Post-PCR Cleanup of Libraries

44 **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 Vortex and invert IPB (stored at room temperature) several times to full resuspend the beads.

46 If plate was retrieved from 4ºC storage, centrifuge plate at 280 x g for 1 minute.

Remove the seal.

47 Place the sample plate on the magnet for 00:05:00

48 Pipette 40 µL of molecular grade water to enough wells on either a new 96-well plate or a set of new wells on the sample plate.

49 Transfer 45 µL of the supernatant (now containing the DNA) to the corresponding wells containing the 40 µl of water.

50 Remove sample plate from the magnet.

51 Vortex IPB thoroughly and add 45 µL to each well containing PCR product and water.

Note: The IPB can be added to a reagent basin and distributed using a multichannel pipet.

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.

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 IPB 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 15 µL of IPB to the supernatant.

Note: The IPB can be added to a reagent basin and distributed using a multichannel pipet.

57 Gently pipet at least 10 times to mix.

58 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 180 µL of the prepared 80% ethanol

Note: Do not add directly to the bead and do not mix.

60.2 Incubate for 00:00:30

60.3 Remove and discard ethanol.

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)

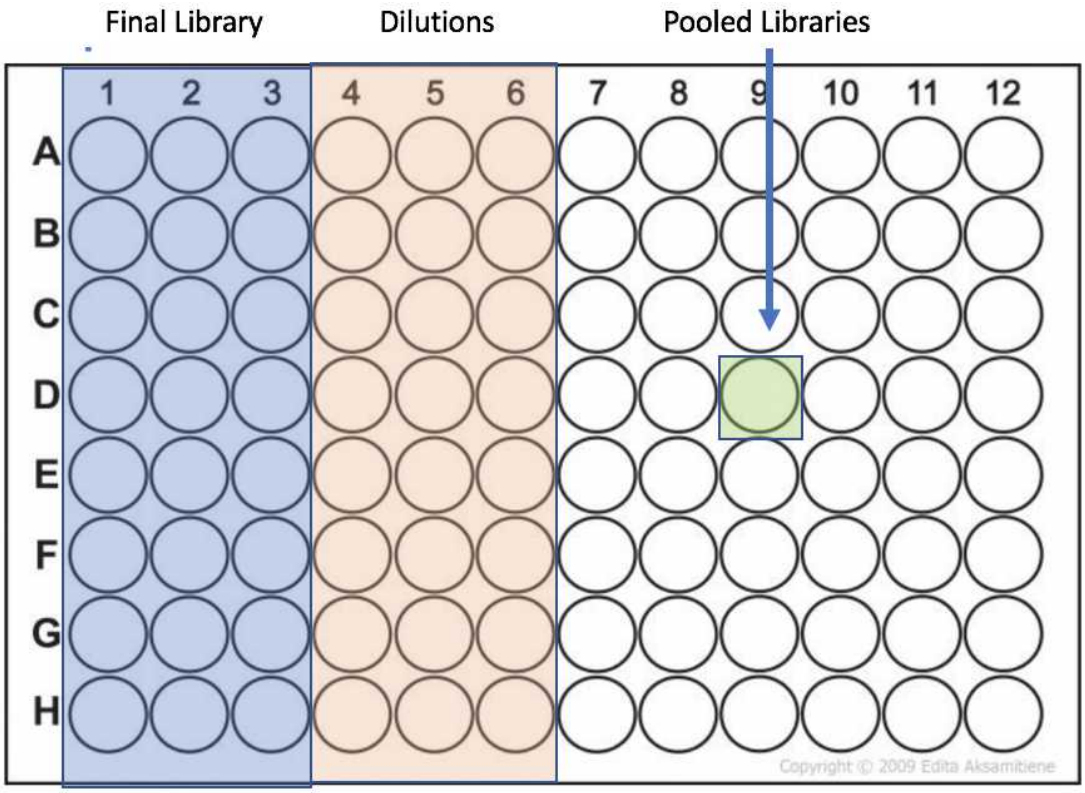
Note: Do not allow beads to over-dry. If bead pellet appears to be cracking, immediately resuspend beads regardless of drying time.

63 Remove the plate from the magnet and add 32 µL of RSB. Pipet thoroughly to mix

64 Incubate at room temperature for 00:02:00

65 Place the plate on the magnet for 00:02:00

66 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. The wells in blue contain the final libraries that have been size-selected. The orange wells contain the libraries diluted to 2nm or 4 nm (based on sequencing chemistry). The well in green would contain the pool of all libraries.

67 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

Note: Setting up your libraries in this fashion will allow for repeat runs if necessary. The libraries are stable for at least 3 months stored at -20 ºC (beyond that time has not been validated by FDA).

Quantification, Normalization and Pooling of Libraries

68 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.

69 Enter the Qubit values into the "Post-Library" section on the DNA Prep Library worksheet (Column K). The DNA Prep worksheet can be found in section 8 of this SOP.

70 For v2 sequencing chemistry, it is recommended to dilute to 2nm; and for v3 sequencing chemistry to dilute to 4nm. Enter the correct value (2 or 4) in Column M.

71 The dilution values will auto populate for a 15 µl volume. This value can be changed in Column N if needed by the individual labs.

Dilute each sample according to the values on the worksheet (Column O and P) in the designated dilution wells on the 96-well plate.

Note: Resuspension Buffer, Molecular Grade Water, or 10mmTris-HCl with 0.1% Tween20 can be used as diluents.

72 Pool 5 µL of each diluted library into the specified well and pipet to mix.

Note: if sequencing *Campylobacter*, pool 2.5 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 990 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.

	Denatured Pool Concentration = 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 µl	800 µl	600 µl	400 µl
9 pM	100 µl	900 µl	550 µl	450 µl
10 pM	NA	NA	500 µl	500 µl
12 pM	NA	NA	400 µl	600 µl
14 pM	NA	NA	300 µl	700 µl
15 pM	NA	NA	250 µl	750 µl
18 pM	NA	NA	100 µl	900 µl
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.

80 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 2 °C - 8 °C until ready for loading (<30 minutes)

82 Proceed to loading 600 µL 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

DNAPrepWorkbook_2024.xlsx19KB

85 **Resources:**

Illumina DNA Prep Reference Guide