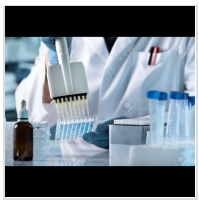

Jan 21, 2022

Modified Illumina DNA Prep (M) Tagmentation Library Preparation for cDNA amplicons from wastewater

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

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This procedure outlines the protocol for sequencing of VarSkip Short SARS-CoV-2 cDNA amplicons using the Illumina DNA Prep library preparation kit for sequencing on an Illumina MiSeq sequencer.

DOI

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Modified Illumina DNA Prep (M) Tagmentation Library Preparation for cDNA amplicons from wastewater, Chris Grim

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

protocol ,

Jan 20, 2022

Jan 21, 2022

57201

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

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

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

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

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

☒ Molecular grade water nuclease-free **Contributed by users**

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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**

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**

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

Chemical Safety Warning: Take proper precautions, and wear appropriate

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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

:

Run theVarSkip Short SARS-CoV-2 cDNA amplicons on TapeStation or Bioanalyzer to determine if clean up is necessary as the next step. Quantify the cDNA using Qubit and determine the input cDNA concentration for the Illumina DNA prep.

Dilute and Tagment Input DNA

1

Based on the viral load in the sample, you will see a spectrum of fragment profiles. If there is a single peak around 400-600 bp, the clean-up step can be omitted and you can proceed directly to library preparation using this protocol.

Quantify the cDNA amplicon using Qubit HS kit. We recommend starting the input cDNA concentration at 100-200 ng. Add 2-30ul DNA, so that the total input amount is 200 ng. If DNA volume is less than 30ul, add nuclease free water to the DNA samples to bring the total volume to 30ul.

The minimum input cDNA recommended is 25 ng. The number of PCR index cycle will vary depending on the starting input.

If there are primer dimers observed on the tape station profile of cDNA amplicons and you still would like to proceed with the Illumina DNA prep library, we suggest

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a clean up before starting the library prep, using AMPure XP, SPRI, or Sample Purification beads, "SPB", at 0.8 to 1x, as recommended by Illumina: Adapter dimers: causes, effects, and how to remove them (illumina.com). For more detailed steps, you can follow this protocol, AMPure Purification Protocol (protocols.io), using the 0.8 to 1.0x bead to amplicon volume ratio. Quantify using Qubit after the cleanup and proceed based on the total input quantity.

2 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

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

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

5 Add gDNA to the molecular-grade water (per volume in Column C) and mix well by gently pipetting 5 - 10 times.

6 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

7 Vortex the TB1 and spin down the tube.

8 *****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

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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

9 Vortex the tagmentation master thoroughly to make sure the BLT beads are evenly resuspended in the buffer.

10 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

11 Pipette up and down 10 times mix the 50 µl reaction to resuspend the beads.

12 Apply an adhesive PCR plate seal to the plate.

13 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**

14 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.

15 Upon completion of the incubation, remove the plate from the thermocycler. Proceed to the Post Tagmentation Cleanup step.

Post Tagmentation Clean Up

16 Remove the plate seal.

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17 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.

18 Apply an adhesive PCR plate seal to the plate.

19 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**

20 While samples are incubating, thaw EPM (stored in freezer) on ice and thaw indices at room temperature.

21 Remove the plate from the thermocycler, quick spin the plate and remove the seal.

22 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.

23 Using a multichannel pipette, remove the supernatant and discard.

24 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.

25 Place the plate on the magnet for **00:03:00** or until solution is clear.

26 Remove the supernatant and discard.

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27 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.

28 Place the plate on the magnet for **00:03:00** or until solution is clear.

29 Remove the supernatant and discard.

30 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.

31 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

32 Invert the EPM to mix, then briefly centrifuge.

33 Briefly centrifuge the Index plate.

34 *****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

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

35 Vortex and spin down the PCR master mix.

36 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.

37 Remove the plate from the magnet and immediately proceed to adding the master mix.

38 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

39 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.

40 Use a pipette to gently mix a minimum of 10 times to ensure thorough mixing.

41 Apply an adhesive PCR plate seal to the plate.

42

NOTE: Depending on the initial concentration of cDNA amplicon that was used as the input, the number of cycles for index PCR WILL vary.
Aim for at least 100-200 ng of input cDNA amplicon in 30ul as recommended by Illumina.

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If the input cDNA is around 100-200 ng, the index PCR cycle has to be set at 7/8 cycles.

If the cDNA amplicon input is from 25-99 ng, the index PCR cycle has to be set at 9/10 cycles.

43 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 - change # of cycles accordingly)

Step 1: **68 °C** for **00:03:00**

Step 2: **98 °C** for **00:03:00**

Step 3: 7 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**

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

45

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

46 **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.*

47 Before starting, prepare reagents:

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47.1 Prepare fresh 80% ethanol sufficient for all samples.

47.2 Bring RSB to room temperature (from freezer) and vortex to mix.

47.3 Bring SPB to room temperature (at least 30 minutes) from refrigerator. Vortex and invert SPB several times to fully resuspend the beads.

48 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.

