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

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1 *Works for me* dx.doi.org/10.17504/protocols.io.bcbnisme

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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

DOI

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PROTOCOL CITATION

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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^{O}C)
- TSB (Store at room temperature)
- TWB (Store at room temperature)

Box 2 of 3: (Store at -25 to -15^{O}C)

- RSB
- TB1
- EPM

Box 3 of 3: (Store at 2-8^{O}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

NAME	CATALOG #	VENDOR
Ethanol (100%, Molecular Biology Grade)	BP2818500	Fisher Scientific
Qubit® dsDNA HS assay kit, 100 reactions	Q32851	Life Technologies
Nextera DNA CD Indexes (96 samples)	20018708	Illumina, Inc.
Sodium Hydroxide 1N	S2770-100ml	Sigma Aldrich
Molecular grade water nuclease-free		
Illumina DNA Prep (M) Tagmentation (96 Samples)	20018705	Illumina, Inc.
Illumina DNA Prep (M) Tagmentation (24 Samples)	20018704	Illumina, Inc.

MATERIALS TEXT

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)

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- Micropipettes (Single and Multichannel)
- Ice bucket
- Microcentrifuge

SAFETY WARNINGS

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.

Illumina 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
- Gray fields are optional
- Blue fields contain formulas, which will auto-populate, and should not be altered

1. Enter sample IDsfor all isolates in column A.
2. Designate indices for each sample. Select the set of indices that will be used from the dropdown menu in E2.
3. Enter the concentration of the gDNA in column H.
4. Enter the volume of extracted DNA to be used for library preparation in column J. 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 100-500ng. Our recommendation is to use at least 100ng 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.

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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 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 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.

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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 (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 **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.

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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 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 **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

Nte: 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.

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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

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8 °C for up to 3 days.

Clean up 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 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 ration 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 **00:05:00**

49 Transfer **45 µl** of supernatant (now containing the DNA) to a set of new wells on the sample plate.

50 Remove sample plate from the magnet.

51 Vortex SPB master mix thoroughly and add **85 µl** to each PCR product.

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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 **15 µl** of stock SPB to the supernatant.

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.

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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)

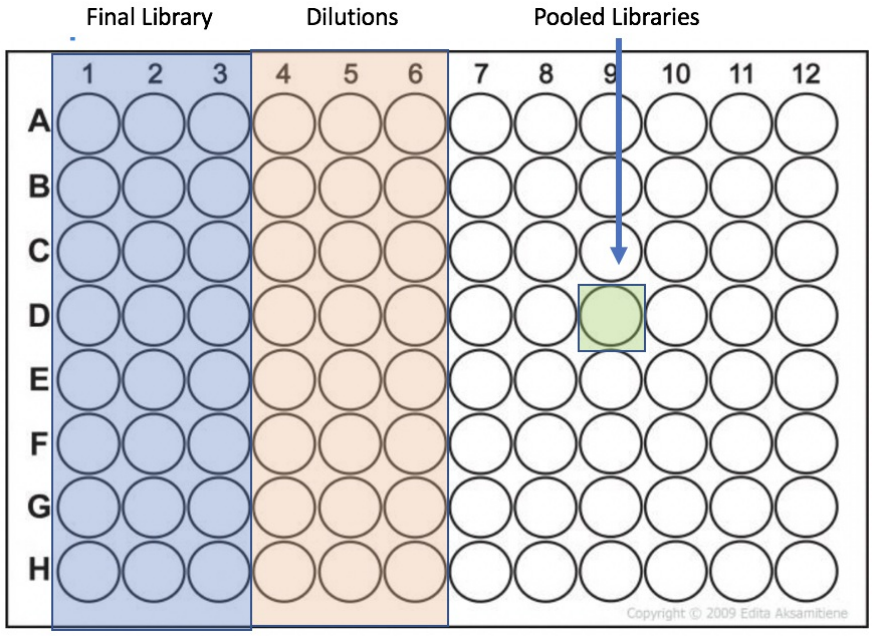
Note: Do not allow beads to over-dry. If bead pellet appears to be cracking, immediately resuspend beads regardless of drying time.

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

67

If ready to proceed go to the Pooling Libraries step. Otherwise, this is a safe stopping point. The plate may be sealed

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and stored at **-20 °C** for up to 30 days.

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 “Normalization and Pooling” tab on the DNA Flexwork sheet. The DNA Flex 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. This value can be adjusted by the dropdown cell H4.

71 Dilute each sample according to the values on the worksheet in the designated dilution wells on the 96-well plate.

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

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.

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	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 Flex Library Prepartion Workbook

GT_LIBRARYPREP_Appendix 1 DNAFlex Worksheet.xlsx

85 **Appendix 2**

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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
A	H505-H701	H506-H702	H517-H703	H505-H705	H506-H707	H517-H723	H505-H706	H506-H712	H517-H720	H505-H710	H506-H711	H517-H714
B	H517-H702	H505-H703	H506-H701	H517-H707	H505-H723	H506-H705	H517-H712	H505-H720	H506-H706	H517-H711	H505-H714	H506-H710
C	H506-H703	H517-H701	H505-H702	H506-H723	H517-H705	H505-H707	H506-H720	H517-H706	H505-H712	H506-H714	H517-H710	H505-H711
D	H503-H705	H503-H707	H503-H723	H503-H706	H503-H712	H503-H720	H503-H710	H503-H711	H503-H714	H503-H701	H503-H702	H503-H703
E	H516-H706	H516-H712	H516-H720	H516-H710	H516-H711	H516-H714	H516-H701	H516-H702	H516-H703	H516-H705	H516-H707	H516-H723
F	H522-H710	H510-H711	H513-H714	H522-H701	H510-H702	H513-H703	H522-H705	H510-H707	H513-H723	H522-H706	H510-H712	H513-H720
G	H513-H711	H522-H714	H510-H710	H513-H702	H522-H703	H510-H701	H513-H707	H522-H723	H510-H705	H513-H712	H522-H720	H510-H706
H	H510-H714	H513-H710	H522-H711	H510-H703	H513-H701	H522-H702	H510-H723	H513-H705	H522-H707	H510-H720	H513-H706	H522-H712

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