49 If plate was retrieved from 4^{o}C storage, centrifuge plate at 280 x g for 1 minute.

Remove the seal.

50 Place the sample plate on the magnet for **00:05:00**

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

52 Remove sample plate from the magnet.

53 Vortex SPB master mix thoroughly and add **85 µL** to each PCR product.

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

Pipette to mix a minimum of 10 times or until thoroughly mixed.

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54

55 Incubate at room temperature for **00:05:00**

56 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.

57 With the plate still on the magnet, transfer **125 µL** of supernatant (containing the DNA) to a new set of wells.

58 Remove the plate from the magnet and add **15 µL** of stock SPB to the supernatant.

59 Gently pipet at least 10 times to mix.

60 Incubate at room temperature for **00:05:00**

61 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

62 Perform the steps below twice (for a total of two washes)

62.1 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.

62.2 Incubate for **00:00:30**

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62.3 Remove and discard ethanol.

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

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

64 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.

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

66 Incubate at room temperature for **00:02:00**

67 Place the plate on the magnet for **00:02:00**

68 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.

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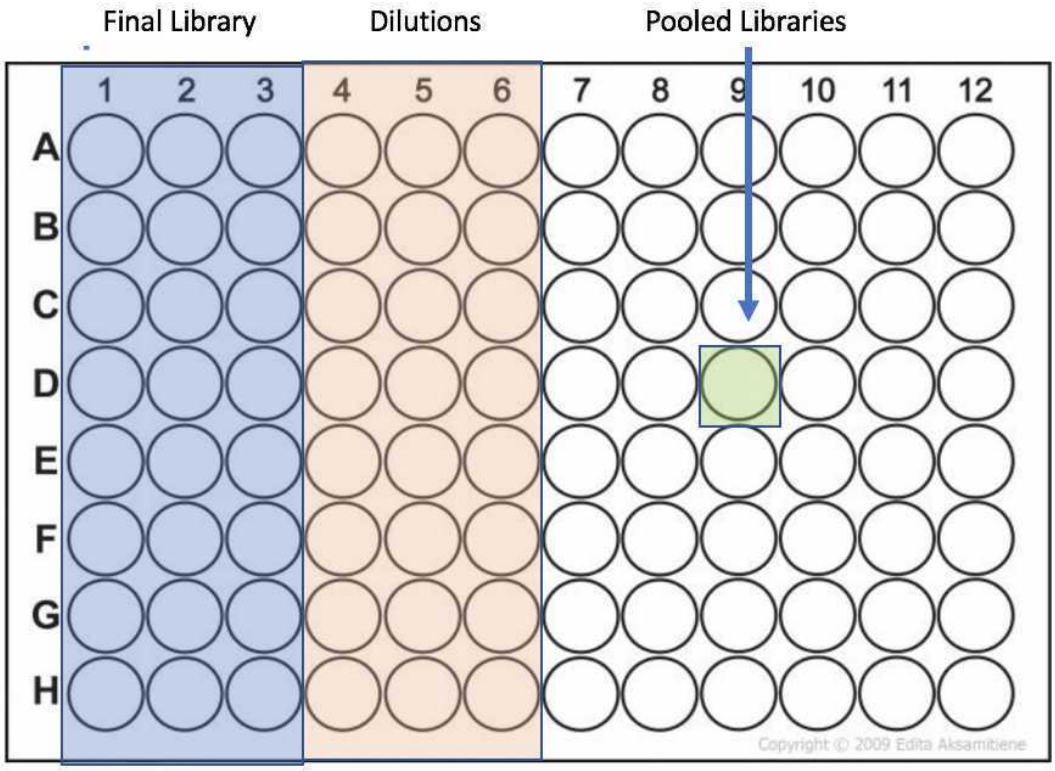

Example 96-well plate set up

69

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

70 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.

71 Enter the Qubit values into the column labeled "Post Library Prep Qubit Conc (ng/ul)" of the Prelibrary_postlibrary worksheet. The normality and dilution values can be calculated. If the libraries are run on TapeStation or BioAnalyzer, use the size reported, otherwise, you can use a value of 600 bp for DNA prep libraries.

72 For v2 sequencing chemistry, it is recommended to dilute to 2nm; and for v3 sequencing chemistry to dilute to 4nm.

Dilute each sample according to the values on the worksheet in the designated dilution wells on

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73 the 96-well plate.

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

Denaturing Pooled LIbrary

75 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.

76 Transfer **5 µL** of the diluted library to a new Eppendorf LoBind tube.

77 Add **5 µL** of 0.2N NaOH and pipette to mix.

78 Incubate at room temperature for **00:05:00** to denature the dsDNA.

79 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.

80 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.

81 Mix by repeated inversion of sample tube.

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

82 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.

83 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)

84 Proceed to loading **600 µL** of the denatured library to the thawed cartridge.

85 Illumina MiSeq Operation and Maintenance: See "**Procedure for Operation and Maintenance of the Illumina MiSeq for Whole Genome Sequencing**" SOP for further instructions.

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86 Appendix1 **Prelibrary_postlibraryworksheet.xlsx**

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